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DETERMINATION OF ISOSORBIDE DINITRATE AND ITS MONONITRATE METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—THERMAL ENERGY ANALYSIS

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SUMMARY

An accurate and sensitive method for the simultaneous determination of isosorbide dinitrate and its 2- and 5-mononitrates in human plasma has been developed. Following extraction of 3.0 ml of plasma with 12.0 ml of dichloromethane—ethyl acetate (1:1) the extract is subjected to high-performance liquid chromatography employing a Zorbax NH_2 column. The eluent stream is introduced into a thermal energy analyser, employing chemiluminescence as a specific means of detection. The minimum quantifiable level of the compound in plasma is 200 pg/ml allowing the quantitation of isosorbide dinitrate in human plasma following single oral administration. Nitroglycerin is employed as internal standard.

INTRODUCTION

Isosorbide dinitrate (ISDN) is widely used for the prophylactic treatment of angina pectoris and for the treatment of refractory congestive heart failure. Following the single oral administration of therapeutic doses of ISDN, plasma levels in the sub-nanogram to low-nanogram per ml range are encountered for the parent compound and a number of gas—liquid chromatographic (GLC) procedures [1—3] have been developed which are sensitive to these levels, using electron-capture detection. Although these methods are suitable for the parent compound, high background responses interfere with the analysis of the metabolites despite the use of elaborate clean-up procedures. A capillary column GLC—electron-capture procedure has been reported [4] which does not suffer this interference, but in our hands the sensitivity for ISDN was

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inadequate and the claimed detection limits of 0.5 ng/ml can rarely be attained in routine use.

High-performance liquid chromatography (HPLC) with thermal energy analysis (TEA) detection has been employed [5] to determine another polynitric ester, nitroglycerin in dog plasma using normal-phase silica columns and gradient elution.

The thermal energy analyser uses the chemiluminescence of nitrogen dioxide radicals [6] produced by pyrolysis of the organic nitrates as the basis of detection.

A preliminary method for the determination of ISDN and its metabolites using HPLC–TEA with gradient elution has been reported [7]. This method employs Preptube extraction cartridges (Thermo Electron Corp., Waltham, MA, U.S.A.) for sample preparation which is time-consuming and costly and gave variable recoveries in our hands.

This present paper describes an HPLC–TEA procedure for ISDN and its 2- and 5-mononitrates which is highly sensitive and reproducible without the necessity for elaborate clean-up procedures and redistillation of reagents. This method, using a polar bonded phase and isocratic elution is thus eminently suitable for routine use.

The developed procedure has been used for the analysis of plasma ISDN and mononitrate concentrations in pharmacokinetic studies with adult healthy subjects of ISDN sublingual tablets, sustained-release tablets and capsule formulations and creams.

EXPERIMENTAL

Reagents and materials

All reagents used were of analytical grade and aqueous based reagents were prepared using double glass distilled water. *n*-Hexane and methanol were of far-UV S grade (Rathburn Chemicals, Walkerburn, Great Britain) and the ethyl acetate used was Nanograde (Mallinckrodt, St. Louis, MO, U.S.A.). All glassware was treated with Surfasil [Pierce and Warriner (UK), Chester, Great Britain] and allowed to dry prior to use. Standard solutions of ISDN were prepared freshly each day at a concentration of 10 mg per 100 ml in *n*-hexane, whereas the isosorbide 2-mononitrate (I-2-MN) and isosorbide 5-mononitrate (I–5-MN) were prepared in similar concentrations in dichloromethane–ethyl acetate (1:1). The internal standard, nitroglycerin, was prepared at a concentration of 10 mg per 100 ml in *n*-hexane. Working standard solutions were then prepared at a concentration of 0.1 mg per 100 ml for each component, diluting in the appropriate solvent used for the stock standard solutions.

Pure, authentic samples of ISDN, I-2-MN, I-5-MN and nitroglycerin were kindly donated by Professor A.H. Beckett, Department of Pharmacy, Chelsea College, London, Great Britain.

Collection of blood samples

Blood samples (10 ml) were taken into glass collection tubes (pretreated with Surfasil) containing 500 units of lithium heparin and 25 μl of 0.002 *M* silver nitrate solution. Following centrifugation the plasma was withdrawn and

stored in glass tubes at −20°C.

Extraction procedure

Aliquots (3.0 ml) of plasma samples were pipetted into 15.0-ml glass extraction tubes fitted with screw caps provided with PTFE liners. Nitroglycerin internal standard solution (30.0 μl, 30 ng) was added to all tubes giving an internal standard concentration of 10 ng/ml plasma. Then 12.0 ml of dichloromethane—ethyl acetate (1:1) were added, the tubes capped tightly and shaken mechanically for 5 min at 250 cycles/min. Following centrifugation at 550 *g* for 5 min at 4°C the organic phases were transferred to 20-ml glass vials using pasteur pipettes. The organic phases were evaporated to approximately 20-μl volumes under a gentle stream of nitrogen at room temperature. The vials were stored frozen at −20°C until just prior to chromatography.

Chromatography

Chromatography was performed on a 25 cm × 0.04 cm I.D. stainless-steel column, prepacked with Zorbax NH_2 polar bonded phase material (10 μm particle size) [DuPont Instruments (UK), Hitchin, Great Britain]. The mobile phase consisted of 5.0% methanol in *n*-hexane maintained at a flow-rate of 5.0 ml/min using an LDC Constametric IIC pump (Milton Roy Corporation, FL, U.S.A.). The eluate stream was directly coupled to a thermal energy analyser, Model 502A (Thermo Electron Corp.) with a furnace temperature of 575°C, argon flow-rate of 15 ml/min, oxygen flow-rate of 25 ml/min, with a slush bath of methanol and solid carbon dioxide pellets maintained at −77°C.

The sample extracts were introduced into the chromatograph using a Rheodyne 100-μl loop injector valve (Rheodyne, CA, U.S.A.).

RESULTS AND DISCUSSION

Calibration

Calibration samples were prepared using pooled control (drug-free) plasma. Aliquots (3.0 ml) were spiked by the addition of ISDN, I-2-MN, and I-5-MN working standard solutions to produce concentration ranges of 0.5—20.0 ng/ml, 1.0—40.0 ng/ml and 5.0—80.0 ng/ml, respectively.

Standard calibration lines were constructed from plotting the peak height ratios of the compound and its metabolites versus internal standard against the concentrations in the calibration standards. Linear regression analysis showed that the calibrations were linear over the concentration ranges: ISDN: $Y = 0.093X + 0.017$; I-2-MN: $Y = 0.0576X + 0.028$; and I-5-MN: $Y = 0.3523X + 0.0674$.

Table I shows typical values of the peak height ratios obtained on different occasions over a period of several weeks indicating good precision for each compound.

Further, calibrations over the standard range assayed in triplicate on the same day and on two further days consecutively, gave coefficients of variation (C.V.) for the slopes after linear regression analysis which again indicates good reproducibility (Table II).

The correlation coefficients of the linear regression analysis were found to be

TABLE I

PRECISION OF REPLICATE CALIBRATIONS OF THE HPLC—TEA ASSAY FOR ISDN AND ITS MONONITRATE METABOLITES IN PLASMA

Each value is the mean of five replicates.

Isosorbide dinitrate			Isosorbide-2-mononitrate			Isosorbide-5-mononitrate		
Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)	Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)	Value (ng/ml)	Mean peak height ratio (± S.D.)	Coefficient of variation (%)
0.5	0.03 (0.0049)	16.35	1.0	0.07 (0.015)	21.6	2.5	0.11 (0.015)	13.6
1.0	0.08 (0.009)	11.8	2.5	0.16 (0.197)	12.3	5.0	0.22 (0.02)	9.09
2.5	0.20 (0.019)	9.7	5.0	0.37 (0.035)	9.6	10.0	0.44 (0.08)	18.2
5.0	0.37 (0.026)	7.04	10.0	0.60 (0.044)	7.4	15.0	0.59 (0.07)	12.2
10.0	0.68 (0.036)	5.29	20.0	1.15 (0.08)	7.7	20.0	0.88 (0.18)	11.5
20.0	1.41 (0.075)	5.35	30.0	1.84 (0.11)	6.2	40.0	1.57 (0.2)	9.4
40.0	2.75 (0.132)	4.8	40.0	2.54 (0.13)	5.3	60.0	2.12 (0.16)	5.5

TABLE II

REPRODUCIBILITY OF CALIBRATION CURVES

Compound	C.V. (%)			
	Day 1	Day 2	Day 3	Mean
ISDN	2.93	0.17	1.33	1.48
I-2-MN	1.84	3.26	2.08	2.39
I-5-MN	13.86	0	4.02	5.96

0.999, 0.9986 and 0.9985 for ISDN, I-2-MN and I-5-MN, respectively.

Reproducibility

The reproducibility of the analysis for each compound was determined by assaying control spiked plasma samples with each batch, the data for which are found in Table III. This shows excellent reproducibility with coefficients of variation of 9.97, 13.45 and 9.9% overall for ISDN, I-2-MN and I-5-MN, respectively.

Recovery

Recovery of the compound and its metabolites was calculated over the

TABLE III

RECOVERY OF ISDN AND ITS MONONITRATE METABOLITES FROM SPIKED PLASMA CONTROL SAMPLES ASSAYED SINGLY OVER A PERIOD OF FOURTEEN DAYS

ISDN (ng/ml)		I-2-MN (ng/ml)		I-5-MN (ng/ml)	
Spiked value	Assayed value	Spiked value	Assayed value	Spiked value	Assayed value
2.0	2.2	10.0	11.0	10.0	11.2
6.0	5.7	20.0	18.5	30.0	30.5
2.7	3.5	12.0	13.4	10.0	9.0
20.0	19.8	2.5	2.5	47.0	43.0
2.5	2.7	13.3	11.8	60.0	58.0
10.0	8.9	60.0	62.5	10.0	9.5
20.0	19.4	6.7	5.5	40.0	39.0
13.3	14.0	13.3	14.5	6.0	8.0
6.7	6.5	33.0	32.0	10.0	9.0
20.0	20.5	6.0	6.5	60.0	58.0
3.3	3.2	3.0	4.5	15.0	15.0
3.0	3.0	10.0	9.0	30.0	32.0
7.0	7.5	60.0	62.0	70.0	68.1
2.5	2.2	2.5	2.5	10.0	10.0
1.7	1.5	10.0	12.0	20.0	22.0
5.0	5.0	25.0	28.5	40.0	37.5
30.0	32.0	60.0	63.0	10.0	9.5
2.5	2.8	3.3	3.0	20.0	19.0
5.0	4.6	10.0	10.5	50.0	53.0
7.0	6.5	13.3	12.5	12.0	13.0
20.0	19.0	20.0	20.0	66.0	63.5

calibration range by comparing the peak height ratios for non-extracted standards to those taken through the complete analytical procedure, including extraction. After correcting for the recovery of the internal standard (64.5%) the mean overall recoveries for the drug and its 2- and 5-mononitrate metabolites were found to be 98.2, 78.3 and 78.0%, respectively, comparing very favourably with other published data.

Chromatography

Typical chromatograms of a blank plasma sample and of a plasma sample from a subject in receipt of a single oral dose of 40 mg sustained release ISDN formulation are shown in Fig. 1. Under the conditions described in the Experimental section, the retention times of ISDN, nitroglycerin internal standard, I-2-MN and I-5-MN are 3.3 min, 5.0 min, 5.8 min and 8.4 min, respectively.

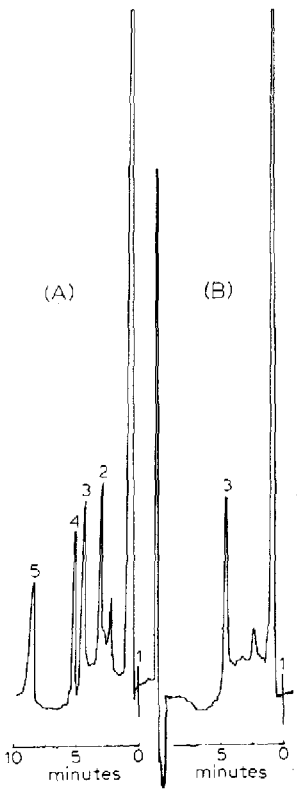

Fig. 1. (A) Chromatogram of human plasma from a subject in receipt of 40 mg ISDN. Peaks: 1 = injection point; 2 = isosorbide dinitrate; 3 = nitroglycerin internal standard; 4 = isosorbide-2-mononitrate and 5 = isosorbide-5-mononitrate. (B) Chromatogram of a control (drug-free) plasma extract.

Detection limits

The observed detection limits, based on the minimum peak to give a signal-to-noise ratio of 2:1 over baseline was 0.25 ng/ml for ISDN, 0.5 ng/ml for I-2-MN and 1.0 ng/ml for I-5-MN. The detection limits did vary from day to day depending on a number of factors: fluctuation in slush bath temperature,

fluctuation in detector vacuum, gradual contamination of the detector filter and the photomultiplier cooling temperature.

Fluctuations in slush bath temperature and photomultiplier temperature can easily be regulated since these parameters are continuously monitored. However, changes in detector vacuum and contamination of the detector filter can cause gradual loss of sensitivity. In order to control these variables a standard solution is injected daily to monitor sensitivity and when significant losses are found the detector is taken down and cleaned.

The values quoted above represent the best possible limits when conditions were optimal and more typically the detection limits on a routine basis were 0.5 ng/ml for ISDN, 0.8 ng/ml for I-2-MN and 1.2 ng/ml for I-5-MN. This is confirmed by calculating the minimum quantitation levels (MQL) by computer program based on the 80% confidence limits of a value being differentiated from zero. From the data presented in Table I, the MQL values for ISDN, I-2-MN and I-5-MN were found to be 0.56 ng/ml, 0.86 ng/ml and 1.66 ng/ml, respectively.

Human studies

The HPLC—TEA procedure has been employed in the study of ISDN pharmacokinetics with a number of different dosage forms. Fig. 2 illustrates two dose—response curves for ISDN in plasma, (A) mean plasma concentrations from twelve adult healthy male subjects in receipt of a 40-mg sustained release ISDN tablet showing mean peak concentrations of plasma ISDN at 6 h at 4.5 ng/ml with mean concentrations of 1.15 ng/ml persisting at 12 h post dosing, and (B) for comparison the mean plasma concentrations from the same twelve subjects following a 5-mg sublingual tablet administration with a peak ISDN concentration of 18.2 ng/ml 20 min following dosing.

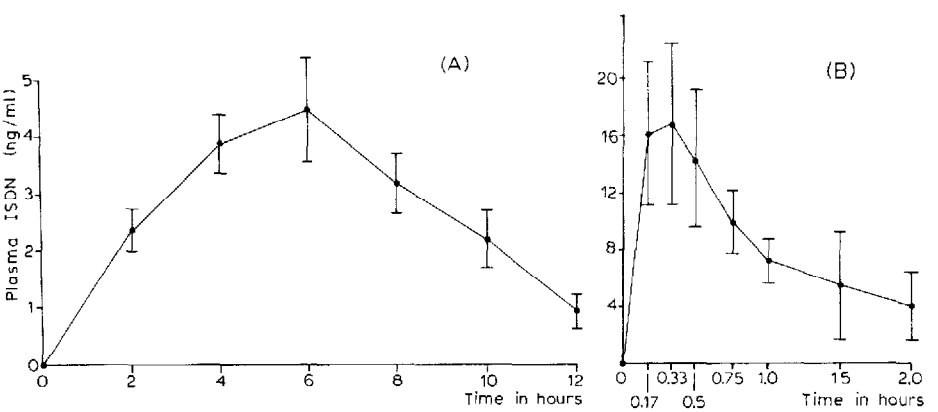

Fig. 2. (A) Mean plasma ISDN concentrations in twelve subjects following a single oral dose of a 40-mg ISDN sustained release tablet. (B) Mean plasma ISDN concentrations in the same twelve subjects following a single dose of a 5-mg sublingual ISDN tablet.

CONCLUSION

An accurate, sensitive and rapid procedure for determining ISDN and its mononitrate metabolites was developed using HPLC with TEA detection.

The assay is eminently suitable for the analysis of large numbers of samples, each chromatography run taking only 10 min and the procedure involves a simple extraction step with the minimum of reagent preparation, e.g. redistillation of solvents, etc. In our hands, the HPLC—TEA system is used manually. However, the system could be fully automated if some method could be devised to flush the cold trap of mobile phase periodically. In addition to ISDN the method allows for the sensitive analysis of its mononitrate metabolites during the same chromatographic run, and in this respect this reported procedure is superior to published methods employing GLC with electron-capture detection.

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