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Note

Gas—liquid chromatographic determination of isosorbide-5-mononitrate derivatized with perfluorinated anhydrides for pharmacokinetic and bioavailability investigations*

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Isosorbide-5-mononitrate (IS-5-MN) and isosorbide-2-mononitrate (IS-2-MN) are active metabolites of isosorbide-2,5-dinitrate (IS-2,5-DN). They all display vasodilating activity and are therapeutically employed in angina pectoris, myocardiocoronarosclerosis and in the prevention of myocardial infarction.

All three substances are strongly metabolized by a first-pass effect in the liver, at a decreasing rate in the order IS-2,5-DN, IS-2-MN, IS-5-MN. In spite of decreasing activity, following the same order, IS-5-MN seems to be preferable to the other two substances because its long-lasting plasma levels allow a therapeutic regime of two to three daily doses to be adopted and, in the case of sustained-release formulations, a twice- or even once-a-day administration is sufficient [1, 2].

The use of organic nitrates involves serious analytical difficulties because of their thermodynamic instability and the low detection limits required, particularly for IS-2,5-DN (< 1 ng/ml) and for IS-2-MN (2—5 ng/ml). Several approaches have been tried by many investigators in later years, in order to improve the reproducibility [3, 4], including the use of capillary columns

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[5—7] and an interesting high-performance liquid chromatographic (HPLC) method employing thermal energy analysis (TEA) as detector which was recently published by Maddock et al. [8].

This paper reports a new analytical method for the evaluation of IS-5-MN in plasma after administration of the substance, with an improved reproducibility due to a derivatization which stabilizes the molecule. The method can be easily applied to pharmacokinetic investigations in humans.

EXPERIMENTAL

Drugs, chemicals and instruments

Solvents and reactants, all of analytical-reagent grade, were supplied by Merck (Darmstadt, F.R.G.). The supports and stationary phases for gas—liquid chromatography (GLC) columns were supplied by Supelchem (Milan, Italy). A Varian 3700 gas chromatograph was employed for the analysis. The statistical evaluation was performed on a Hewlett-Packard HP-86 personal computer.

Sample preparation

A 5-ml aliquot of a mixture consisting of diethyl ether–heptane (8:2) was added to 0.5 ml of plasma in a glass-stoppered test tube. The mixture was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 5 min. A 4-ml aliquot of the upper phase was collected and evaporated to dryness under a gentle nitrogen stream at room temperature. Then 200 μl of a solution containing 5% trifluoroacetic anhydride in diethyl ether—heptane (8:2) were added as a derivatizing agent and left at room temperature for 30 min. The mixture was evaporated to dryness under a nitrogen stream and the residue was redissolved in 0.5 ml of the above mixture of diethyl ether–heptane containing the internal standard (I.S.). A series of substances were employed usefully as internal standards: 2,4-dinitrochlorobenzene, fluorenone, IS-2,5-DN, IS-5-MN pentafluoropropionyl derivative and IS-5-MN heptafluorobutyryl derivative.

GLC conditions

A glass column (1 m × 6 mm O.D., 2 mm I.D.) filled with 30% OV-101 on 80—100 mesh Chromosorb W AW DMCS was used. The temperatures of the injection port, oven and electron-capture detector were maintained at 180, 165 and 200°C, respectively. A mixture of argon—methane (9:1) was used as the carrier gas.

RESULTS AND DISCUSSION

Plasma interference

The retention times observed were 3 min 18 s for trifluoroacetyl-IS-5-MN, 4 min 12 s for heptafluorobutyryl-IS-5-MN, 5 min 27 s for IS-2,5-DN, 4 min 37 s for 2,4-dinitrochlorobenzene and 11 min 36 s for fluorenone.

Fig. 1 gives chromatograms of a blank sample of human plasma, showing no interference with the analytical peaks, the derivatized IS-5-MN or the

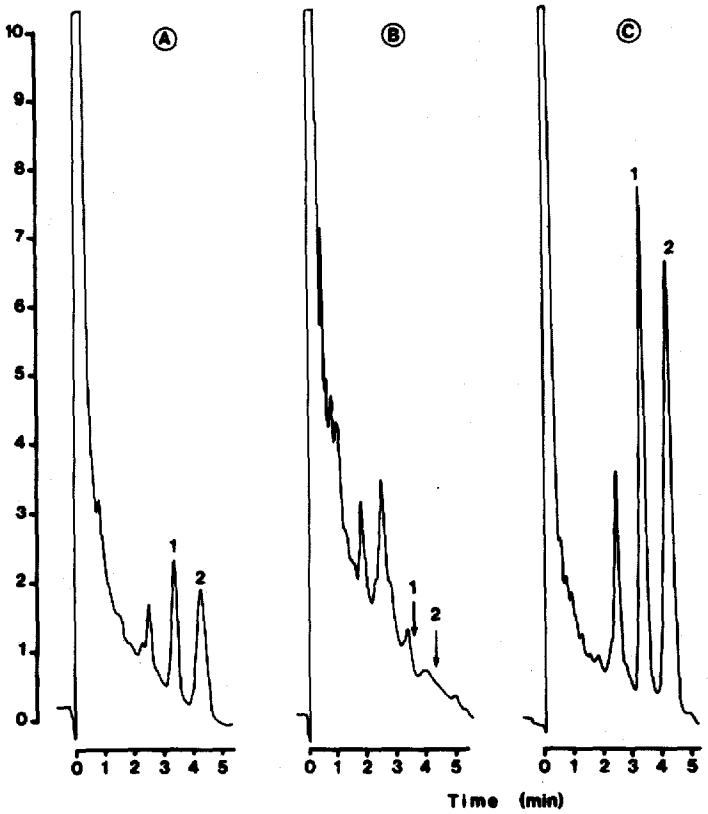

Fig. 1. Chromatograms of trifluoroacetyl-IS-5-MN (1) and the internal standard, heptafluorobutyryl-IS-5-MN (2) as standards (A), of a blank sample of human plasma (B) and of a plasma sample with a concentration of IS-5-MN of about 100 ng/ml (C).

internal standard (IS-5-MN heptafluorobutyryl derivative), and an analysis of a plasma sample from a preliminary pharmacokinetic study with a concentration of IS-5-MN of ca. 100 ng/ml, which underwent all the analytical procedures described.

Determination of the detector response factor

Table I shows the linearity of the method, which was investigated in a range between 10 pg and 2 ng per single injection. Linearity was confirmed by a constant detector response factor (d.r.f.) evaluated as follows:

$$\text{d.r.f.} = \frac{\text{weight IS-5-MN}}{\text{weight I.S.}} \times \frac{\text{peak area I.S.}}{\text{peak area IS-5-MN}}$$

This factor allows the different responses of the detector to the analytical substance and the internal standard to be counter-balanced.

Table II shows the d.r.f. with an IS-5-MN/I.S. ratio ranging from 1:4 to 4:1. The linearity was also confirmed.

Recovery of IS-5-MN from plasma

Table III shows the recovery of IS-5-MN from plasma, which was satisfactory

TABLE I

LINEARITY OF THE DETECTOR RESPONSE TO INCREASING AMOUNTS OF IS-5-MN DERIVATIVE INJECTED AT A CONSTANT RATIO BETWEEN THE IS-5-MN DERIVATIZATION PRODUCT AND THE INTERNAL STANDARD (HEPTAFLUOROBUTYRYL-IS-5-MN) (1:1)

Amount IS-5-MN injected (pg)	Mean d.r.f.* (n = 4)	S.D.
10	1.33	$2.94 \cdot 10^{-2}$
20	1.35	$6.99 \cdot 10^{-2}$
50	1.34	$4.22 \cdot 10^{-2}$
100	1.34	$5.74 \cdot 10^{-2}$
200	1.34	$4.04 \cdot 10^{-2}$
500	1.34	$4.27 \cdot 10^{-2}$
1000	1.33	$3.40 \cdot 10^{-2}$
2000	1.34	$5.07 \cdot 10^{-2}$

*Mean value = 1.34; S.D. = $0.64 \cdot 10^{-2}$; percentage S.D. = 0.48.

TABLE II

LINEARITY OF THE DETECTOR RESPONSE TO IS-5-MN AND I.S. AS PERFLUORINATED DERIVATIVES AT VARIABLE WEIGHT RATIOS

IS-5-MN:I.S. weight ratio	Mean d.r.f.* (n = 4)	S.D.
4:1	1.35	$3.30 \cdot 10^{-2}$
4:2	1.35	$3.16 \cdot 10^{-2}$
4:4	1.34	$3.50 \cdot 10^{-2}$
2:4	1.33	$2.89 \cdot 10^{-2}$
1:4	1.34	$3.16 \cdot 10^{-2}$

*Mean value = 1.34; S.D. = $0.84 \cdot 10^{-2}$; percentage S.D. = 0.63.

TABLE III

RECOVERY OF IS-5-MN FROM PLASMA WITH THE METHOD EMPLOYING DERIVATIZATION

The linear relationship between IS-5-MN added (x) and that found (y) is expressed by: $y = 0.825 + 0.713\,x$; $r^2 = 0.9985$.

Concentration added (ng/ml)	Mean concentration found (n = 4) (ng/ml)	S.D.	Intra-assay variation* (% S.D.)	Recovery** (%)
5	3.6	0.30	11.7	72.0
10	6.9	0.81	8.3	69.0
20	14.1	1.30	9.2	70.5
50	35.9	2.03	5.6	71.8
100	68.6	3.82	5.6	68.6
200	137.2	6.13	4.5	68.6
500	380.0	23.51	6.2	76.0
1000	703.8	36.62	5.2	70.4

*Mean value = 7.0.
**Mean value = 70.9; S.D. = 2.47; percentage S.D. = 3.48.

in the whole range explored (5—1000 ng/ml), with an r^2 value of 0.9985 obtained by linear regression calculations.

Selectivity, reproducibility and limit of quantitation

Reproducibility expressed as S.D. percentage (coefficient of variation) ranged between 2 and 5% if the standards were injected without undergoing extraction from plasma, whereas it was 7% on average when the complete analytical procedure was applied.

The limit of quantitation of this method was about 5 ng/ml for IS-5-MN. Further attempts to improve sensitivity did not succeed using this column, whereas from research now in progress, it seems that the limit of quantitation could be considerably increased by using a capillary column [5—6]. In any case, it should be considered that a concentration of 5 ng/ml IS-5-MN is an acceptable limit, lower plasma concentrations of the drug being commonly regarded as inactive.

The use of the IS-5-MN heptafluorobutyryl derivative as I.S. was preferred by us as it reduces the time for the analysis and it proved not to interfere with endogenous peaks. This choice was facilitated in that the extraction procedure of IS-5-MN was particularly simple and allowed the I.S. to be added after extraction and derivatization procedures. The use of IS-2,5-DN as I.S. involved its addition after the derivatization procedures, as IS-2,5-DN undergoes trans-esterification processes; fluorenone and 2,4-dinitrochlorobenzene can be added both before or after extraction procedures, but in the first case recovery must be carefully evaluated.

The method is not applicable to simultaneous evaluation of IS-2,5-DN, IS-5-MN and IS-2-MN, in that IS-2,5-DN interferes with derivatization.

The following considerations of this method are to be stressed: linearity proved to be very good and day-to-day variations were practically negligible over at least a thousand injections. From a practical point of view, the method employing the IS-5-MN heptafluorobutyryl derivative as internal standard allows a mean rate of ten injections per hour to be attained and a skilful operator can easily perform 30 analyses a day with manual area evaluation and about 50 analyses a day with an automatic integrator. The method suggests a series of possible internal standards, all of them easily available.

In conclusion, our method is suitable for pharmacokinetic and bioavailability investigations, it guarantees good linearity and is fairly inexpensive.

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