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DETERMINATION OF ISOSORBIDE AS A METABOLITE OF ISOSORBIDE DINITRATE IN HUMAN URINE BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A method for the determination of isosorbide as a metabolite of isosorbide dinitrate at concentrations down to 200 ng/ml in human urine is described. After addition of a known amount of isomannide as internal standard to 50 μl of urine, both compounds are extracted at basic pH into chloroform—isopropanol (4:1, v/v), which is then evaporated to dryness. They are then derivatized with heptafluorobutyric anhydride, and isosorbide is quantitated by capillary gas chromatography with electron-capture detection. A conjugate of isosorbide is determined in urine after enzymatic hydrolysis.

INTRODUCTION

Isosorbide dinitrate (ISDN) is a vasodilative organic nitrate. 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].

Several authors have reported that the major urinary metabolite of ISDN is the glucuronide of isosorbide. Rosseel and Bogaert [2] studied the urinary excretion of ISDN in rats and found (without quantitation) isosorbide before and after incubation of the urine for 20 h with β-glucuronidase—arylsulphatase. Sisenwine and Ruelius [3] measured plasma concentrations and urinary excretions of ISDN and its metabolites in the dog after administration of either an oral or intravenous dose of [^{14}C]ISDN; isosorbide and its polar conjugate in urine were measured after thin-layer chromatography and determination of radioactivity. Down et al. [4] studied the biotransformation of ISDN after oral administration of 5 mg of [^{14}C]ISDN and showed that nearly 50% of the drug was transformed to an isosorbide conjugate; concentrations of isosorbide were measured in urine after thin-layer chromatography and determination of radioactivity.

This present paper describes a procedure which permits the quantitative
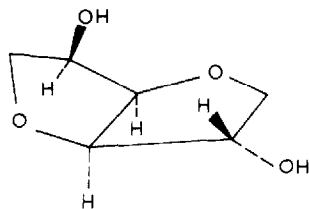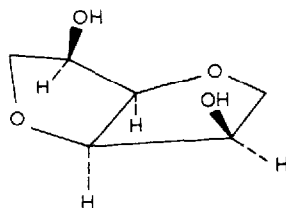
ISOSORBIDE   ISOMANNIDE

gas chromatographic determination of free isosorbide in human urine using isomannide as internal standard.

Conjugated isosorbide may also be assayed in urine after enzymatic hydrolysis. The limit of quantitation is 200 ng isosorbide per ml of urine.

EXPERIMENTAL

Chemicals and reagents

Isosorbide and isomannide were synthesized in our laboratories according to the method of Hockett and Fletcher [5] from D-sorbitol and D-mannitol, respectively. The solvents and reagents used are all of analytical grade: chloroform (Uvasol 2447; Merck, Darmstadt, F.R.G.), n-heptane (Merck, Uvasol 4366), isopropanol (Prolabo 20837; Rhône-Poulenc, Paris, France). Pyridine (Fluka 82702; Fluka, Buchs, Switzerland) was distilled at 115—116°C with potassium hydroxide pellets and stored over the same reagent. Heptafluorobutyric anhydride was purchased from Ventron (Ref. 1300-3; PCR, Karlsruhe, F.R.G.) and stored in 1-ml glass ampoules. Potassium carbonate (Merck 4928)—potassium hydrogen carbonate (Merck 4854) (2:3, w/w) was used as solid buffer. The extraction solvent is chloroform—isopropanol (4:1, v/v). Potassium dihydrogen phosphate (Merck 4873) was used as a saturated solution. 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 up to 100 ml with water. The enzyme solution (β-glucuronidase—arylsulphatase) (Calbiochem, Los Angeles, CA, U.S.A.; B grade, 6.66 IU/ml β-glucuronidase, 3.41 IU/ml arylsulphatase) is diluted to one-tenth concentration with pH 5 buffer.

Calibration solutions

The methanolic solutions of isosorbide contain 10—250 ng per 25 μl. The methanolic solution of internal standard contains 250 ng of isomannide per 25 μl.

Equipment

A Hewlett-Packard Model 5880 A gas chromatograph equipped with computing integrator, electron-capture detector and automatic sampler is used. The column is a 12 m × 0.2 mm fused-silica capillary column coated with dimethylsilicone (OV-101) (Hewlett-Packard No. 19091-60010). Splitless injection is used with a 72-sec splitless period. The column is flushed with the carrier gas (argon—methane, 90:10) at a flow-rate of 2 ml/min; the septum purge is 5 ml/min and the auxiliary gas flow to the detector 30 ml/min. The injector temperature is 250°C and the detector is set at 300°C. The column

is at 80°C initially for 0.3 min and the temperature is then increased by 5°C/min up to 120°C; to wash out urine residues, it is held at 120°C for 1 min, then raised rapidly by 30°C/min up to 200°C for 15 min.

Enzymatic hydrolysis of isosorbide conjugates in urine

Twenty-five microliters of the internal standard solution are measured into a 10-ml glass tube, to which 50 μl of urine, 200 μl of pH 5 acetate buffer and 2.5 μl of β-glucuronidase—arylsulphatase mixture are added. This preparation is then kept for 24 h at 37°C and fresh enzyme (2.5 μl) is added four times during the incubation. The extraction is then performed as described below for free isosorbide.

Extraction of free isosorbide from urine

A 25-μl aliquot of the internal standard solution is measured into a glass tube, into which 50 μl of urine, 1 ml of distilled water and around 300 mg of potassium carbonate—potassium hydrogen carbonate (2:3, w/w) are then introduced. The stoppered tube is shaken on a Vortex mixer for 1 min, then 5 ml of chloroform—isopropanol (4:1, v/v) are added. The tube is restoppered and shaken mechanically for 15 min at 300 rpm with an Infors shaker, then centrifuged for 3 min at 2500 g. An aliquot of the organic phase is transferred to another tube and dried under a nitrogen stream at room temperature. Evaporation must be done very carefully and stopped just as the tube reaches dryness.

Derivatization and chromatography

To the dry residue are added 1 ml of heptane containing 0.5% pyridine and 10 μl of heptafluorobutyric anhydride. The medium is thoroughly mixed (Vortex mixer) for 20 sec and allowed to stand for 10 min at room temperature. Then 1 ml of potassium dihydrogen phosphate saturated solution is added, and the tube is shaken for 30 sec and centrifuged. The aqueous phase is frozen by immersing the tube in a methanol bath containing dry ice. An aliquot of heptane is transferred into a 250-μl conical glass flask (Hewlett-Packard 5080-8779) and a 1-μl portion is injected into the gas chromatograph using the splitless injection technique.

Calibration curves

Calibration samples are prepared by introducing 25 μl of a suitable methanolic isosorbide solution (10—250 ng) into 10-ml glass centrifuge tubes containing 50 μl of urine and a constant amount of internal standard (250 ng). The calibration curve is obtained from the ln—ln plot of the peak-area ratios versus the urine concentrations. The equation is calculated by the least-squares method. A calibration curve is prepared every day.

Human study

A healthy male subject, who had been advised to take no drugs during the fourteen days preceding the experiment and none besides ISDN throughout the duration of the study, received 5 mg of ISDN as one tablet of Isoket® . Urine was collected at the following time intervals: 0—4, 4—8, 8—12,

12—24, 24—48, 48—72, 72—96 and 96—104 h. The volume of each sample was measured and an aliquot was stored at −20°C.

RESULTS AND DISCUSSION

Reaction time

The derivatization was applied to isosorbide and isomannide, and the reaction time was varied from 5 to 60 min. Derivative formation was evaluated by peak areas; derivatization was almost immediate and the yield of isosorbide and isomannide was the same over the time range of 5—60 min. For convenience and safety, the suggested derivatization time is 10 min.

Capillary gas chromatography

The quantitative isosorbide determination by conventional packed column gas—liquid chromatography was difficult because of interfering peaks from the urine. The capillary column used here provides a suitable means of analysing isosorbide with the required efficiency.

Splitless injection

Splitless injection (solvent effect) is the appropriate technique for analysing samples containing trace-level components. To obtain a good solvent effect, the oven temperature must be low enough that the solvent does not leave the head of the column too quickly. This generally means that the oven should be 10—30°C below the boiling point of the solvent. In this technique, the sample components which are less volatile than the solvent (heptane) are reconcentrated at the beginning of the column during a 72-sec splitless period.

Urine hydrolysis of conjugated isosorbide

Preliminary experiments were carried out to determine the best conditions for the hydrolysis of the isosorbide conjugates in human urine. Stability tests showed that isosorbide and isomannide are stable for 24 h in human urine at 37°C. Samples of the same urine fraction, containing the conjugate of isosorbide, from a volunteer treated with ISDN were subjected to enzymatic hydrolysis. Enzymatic hydrolysis was studied in aliquots of this urine incubated with enzyme (β-glucuronidase—arylsulphatase) and acetate buffer (pH 5) for 2, 4, 6, 8, 22 and 24 h at 37°C; fresh enzyme (2.5 μl) was added four times during incubation. A maximum yield of isosorbide was obtained after incubation for 22 h.

Extraction

Isosorbide and isomannide are not extracted into organic solvents such as toluene or ethyl acetate. Mixed systems employing a non-polar solvent in combination with a highly polar solvent have, however, been used (chloroform containing isopropanol). Better isosorbide and isomannide extraction was obtained after saturation of urine samples with a basic solid buffer (potassium carbonate—potassium hydrogen carbonate, 2:3). The formation of emulsions with the extraction solvent was avoided by use of a solid buffer.

Linearity and reproducibility of calibration curves

Straight lines were obtained for calibration curves in the concentration range of 200—5000 ng/ml, and the method was validated over this concentration range (Table I). The day-to-day reproducibility of the standard curves was shown in three consecutive experiments carried out on separate days. On each occasion, the peak area ratio of isosorbide versus the internal standard (mean of duplicate analysis) plotted against five concentrations of isosorbide gave straight lines. A least-squares ln—ln regression line was generated from the fifteen data points of the three standard curves (Table I). It corresponds to the regression equation $\ln Y = 1.23276 \ln X - 10.7024$. A test of day-to-day reproducibility was made by expressing each data point as a percentage of the value read off in the ln—ln line for the corresponding concentration (Table I). The distribution of these normalized (concentration-independent) data had an overall average ($\pm$ C.V.) of 100.9 $\pm$ 8.9%, demonstrating a good reproducibility between experiments. However, a variability between the curves is sometimes noted which is the reason why the calibration curve was generated daily.

TABLE I

LINEARITY AND REPRODUCIBILITY OF CALIBRATION CURVES

Concentration added to plasma (ng/ml)	Peak area ratio				$100 \times \dfrac{E}{C}$ (%)
	Experimental (E)			Calculated from ln—ln regression line (C)	
	Day 1	Day 2	Day 3		
200	0.015				100.0
		0.018		0.015	120.0
			0.012		80.0
500	0.051				106.3
		0.051		0.048	106.3
			0.044		91.7
1000	0.120				107.1
		0.119		0.112	106.3
			0.114		101.8
2000	0.275				104.2
		0.279		0.264	105.7
			0.260		98.5
5000	0.773				94.7
		0.798		0.816	97.8
			0.765		93.8
Average					100.9
$\pm$ C.V. (%)					8.9

Precision and recovery

The repeatability of the method was checked by determining six urine samples spiked with several different concentrations. Table II shows the good reproducibility obtained with concentrations down to 200 ng/ml isosorbide. This concentration (200 ng/ml) may be taken as the limit of quantitation of the method.

TABLE II

PRECISION AND RECOVERY IN THE DETERMINATION OF ISOSORBIDE IN SPIKED HUMAN URINE

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (coefficient of variation)	Recovery (%)
200	196	4.6	98.2
500	500	2.7	100.0
1000	1010	4.2	101.0
2000	1946	4.1	97.3
5000	4730	0.6	94.6
Mean			98.2
± C.V. (%)			2.5

Interference from urine and the precursors of isosorbide

Fig. 1 shows the chromatograms of an extract of human urine (50 μl) and of the same extract spiked with 10 ng of isosorbide (200 ng/ml) and 250 ng of internal standard (isomannide). Isosorbide and the internal standard
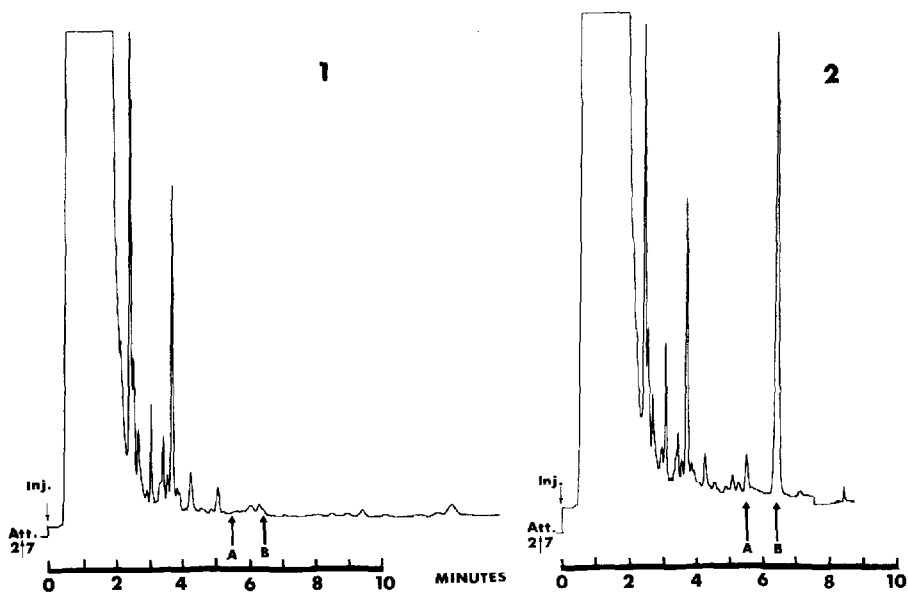

Fig. 1. Chromatograms of (1) human urine blank (50 μl urine); and (2) the same urine spiked with 10 ng of isosorbide (200 ng/ml) (A), and 250 ng of internal standard (B).
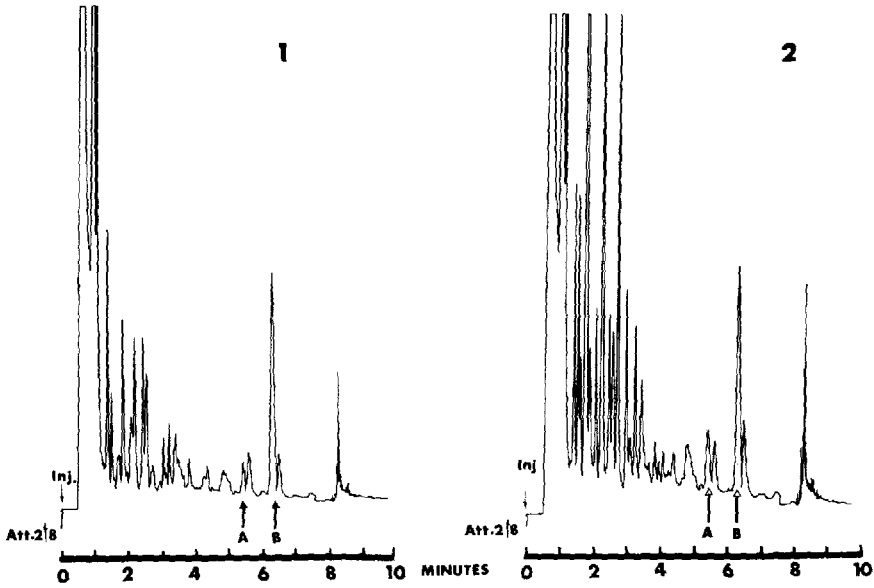
Fig. 2. Chromatograms of a urine aliquot (50 µl) collected between 4 and 8 h from a healthy subject treated with 5 mg of ISDN, (1) containing 696 ng/ml free isosorbide (A), and (2) containing 1296 ng/ml total isosorbide (free + conjugated, A) after enzymatic hydrolysis, both with 250 ng of internal standard (B).

are well separated from the normal components of the urine extract. Fig. 2 shows chromatograms of a urine aliquot (50 µl) before and after enzymatic hydrolysis; this urine was collected between 4 and 8 h from a healthy subject treated with 5 mg of ISDN. Isosorbide dinitrate and the two isosorbide
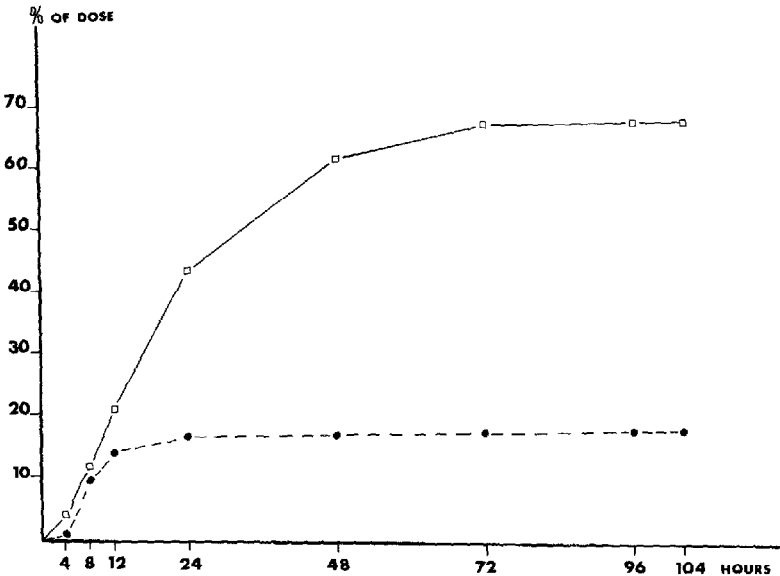

Fig. 3. Cumulative urinary excretions of free and conjugated isosorbide in one healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (●), Conjugated isosorbide; (□), free isosorbide.

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mononitrate metabolites (2-ISMN and 5-ISMN) were injected under the same conditions as isosorbide. These compounds were not recorded.

Application

The present method was used to determine the urinary excretion of the metabolite of ISDN after oral administration to one healthy subject. The corresponding plasma concentrations of the parent drug (ISDN) have also been determined [1]. Fig. 3 shows the curves of urinary free and conjugated isosorbide excretion in the subject given 5 mg of ISDN as one Isoket tablet. In the 0—104 h urine, 66.2% of the administered dose was recovered as free isosorbide and 15.8% as conjugated isosorbide.

CONCLUSION

The proposed gas chromatographic technique permits the quantitative determination of isosorbide in human urine at concentrations down to 200 ng/ml. This method can be applied to the determination of free and conjugated isosorbide as a metabolite of ISDN in urine.

ACKNOWLEDGEMENTS

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