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DETERMINATION OF ISOSORBIDE DINITRATE AND ITS MONONITRATE METABOLITES IN HUMAN PLASMA USING EXTRELUT® PURIFICATION AND CAPILLARY COLUMN GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, accurate and sensitive method for the determination of isosorbide dinitrate (internal standard: isomannide dinitrate) and its 2- and 5-mononitrate metabolites (internal standard: isoidide mononitrate) in 1.0 ml of human plasma has been developed. Before chromatographic quantitation by gas—liquid chromatography with electron-capture detection, isosorbide nitrates were purified by Extrelut® chromatography (recovery about 90%), eliminating most of the endogenous interferences. Routine limit of quantitation and reproducibility were 0.5, 2.0 and 10.0 ng/ml and 6, 8 and 7% for isosorbide dinitrate and the 2- and 5-mononitrates, respectively. This method allowed the behaviour of this vasodilating drug and its metabolites to be studied in humans.

INTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate widely used for its vasodilating properties in the treatment of angina pectoris and refractory congestive heart failure. ISDN is rapidly metabolized in humans to 2-isosorbide mononitrate (2-ISMN) and 5-isosorbide mononitrate (5-ISMN) which are pharmacologically active, sharing the action of the unchanged drug [1, 2]. Pharmacokinetic studies of isosorbide nitrates (ISN) therefore require the plasma determination of ISDN, 2-ISMN and 5-ISMN. Available methods for determination of ISDN [3—8] or its metabolites [9] or the three products [10—14] in plasma following administration of therapeutic doses of ISDN (ng/ml range) use gas—liquid chromatography with electron-capture detection (GLC—ECD) or high-performance liquid chromatography with detection by

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thermal energy analysis [15, 16]. The methods whose technological features have been provided are highly sensitive and accurate, although time consuming [11] and/or poorly reproducible in the lower concentration range [10, 12] and/or require a large plasma sample [10, 11, 15, 16].

For determination of ISN by GLC–ECD, the use of packed columns is hampered by the high background response interfering with the analysis of the metabolites and, in our hands, by irreproducibility of mononitrates. GLC capillary columns appear to overcome these drawbacks although data available from the literature are scarce [11, 17].

The present paper describes a GLC–ECD capillary column procedure for the determination of ISDN and its 2- and 5-mononitrate metabolites in a 1.0 ml plasma sample. The extraction procedure involves chromatography on an Extrelut® column eliminating laborious extraction and most of the background response but keeping a good extraction yield.

EXPERIMENTAL

ISDN, isomannide dinitrate (IMDN), 2-ISMN, 5-ISMN and isoidide mononitrate (IIMN) were provided by Merrell, Paris, France. All reagents used were analytical grade and water was double glass distilled. Hexane and ethyl acetate were from Carlo Erba, Milan, Italy. Extrelut was obtained from E. Merck, Darmstadt, F.R.G., and washed with hexane–ethyl acetate mixture (1:1) and then dried at 80°C for 48 h. Stock standard solutions of ISDN and IMDN (the internal standard for ISDN) were prepared at a 10 mg per 100 ml concentration in hexane. 2-ISMN, 5-ISMN and IIMN (the internal standard for mononitrates) stock standard solutions were prepared at a 10 mg per 100 ml concentration in ethyl acetate. Working standard solutions were prepared in hexane at 0.1 mg/ml concentration for ISDN, IMDN, IIMN and 2-ISMN and 1 mg/ml for 5-ISMN by dilution of the respective standard solutions. All these solutions were stored at −20°C. Under these conditions, ISN were found to be stable for several weeks. All glassware (columns and conical tubes) were washed with an ionic detergent, rinsed with double glass distilled water and silanized by a toluene solution of trimethylchlorosilane (5%).

Blood collection

Blood samples (10 ml) were drawn into Vacutainer® tubes (Becton-Dickinson A3200 XF 713) and immediately centrifuged at 4500 *g* for 10 min at 4°C. Separated plasmas were frozen and stored as 1.1 ml aliquots at −20°C until processing.

Extraction procedure

Aliquots (1.0 ml) of plasma samples were introduced into 10 ml conical extraction tubes fitted with glass caps. Internal standard solutions were added to all tubes giving a concentration of 20 ng/ml plasma for IMDN and 25 ng/ml plasma for IIMN. Then 1.0 ml of water was added. The tubes were capped and mixed for 10 sec. Diluted plasma was transferred with a silanized Pasteur pipette to the top of a column prepared as follows: 1.2 g of washed Extrelut were packed into a silanized glass column (height 30 cm, internal diameter 0.6 cm;

solvent tank 15 ml) fitted with silane glass wool and left to equilibrate for 20 min. Dinitrate compounds were eluted from the column by 10 ml of hexane (elution time was about 10 min). Thereafter, mononitrates were eluted by 9 ml of ethyl acetate—hexane mixture (70:30); elution time was about 5 min. The respective fractions were evaporated under a gentle nitrogen stream at room temperature until approximately 15 μl of dinitrate fraction and 75 μl of mononitrate fractions were obtained. The tubes were immediately frozen and stored at −20°C prior to chromatography; 1.0 μl was injected into the chromatograph.

Chromatography

Chromatography was performed on an HP 5700 gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a ^{63}Ni electron-capture detector, direct silanized glass injector (length 11 cm, internal diameter 1 mm) and an OV-17/01 wall-coated open tubular fused-silica capillary column (length 12.5 m, I.D. 0.32 mm) obtained from Girdel, Suresnes, France. Chromatographic conditions were different for dinitrate or mononitrate analysis. Temperature settings were detector 200°C, injection port 150°C (dinitrates) or 200°C (mononitrates), oven 140°C (dinitrates) or 130°C (mononitrates). Helium was used as carrier gas at a flow rate of 6 ml/min. Flow rate of make-up gas (argon—methane, 90:10) was 25 ml/min.

RESULTS AND DISCUSSION

Calibration

Calibration samples were prepared using drug-free plasma. Aliquots (1.0 ml) were spiked by addition of ISDN, 2-ISMN and 5-ISMN working standard solutions to produce concentration ranges of 1—60 ng/ml, 5—60 ng/ml and 25—250 ng/ml, respectively. IMDN (20 ng/ml) and IIMN (25 ng/ml) were added according to the routine extraction procedure. Standard calibration curves were obtained by plotting the peak height ratios (measured by a 3388 A Hewlett-Packard integrator) of ISDN and its metabolites versus internal standard against the concentration in the calibration standards. Linear regression analysis of typical calibration curves were found to be:

$y = 0.0686x + 0.035$ ($r = 0.9994$) for ISDN

$y = 0.0796x + 0.071$ ($r = 0.9980$) for 2-ISMN

$y = 0.0263x + 0.109$ ($r = 0.9998$) for 5-ISMN

These results demonstrate that the calibrations are linear over the concentration range. Day-to-day chromatographic variations may occur spontaneously, thus inducing changes in the parameters of the calibration curves. However, these changes do not influence accuracy since calibration curves were run every day.

Reproducibility and accuracy

The reproducibility and accuracy were determined for the three compounds in series consisting of five, seven or ten spiked plasma samples with respect to

a standard calibration curve (three or five points of increasing concentration and a blank). From results shown in Tables I, II and III, coefficients of variation were found not to exceed 6%, 7% and 6.5% for ISDN, 2-ISMN and 5-ISMN, respectively. However, the coefficient of variation for ISDN was 8.0% at the routine limit of detection. As shown in Tables I–III, accuracy did not exceed 10%, whatever the ISN molecule.

TABLE I

REPRODUCIBILITY AND ACCURACY OF ISOSORBIDE DINITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
0.5	5	0.62 ± 0.05	8.0
1	10	0.97 ± 0.04	4.1
2	5	2.00 ± 0.04	2.0
5	5	5.50 ± 0.09	1.6
10	10	10.22 ± 0.40	3.9
10	7	10.56 ± 0.41	3.9
20	10	20.38 ± 0.44	2.2
20	7	20.79 ± 0.67	3.2
20	5	19.70 ± 0.50	2.5
Day-to-day			
2	5	1.98 ± 0.08	4.0
4	5	4.21 ± 0.22	5.2
6	5	6.43 ± 0.22	3.4

TABLE II

REPRODUCIBILITY AND ACCURACY OF 2-ISOSORBIDE MONONITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
2	5	1.7 ± 0.1	5.2
5	10	4.8 ± 0.2	4.1
5	5	5.5 ± 0.1	1.8
10	10	10.5 ± 0.7	6.6
10	7	10.9 ± 0.5	4.6
10	5	10.4 ± 0.4	3.8
25	10	23.8 ± 1.5	6.3
40	7	38.7 ± 1.4	3.5
40	5	41.1 ± 1.1	2.6
Day-to-day			
5	5	4.9 ± 0.3	6.1
10	5	10.1 ± 0.7	6.9
20	5	19.9 ± 1.3	6.5

TABLE III

REPRODUCIBILITY AND ACCURACY OF 5-ISOSORBIDE MONONITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
10	5	9.3 ± 0.5	5.3
25	10	24.9 ± 1.4	5.6
25	5	22.8 ± 0.7	3.0
50	5	51.5 ± 1.7	3.3
100	10	101.4 ± 3.9	3.8
100	7	108.5 ± 7.1	6.5
200	10	197.7 ± 6.3	3.2
250	7	242.6 ± 3.2	1.3
250	5	246.5 ± 2.8	1.1
Day-to-day			
25	5	22.5 ± 0.9	4.0
50	5	49.1 ± 2.5	5.1
200	5	205.3 ± 5.9	2.9

Extraction yield

Extraction yield was evaluated as follows (Table IV). Internal standards were added to ten plasma samples as described above. Of these, seven were spiked with ISDN, 2-ISMN and 5-ISMN before extraction and three received the three organic nitrates just before evaporation. Extraction yield was calculated as the ratio of mean peak height ratio (PHR) of the seven plasma samples over mean PHR of the three samples. It should be noted that a similar recovery (around 90%) was obtained for each of the isosorbide nitrates.

TABLE IV

EXTRACTION YIELD OF ISDN, 2-ISMN AND 5-ISMN FROM SPIKED PLASMA SAMPLES

ISDN			2-ISMN			5-ISMN		
Spiked value (ng/ml)	Number of samples	Extraction yield (%)	Spiked value (ng/ml)	Number of samples	Extraction yield (%)	Spiked value (ng/ml)	Number of samples	Extraction yield (%)
2	7	92.0	5	7	90.4	25	7	89.9
10	7	91.7	10	7	89.8	100	7	87.8
20	7	92.6	40	7	90.5	250	7	92.6

Chromatography

Typical chromatograms of healthy subjects receiving a single oral dose of a 40 mg sustained release form of ISDN are shown in Fig. 1. Retention times of ISDN and IMDN were 5.5 and 7.6 min, respectively, and 2.6, 5.0 and 7.1 min for 2-ISMN, IIMN and 5-ISMN, respectively.

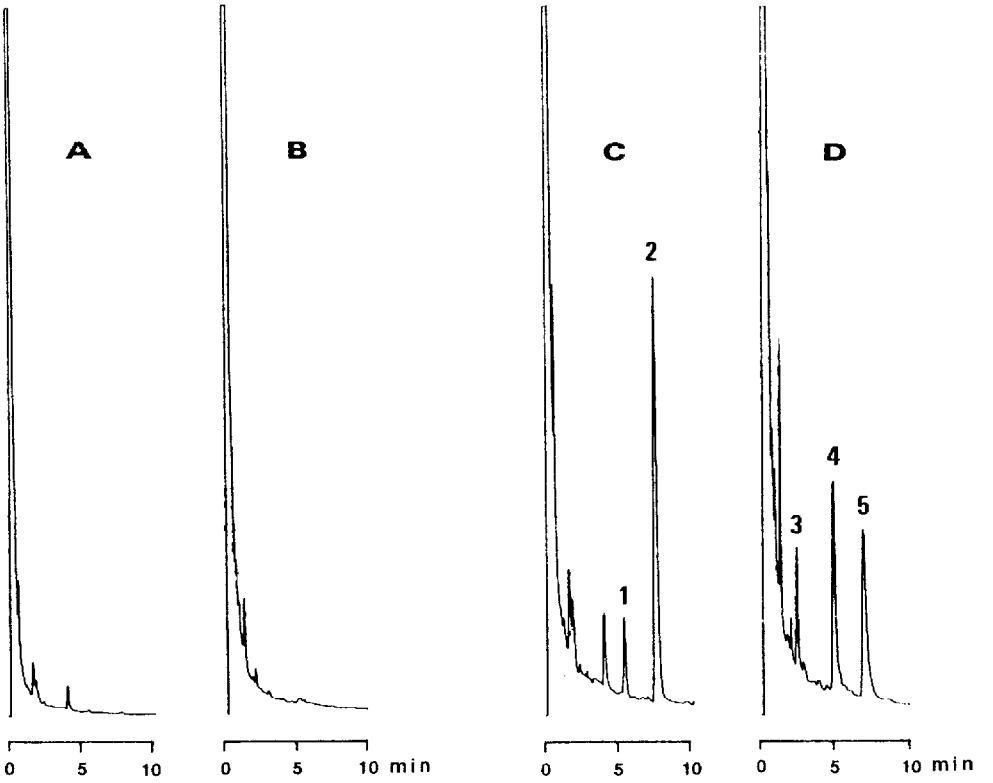

Fig. 1. Chromatograms of plasma extracts from a healthy subject (LAP. . .) before administration (A, B) and in receipt of 40 mg of ISDN (C, D). A = dinitrate fraction. B = mononitrate fraction. C = dinitrate fraction: 1 = isosorbide dinitrate (2 ng/ml); 2 = isomannide dinitrate (20 ng/ml), internal standard. D = mononitrate fraction: 3 = 2-isosorbide mononitrate (6.5 ng/ml); 4 = isoidide mononitrate (25 ng/ml), internal standard; 5 = 5-isosorbide mononitrate (30 ng/ml).

Limits of quantitation

The limit of quantitation of routine assays was 0.5, 2 and 10 ng/ml for ISDN, 2-ISMN and 5-ISMN, respectively, with reproducibility better than 10% at these concentrations. It should be emphasized that detection limits were often half of these values.

Kinetic studies in healthy human subjects

The proposed method was used for kinetic studies of ISDN and metabolites using sustained release forms of ISDN with different dosages (40, 60, 80 mg). Fig. 2 shows a concentration—time curve after administration to a healthy subject.

CONCLUSION

This paper describes a new capillary GLC—ECD method for the determination of ISDN and its two mononitrate metabolites, which represents a significant advance over previous techniques [3—17]. The Extrelut extraction

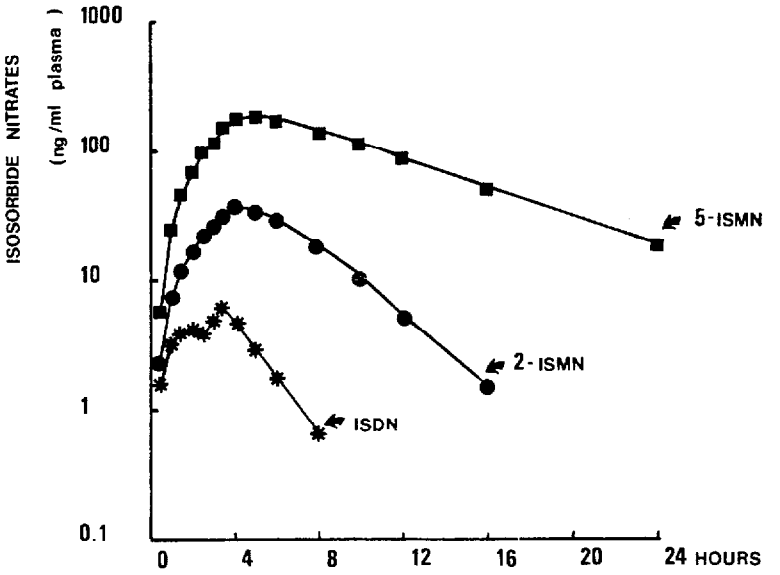

Fig. 2. Plasma ISDN, 2-ISMN and 5-ISMN concentration—time curves in a healthy subject (REV.) following a single oral dose of a 40 mg sustained release form (Langoran® 40 mg, Merrell).

procedure permits a high extraction yield (90% and over) for the three ISN molecules with only a 1.0 ml plasma sample. The specific quantitation of the mononitrate derivatives has been performed eliminating significantly the endogenous interferences. Furthermore, this method is very sensitive, specific, and reproducible, and the determination of the unmetabolized drug and its metabolites allows a study of the kinetic behaviour of this vasodilating drug. In addition, it should be noted that this technique can be used routinely and 15—20 plasma samples can be easily analyzed every day.

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