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AS CHROMATOGRAPHIC DETERMINATION OF ISOSORBIDE INITRATE IN HUMAN PLASMA AND URINE

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JMMARY

A rapid, simple and sensitive method for the specific determination of isosorbide dinitrate concentrations down to 0.5 ng/ml in human plasma and urine is described. Following ;traction (with or without internal standard) of isosorbide dinitrate into toluene, the com-)und is determined by gas chromatography using a ^{63}Ni electron-capture detector.

NTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate vasodilator. Many methods ave been already proposed for its quantitative assay in biological fluids. Con-entrations of [^{14}C]ISDN [1] were measured in blood after thin-layer chroma-ography and determination of radioactivity, the limit of accurate measurement eing 2 ng/ml.

Many gas chromatographic (GC) methods have been described [2—10]. ome methods lack sensitivity [2, 3, 6, 7] and require a large sample volume: etween 4 and 10 ml. Several procedures use a laborious extraction [4, 7, 10] r a chromatography which takes around 30 min [4, 8]. In two procedures, o internal standard is required [6, 7]. In the others, nitroglycerin [4, 8, 9], initrobenzene [2, 3] or isomannide dinitrate [10] are used as internal tandard. In most of the published assays, the internal standard is dissolved in solvent before extraction [8, 9] or is added just before injection [2—4] and he incomplete extraction recovery of ISDN is not corrected. The methods ited above have a sensitivity between 0.5 and 50 ng/ml.

Owing to the volatility of ISDN, it is advisable to avoid evaporation of the xtraction solvent, which is required in most of the above-mentioned procedures.

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This paper describes a rapid and sensitive procedure which permits the determination of ISDN (with or without isomannide dinitrate as internal standard) down to 0.5 ng/ml (without internal standard) in human plasma and urine using GC and an electron-capture detector. This technique is faster than the existing assay procedures, it requires a one-step extraction and avoids solvent concentration. The method is particularly well suited for routine determinations of large numbers of samples with convenient sensitivity.

EXPERIMENTAL

Chemicals and reagents

ISDN on lactose was supplied by Sanol (Monheim, G.F.R.). Its content is determined following the colorimetric method of USP XIX [11]. The ISDN solutions are prepared in acetone. Isomannide dinitrate (IMDN) was synthesized from isomannide in our laboratories according to the method of Jackson and Hayward [12]. Isomannide was obtained from D-mannitol according to the method of Hockett and Fletcher [13]. Toluene (Pestipur, SDS, Peypin, France) is of analytical grade. The methanolic solution of internal standard contains 10 ng of isomannide dinitrate per 25 μl. The ISDN calibration solutions contain 0.5—500 ng per 25 μl of acetone.

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

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a Hewlett-Packard electron-capture detector (Model 18 713A) is used. The temperature controllers of detector and injection port have a temperature range from 100 to 400°C in 50°C steps but have been modified for a continuous adjustment of temperature. The peak areas are given by a Hewlett-Packard computing integrator (Model 3388A). The column is operated at 147°C, the injector temperature is 165°C and the detector is set at 215°C with argon—methane (90:10) at a flow-rate of 75 ml/min. The glass column (1 m × 2 mm I.D.) is washed [14], and packed with 3% QF-1 on Gas-Chrom Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature is held overnight at 150°C and after some injections of ISDN the column is ready for use.

Extraction

Twenty-five microlitres of internal standard solution (if an internal standard is used) are measured into a 5-ml glass centrifuge tube. Then, 1 ml of the sample (plasma or urine) and 250 μl of toluene are added. The tube is stoppered and shaken mechanically (Infors shaker) for 10 min at 200 rpm, then centrifuged for 5 min at 2500 *g*.

Gas chromatography

A 3-μl aliquot of the organic layer is injected into the gas chromatograph.

The ISDN content is calculated from the peak area (peak-area ratio if an internal standard is used) by reference to a calibration curve. This curve is obtained by extraction of plasma or urine spiked with increasing amounts of ISDN (from 0.5 to 500 ng/ml) and a constant amount of internal standard (10 ng/ml plasma or urine if an internal standard is used). A calibration curve is prepared 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 tablet of Isoket®. 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 and internal standard

Without internal standard, numerous samples spiked with ISDN in plasma and urine (within-day and day-to-day) have been determined for the validation of the method. The one-step extraction with toluene was reproducible. But it is possible to use isomannide dinitrate as internal standard. The chromatographic separation of ISDN and IMDN is difficult at 147°C; a better resolution is obtained at 138°C but the sensitivity limit of the method will be lower and the retention time of ISDN longer.

TABLE I

RECOVERY IN THE WITHIN-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA AND URINE SAMPLES WITHOUT INTERNAL STANDARD

Amount added (ng/ml)	Mean amount found in plasma (ng/ml) (n = 6)	Recovery in plasma (%)	Mean amount found in urine (ng/ml) (n = 6)	Recovery in urine (%)
0.50	0.51	102.0	0.51	102.0
0.75	0.74	98.7	—	—
1	0.97	97.0	0.93	93.0
5	5.0	100.0	5.3	106.0
25	25.0	100.0	25.7	102.8
50	51.2	102.4	51	102.0
100	—	—	98	98.0
250	262	104.8	—	—
500	529	105.8	—	—
Mean		101.3		100.6
C.V. (%)		± 2.9		± 4.5

Precision and recovery without internal standard

The within-day reproducibility of the method was checked in plasma and urine by determining six samples spiked with several different concentrations. Table I gives the results obtained when the described procedure was applied to spiked plasma and urine. The day-to-day reproducibility was checked in plasma and urine by determining during one week, on each day, two concentrations in duplicate: 1 and 50 ng/ml ISDN. Table II shows the day-to-day reproducibility

TABLE II

PRECISION AND RECOVERY IN THE DAY-TO-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA AND URINE SAMPLES WITHOUT INTERNAL STANDARD

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 (%)
1	0.95	0.098	95.0	1.05	0.081	105.0
50	52.4	3.6	104.8	49.8	3.0	99.6

of the method in plasma and urine. These tables demonstrate the good reproducibility of the assay without internal standard down to concentrations of 0.5 ng/ml of plasma or urine. This concentration (0.5 ng/ml) may be taken as the quantitation limit of the method; lower concentrations could still be detected.

Precision and recovery with internal standard

Table III gives the results obtained when the described procedure with internal standard was applied to spiked plasma samples. As seen in the table, a good reproducibility was obtained with concentrations down to 1 ng/ml.

TABLE III

RECOVERY IN THE WITHIN-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA WITH ISOMANNIDE DINITRATE AS INTERNAL STANDARD

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 8)	Recovery (%)
1	1.05	105.0
5	5.03	100.6
25	24.0	96.0
Mean		100.5
C.V. (%)		± 4.5

Plasma and urine interference

Fig. 1.1 shows chromatograms of an extract of human plasma and of the same extract spiked with 25 ng of ISDN. Fig. 1.2 shows chromatograms of a human plasma extract and of the same extract spiked with 25 ng of ISDN and 10 ng of internal standard. No interference from normal plasma components was recorded. ISDN and the internal standard are well separated from the normal components of the urine extract.

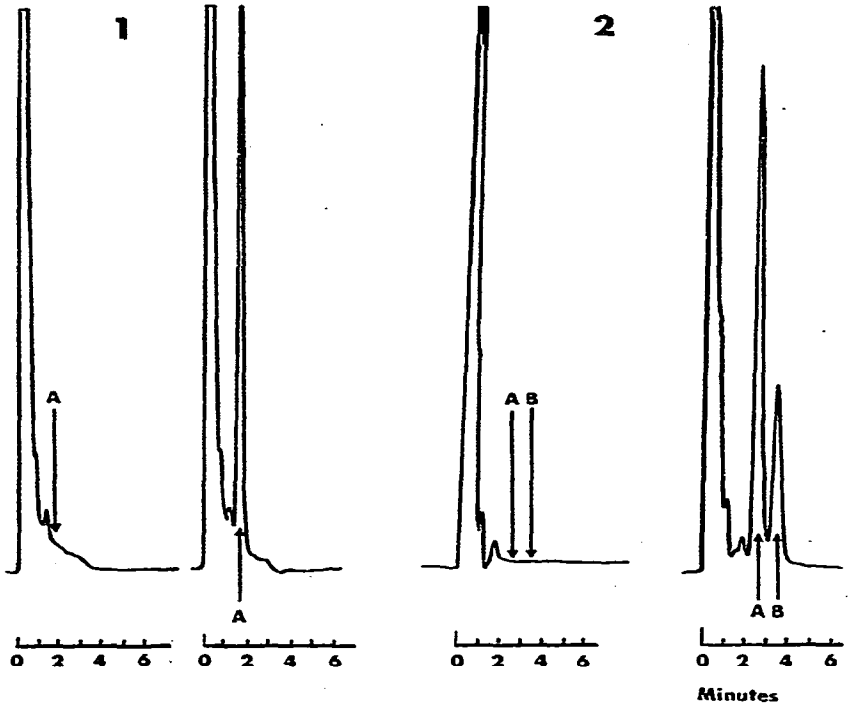

Fig. 1. Examples of chromatograms. (1) Human plasma blank (1 ml plasma), and same plasma spiked with 25 ng of isosorbide dinitrate (A); the column temperature is 147°C. (2) Human plasma blank (1 ml plasma), and same plasma spiked with 25 ng of isosorbide dinitrate (A), and 10 ng/ml internal standard (B); the column temperature is 138°C.

Speed of analysis

The analytical technique is fast: one single extraction is needed (15 min) before chromatography, which takes about 5 min.

Selectivity

The two known isosorbide mononitrate metabolites were injected under the same conditions as isosorbide dinitrate. These compounds were detected with retention times of 1 min and 1.5 min, respectively, for the 2-mononitrate and the 5-mononitrate, the retention time of ISDN being 3 min. ISDN is clearly separated from its two known mononitrate metabolites.

The procedure described here does not permit the simultaneous determination of ISDN and its mononitrate metabolites because these metabolites are poorly extracted by toluene under the conditions described above.

Storage stability of ISDN in human plasma

Table IV shows a decrease of around 15% in the ISDN content (5 ng/ml and 100 ng/ml) which was observed in plasma samples when stored frozen for 3 months at −20°C; this decrease reaches 30% after 9 months at −20°C.

TABLE IV

STORAGE STABILITY OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AFTER 9 MONTHS AT −20°C

Duration of storage at −20°C	Amount of isosorbide dinitrate added to plasma (ng/ml)	
	5.0	100
	Amount of isosorbide dinitrate found (average of two assays) (ng/ml)	
1 day	4.8	96
8 days	4.8	103
15 days	4.4	98
1 month	4.6	90
3 months	4.2	85
6 months	4.3	80
9 months	3.8	69

Application

The technique was used to study the elimination of ISDN after oral administration to one healthy subject. Fig. 2 shows the curve obtained from the

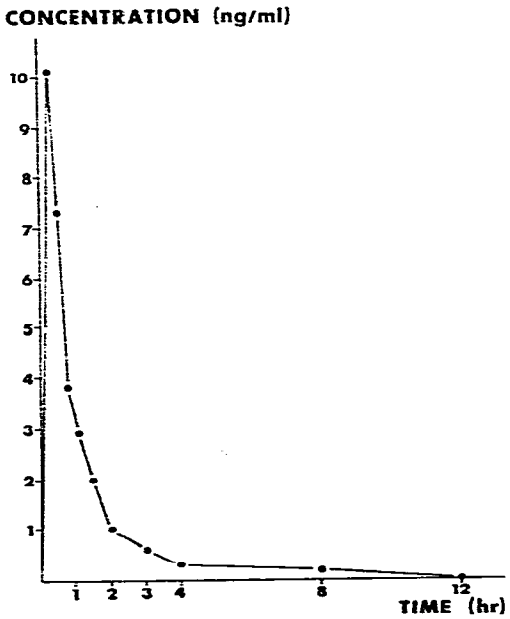

Fig. 2. Isosorbide dinitrate plasma concentrations obtained in one healthy subject after oral administration of 5 mg as one Isoket® tablet.

plasma samples of the subject given 5 mg of ISDN as one Isoket® tablet. ISDN was detected below the limit of quantitation in the 12—24 h urine only.

CONCLUSION

This paper describes a simple and sensitive GC technique for the determination of isosorbide dinitrate in human plasma and urine. This technique is faster than the existing assay procedures. The use of internal standard is not recommended because the one-step extraction is reproducible and solvent concentration is avoided.

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