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DETERMINATION OF THE PARTITIONING, STABILITY, AND METABOLITE FORMATION OF ISOSORBIDE DINITRATE IN HUMAN AND RAT BLOOD USING AN IMPROVED GAS—LIQUID CHROMATOGRAPHIC ASSAY

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SUMMARY

A simple and highly sensitive gas—liquid chromatographic method using electron-capture detection has been developed for the simultaneous determination of isosorbide dinitrate (ISDN) and its mononitrate metabolites in rat and human plasma. This method has a limit of quantitation of about 5 ng/ml for the mononitrates and of 1 ng/ml for ISDN using 0.1 ml of plasma, and is thus useful for pharmacokinetic studies of these compounds in small animals, and in humans when the available volume of blood is limited. Using this method, we found the apparent in vitro partitioning ratio of ISDN between erythrocyte and plasma in rat and human blood at 37°C to be 0.22 and 0.13, respectively. In spite of this poor affinity for red blood cells, ISDN degradation in whole blood was mediated primarily via this blood fraction. Loss of ISDN in blood appeared to proceed exclusively through its mononitrate metabolites, resulting in a 6:1 product ratio of the 5-mononitrate to its 2-isomer. These data suggest that although blood degradation of ISDN and erythrocyte partitioning occur per se, these phenomena do not contribute significantly to the very rapid in vivo clearance of ISDN observed in man and in the rat.

INTRODUCTION

Isosorbide dinitrate (ISDN) has been shown to be an effective drug for the management of angina pectoris [1,2], congestive heart failure [3], and acute pulmonary edema [4]. Recently, reliable gas chromatographic techniques for the determination of this drug in plasma have become available and have been used in clinical pharmacology studies of ISDN [5,6]. The denitrated, primary metabolites of ISDN, isosorbide-2-mononitrate (2-ISMN) and isosorbide-5-mononitrate (5-ISMN), have been shown to be pharmacologically active vasodilators in dogs [7,8] and in man [9].

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Assay procedures for ISDN only [5,10—12], mononitrates only [13], or for all three compounds [14—16] in plasma have been described. The simultaneous methods [14—16] are superior when throughput is considered since sample splitting for separate determinations is not required. However, these procedures employed relatively large plasma volumes (2—4 ml) to attain the requisite sensitivity. In pharmacokinetic studies with small laboratory animals, such a sample size is not feasible. In studies with selected patient populations, the availability of blood may also be limited. Thus, it appears desirable to develop a more sensitive procedure for the simultaneous determination of ISDN and its mononitrate metabolites.

Several reports have indicated that organic nitrates, particularly nitroglycerin, are highly unstable in whole blood and in plasma [17—22]. Johson et al. [17] examined the in vitro disappearance rate of ISDN in rat whole blood at 37°C and reported a degradation half-life ($t_{½}$) of 15—20 min. Their study, however, used a relatively high initial ISDN concentration (ca. 2.5 μg/ml) and therefore these results may not apply to the lower drug concentrations typically observed in vivo. ISDN was found to be considerably more stable in human plasma, $t_{½}$ = 55 h at 37°C [5], but its stability and partitioning characteristics in human whole blood has not been investigated. Knowledge of these parameters is necessary for the proper planning and interpretation of ISDN pharmacokinetic studies. In addition, the contribution of in vivo blood metabolism to the total body clearance might also be estimated from this information. This estimation may be important in understanding the reason(s) for the very high in vivo total body clearance observed for ISDN [23].

EXPERIMENTAL

Standard solutions

ISDN was extracted with diethyl ether from a 20% (w/w) mannitol adsorbate supplied by Stuart Pharmaceuticals (Wilmington, DE, U.S.A.), recrystallized with an aqueous ethanol solution, and checked for purity with the United States Pharmacopoeia (USP) [24] method modified to effect separation of metabolites using a celite column with isooctane elution. The isomeric mononitrates, obtained from Ayerst Pharmaceuticals (Rouses Point, NY, U.S.A.), as well as heptachlorepoxide (HCE) and dichlorodiphenyltrichloroethane (DDT), both from Supelco (Bellefonte, PA, U.S.A.), were received as pure solids (> 99%) and were used without further purification.

Cleaning and silanization procedure

All glassware used was acid-washed and consecutively rinsed with distilled water and pesticide-grade methanol and then dried. A 10% (v/v) dimethyldichlorosilane solution in toluene was used to silanize tubes. Tubes were immediately rinsed with toluene to remove excess reagent, and then successively rinsed with toluene—methanol (1:1) and pesticide-grade methanol before being dried.

Effect of gas—liquid chromatographic injection solvent

Four solutions were prepared in which the isoamyl alcohol concentration in

hexane varied from 5 to 62.5% (v/v). In these co-solvents, as well as neat hexane, the concentrations of the three organic nitrates were held constant in ratios similar to those found for in vivo plasma samples (ISDN = 201, 2-ISMN = 107, 5-ISMN = 674 pg/μl). The effect of injection co-solvent composition on the apparent electron-capture detector response was assessed after triplicate 1-μl injections.

Gas—liquid chromatographic assay

Organic nitrates in 0.1 ml of plasma were first stabilized with 0.01 ml of 1 *M* silver nitrate [18] and extracted once with 3 ml of pesticide-grade chloroform containing an appropriate amount of HCE or DDT as internal standard. This tube was then sealed with a PTFE stopper and shaken at slow speed for 30 min. The upper, aqueous phase was aspirated, and a suitable aliquot (0.05—2.0 ml) of the chloroform was transferred to a 3-ml centrifuge tube for evaporation to dryness under pre-purified nitrogen in an ice bath (4°C). As soon as the tube was dry, care was taken to stop the nitrogen flow quickly. The compounds were reconstituted with an appropriate volume of 25% (v/v) isoamyl alcohol in hexane, depending on the anticipated concentration calculated from reported pharmacokinetic parameters [9,23]. These solutions were then vortexed, covered with parafilm, and stored in crushed ice prior to injection of 0.5—3.0 μl on the chromatograph. The glass column used (1.85 m × 4 mm) was custom-made with an extension to the septum so as to facilitate on-column injection. The column was packed with 3% SP-2401 and 100—120 mesh Chromosorb 750 purchased from Supelco. On a daily basis, the column was primed and stabilized for response via multiple injections of the highest and lowest standards. The nitrogen carrier gas flow-rate was approximately 80 ml/min with operating temperatures of 200°C, 160°C and 200°C for the inlet, column, and detector, respectively. The gas—liquid chromatographic (GLC) instruments (Packard Models 7500 and 428, Downers Grove, IL, U.S.A.), equipped with a ^{63}Ni electron-capture detector, maintained a linear response for these compounds over the range of 25—1500 pg injected on-column. The GLC—electron-capture detector (ECD) response for the internal standards was linear over the range of 5—500 pg injected on-column. Calibration curves were constructed daily using standards prepared in 25% (v/v) isoamyl alcohol in hexane containing either HCE or DDT as internal standard.

Recovery from spiked plasma

For recovery from rat plasma, six male Sprague-Dawley rats supplied by Blue Spruce Farms (Altamont, NY, U.S.A.) were fasted overnight and blood was collected via cardiac puncture into heparinized tubes. The separated plasma was then pooled and spiked using aqueous stock solutions of organic nitrates. For recovery from human plasma, blood was withdrawn from a cubital vein, heparinized, and the separated plasma was similarly spiked. Recovery was evaluated over wide ranges of plasma concentrations for rat plasma in order to encompass expected levels in future pharmacokinetic studies. Recovery studies from human plasma covered smaller ranges of the three nitrates but these were sufficient to include concentrations typically seen after therapeutic doses.

Stability studies

Stability of ISDN was studied in heparinized (50 U/ml) rat and human whole blood, and rat plasma incubated at 37°C. Rat fluids were obtained from three rats via cardiac puncture and pooled. Human whole blood was collected from a peripheral arm vein from three healthy volunteers. Incubation trials were conducted in triplicate. Each incubation (ca. 8 ml) was covered and gently stirred to prevent sedimentation of erythrocytes. Serial aliquots (0.25 ml) were collected at 0, 2, 5, 10, 30, 60, 90, 120, 180 and 240 min for whole blood experiments, and plasma (0.1 ml) was separated for analysis. For the study of ISDN stability in rat plasma, an additional sample at 300 min was obtained.

The procedure developed for the assay of plasma concentrations of these organic nitrates was not suitable for determining the concentrations of these compounds in whole blood, since both specificity and precision were unacceptably impaired. Thus, the in vitro whole blood stability of ISDN was determined by monitoring the concentrations of organic nitrates in plasma which was separated from whole blood after serial sampling. This being the case, the disappearance of ISDN from plasma obtained from spiked whole blood samples might be contributed, at least initially, by distribution of drug into the erythrocytes, as well as by degradation. This distribution phase might be avoided if ISDN stability was studied with blood in which erythrocyte-to-plasma equilibrium has been achieved. Thus, ISDN stability in rat whole blood was carried out with spiked samples and with samples obtained from animals which have been infused in vivo to apparent steady state with ISDN.

The spiked blood was prepared by adding 0.5 ml of a normal saline solution of ISDN (5.1 μg/ml) to 25 ml of whole blood. Plasma incubation samples were prepared similarly. Rat whole blood was also prepared to contain ISDN by using a constant-rate in vivo infusion (7 μg/min) into a right jugular vein catheter implanted [25] in the animal. A 2-h infusion duration was sufficient to attain apparent steady state [26] and resulted in plasma concentrations similar to those obtained from in vitro spiking (about 200 ng/ml).

RESULTS AND DISCUSSION

Standardization

Because of its explosive nature, ISDN cannot be handled in its pure form. Primary standards of ISDN have to be prepared via extraction from a mannitol adsorbate mixture. Quantitation of primary standards of ISDN, on which all subsequent determinations are based, is therefore extremely important. When this work was initiated, the USP XIX method [24] quantified ISDN via hydrolytic liberation of inorganic nitrate and nitrate ions, and subsequent reaction with phenoldisulfonic acid to form a stable chromophore. There was no provision for a celite column purification step to remove the possible degradation or contaminant products; viz., the mononitrates, inorganic nitrate and/or nitrite ions. This type of interference has been shown to be present when nitroglycerin is assayed by this method [27]: The USP XIX method [24] was therefore modified in this study by adding a celite column elution step to overcome this deficiency. Under certain circumstances (e.g. equipment or expertise availability), this method may be a useful alternative to the polarographic assay recommended in USP [28].

Choice of internal standard

Nitroglycerin (NTG) can be used as an internal standard for this assay. However, in some therapeutic situations, the concomitant use of NTG and ISDN cannot be avoided, and the use of NTG as an internal standard for assay is obviously inappropriate. Isoidide mononitrate, which has been used for this purpose [16], was shown to be a urinary metabolite of ISDN in the rat [29]. Other organic nitrates used, viz., isoidide dinitrate [11,12] and isomannide dinitrate [16] are not available commercially.

The pesticides used here, HCE and DDT, are useful alternatives to NTG as internal standards. They are available commercially in pure form, are highly sensitive to ECD, and chromatograph at locations which were usually free from interference from peaks which appeared with blank plasma samples. When interference does occur with either one of the internal standards (as we found occasionally with HCE in some human plasma samples), the other internal standard may be employed. Although DDT has a long retention time, we seldom found any interference at its location in patient plasma samples.

Ideally, the internal standard should be added to plasma directly. However, in this case, HCE and DDT are so poorly soluble in water that an alcoholic co-solvent would have been needed; this would then have decreased the extraction efficiency of the polar mononitrates into chloroform. Thus, in this assay, the internal standards were added to plasma via the extraction solvent.

Choice of injection solvent

Mononitrates were found to adsorb to the injection syringe if a non-polar solvent such as hexane was used. Smith and Besic [13] attempted to decrease such adsorptive losses via *tert.*-butylsilyl derivatization of the mononitrate's polar hydroxy groups. This method, which required two back-extractions as well as the derivatization step, was unsuccessful in decreasing the detection limit of 5-ISMN in plasma below 250 ng/ml. LeBel and Williams [30] demonstrated that syringe adsorption in the GLC analysis of polar insecticides could be overcome by using acetone instead of the relatively non-polar hexane as injection solvent. Since acetone was not compatible with the trifluoropropyl liquid phase of our packing material, isoamyl alcohol was added to hexane in order to increase injection solvent polarity, and hence, mononitrate solubility.

The effect of isoamyl alcohol concentration in the injection co-solvent on the assay sensitivity of the three organic nitrates was examined. Dramatic improvements in the absolute detector response and in the coefficient of variation (C.V.) were observed for all three organic nitrates when isoamyl alcohol concentration in hexane was increased (Fig. 1). When compared with neat hexane, an injection co-solvent composition of 63% (v/v) isoamyl alcohol in hexane caused 6-, 15-, and 25-fold increases in apparent detector response for ISDN, 2-ISMN, and 5-ISMN respectively. Additionally, this co-solvent mixture produced an approximate 50% reduction in the C.V. value with triplicate injections. Unfortunately, injections containing high concentrations of isoamyl alcohol produced a more prolonged solvent front, and possibly lessened column stability. The co-solvent composition of 25% (v/v) of isoamyl alcohol in hexane appeared to provide an optimal balance between the need to increase detector response and the desirability to minimize the amount of isoamyl alcohol in-

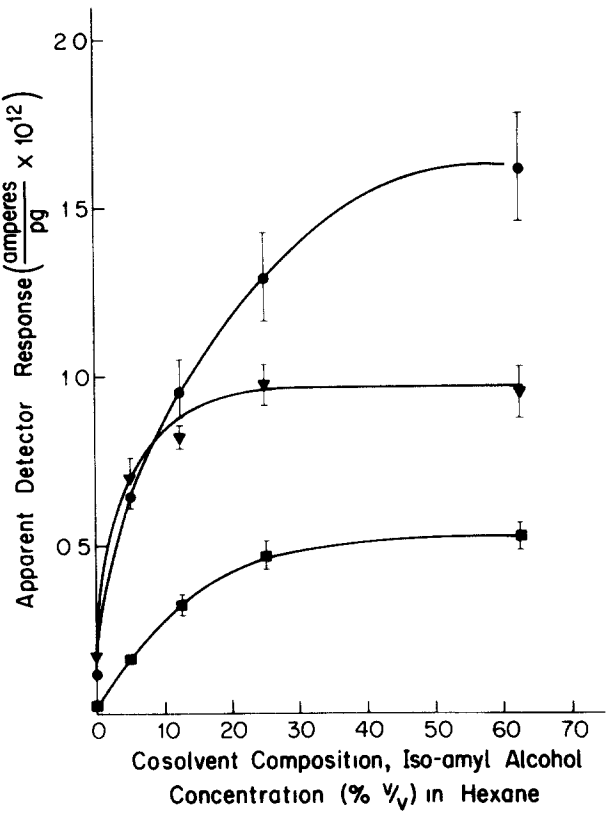

Fig. 1. Effect of co-solvent composition on the mean (± S.D.) GLC—ECD response of ISDN (▾), 2-ISMN (•), and 5-ISMN (▪). Each point represents three determinations

jected; this particular composition was therefore chosen for all subsequent work.

Fig. 2 shows the chromatogram of a standard solution, and those of extracts of spiked and blank rat plasma. The key determinant of limit of quantitation for these compounds was the degree of possible interference from other substances in blank plasma or in the system. Typically, blank values for ISDN, 2-ISMN, and 5-ISMN were at or below 0.5, 1, and 2 ng/ml, respectively. The higher blank values for the mononitrates reduced their limits of quantitation relative to ISDN. Compared to previously published assays, however, this method still represents considerable increase in sensitivity for the mononitrate metabolites.

Choice of extraction solvent

Although ethyl acetate and diethyl ether were both satisfactory in extracting the three organic nitrates, chromatograms of blank rat plasma using these solvents were found to contain numerous interference peaks which limited the sensitivity for mononitrate detection. This problem was absent with chloroform as the extraction solvent. However, high-density polyethylene could not be used with this solvent since a contaminant occurred which co-chromatographed with 5-ISMN. Use of PTFE in place of this plastic removed this

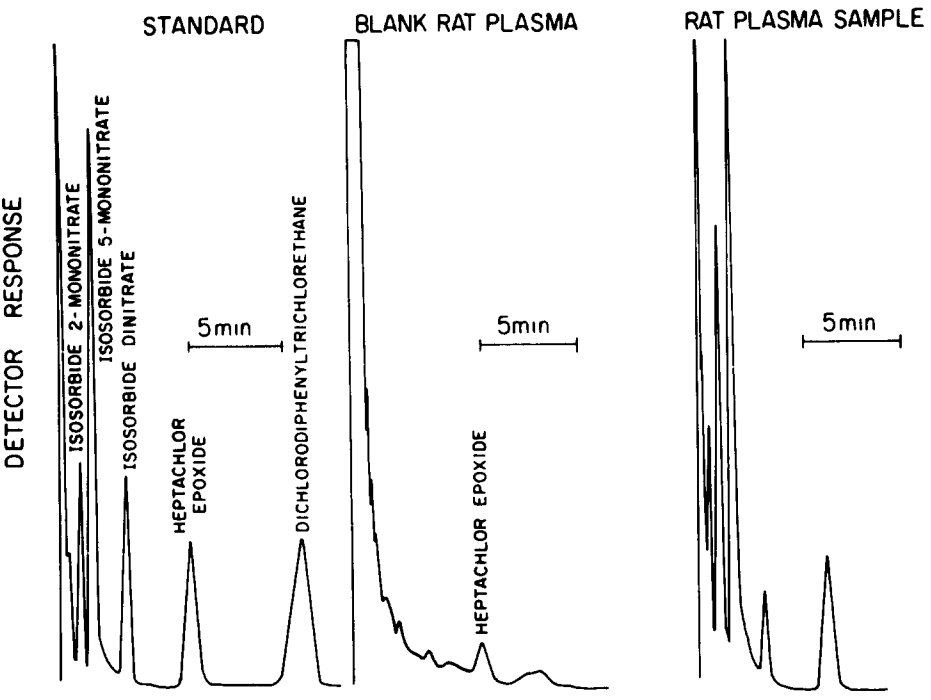

Fig. 2. Chromatograms showing simultaneous determination of ISDN, 2-ISMN, and 5-ISMN in rat plasma. The approximate on-column amounts injected for the standard shown were 2-ISMN (0.1 ng), 5-ISMN (0.5 ng), ISDN (0.2 ng), HCE (0.2 ng) and DDT (0.5 ng).

problem. Chloroform is highly electron capturing and therefore must be evaporated completely before reconstitution with 25% (v/v) isoamyl alcohol—hexane. Recovery was reduced by 80% and 10% for 2-ISMN and 5-ISMN, respectively, if evaporation to dryness was carried out at ambient temperatures rather than in an ice bath (4°C).

Recovery and reproducibility

The extraction efficiency and overall reproducibility of this procedure were examined following multiple determinations on spiked rat and human plasma. For the mononitrates, mean recovery was about 80% and did not appear to depend on the added concentration over a wide range of concentrations studied (Table I). ISDN, however, had significantly higher recovery (analysis of variance, $p < 0.001$, 35 pairs) with increasing spiked rat plasma ISDN concentration. A significant correlation ($r = 0.66$, $p < 0.001$, 35 pairs) was observed between percent recovery and spiked concentration (Fig. 3). Although the absolute difference in percent recovery over this concentration range was not large (ca. 20%), the differences were statistically significant. This concentration dependency in recovery was found not to arise from artifactual factors such as the percentage of water in diluted plasma, aliquot size and the presence of different concentrations of the mononitrate metabolites.

Since the concentration range covered almost four orders of magnitude, a ln—ln plot was used to examine the relationship between the experimentally determined concentration [not corrected for recovery, i.e., $C_{p(\text{assayed})}$] and the

TABLE I

RECOVERY OF ISDN AND ITS MONONITRATE METABOLITES FROM RAT AND HUMAN PLASMA AT DIFFERENT ADDED CONCENTRATIONS

	ISDN			2-ISMN			5-ISMN		
	Concentration* (ng/ml)	Percent recovery (mean)	C.V. (%)	Concentration (ng/ml)	Percent recovery (mean)	C.V (%)	Concentration (ng/ml)	Percent recovery	C.V. (%)
Rat plasma	1.49 (11)	69	13	1.89	78	9	7.71	81	10
	6.88	75	15	3.92 (5)	73	7	18.4	81	8
	68.8	78	10	196	78	8	920	84	12
	1560	87	13	1980	75	11	8080	100	12
	6880	88	7	19,600	83	6	92,000	88	9
Grand mean		**			78	8		87	10
Human plasma	1.56	80	22	2.13	79	12	8.69	80	9
	3.12	79	22	4.26	76	11	17.4	75	11
	31.2	90	11	42.6	79	16	174	81	12
Grand mean		83	18		78	12		79	10

*n = 6 determinations for rat plasma unless otherwise indicated in brackets, n = 4 for human plasma.

**Recovery was concentration-dependent and was therefore not averaged; see Fig. 3 and text.

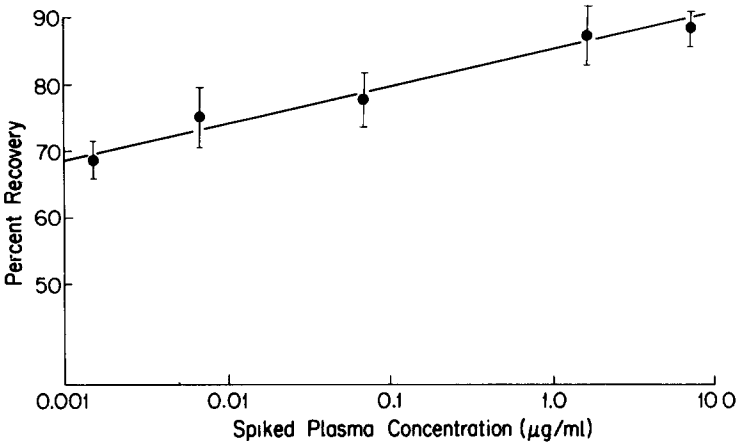

Fig. 3. Percent recovery of ISDN (± S.E.M.) as a function of a spiked rat plasma concentration.

theoretical, spiked concentration, $C_{p(\text{spiked})}$. Regression constants which were generated from individual recoveries (n = 35 pairs) are shown in eqn. 1.

$$\ln C_{p\ (\text{spiked})} = \frac{\ln C_{p\ (\text{assayed})} + 0.366}{1.029} \quad (1)$$

This relationship allows for correction of the concentration dependency of ISDN recovery over the wide range of rat plasma concentrations studied.

The recovery of ISDN from human plasma was studied over a much smaller range (1.5—31 ng/ml) and no significant effect of concentration on recovery was observed (Table I). Over this range of concentrations the mean percent recovery was 83%, 78%, and 79% for ISDN, 2-ISMN, and 5-ISMN, respectively.

Stability and partitioning studies

Fig. 4 shows that ISDN was metabolized rapidly in whole blood from rat and human ($t_{½}$ = 100 min) but was more stable in isolated rat plasma ($t_{½}$ = 8.7 h). A previous report showed ISDN to have a half-life of 55 h in human plasma [20]. Ideally, the stability of ISDN in whole blood should be directly monitored in blood and/or erythrocytes in addition to measurements of drug and metabolite concentrations in plasma. However, the present assay technique did not allow for these direct determinations in either blood or erythrocytes. Thus, the observed decline in plasma ISDN concentration after spiking of intact drug into whole blood may have been contributed, in part, by drug distribution into erythrocytes. Relatively slow uptake of drug into red blood cells has been reported for acetazolamide [31] and lithium [32]. To examine this possibility for ISDN, its stability in whole blood was assessed after incorporation of this drug into rat blood by both in vivo steady-state infusion as well as by in vitro spiking. A previous study [26] indicated that steady-state plasma ISDN levels were attained (ca. 150—200 ng/ml) with a 2-h constant-rate infusion at 7 μg/min. Therefore, it is reasonable to assume that after this infusion regimen in the rat, distributional equilibrium between plasma and erythrocytes has been achieved. No difference in the in vitro stability of ISDN was found between

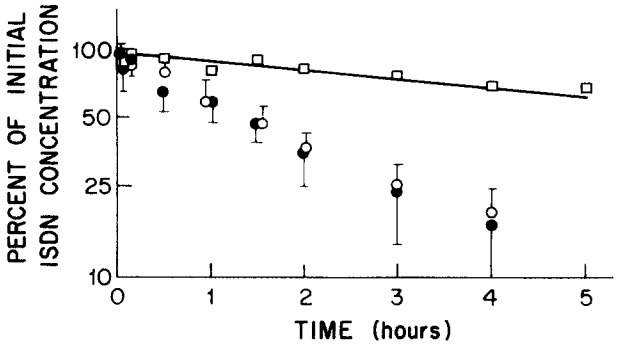

Fig. 4. Stability of ISDN following incubation (37°C) in whole blood (mean ± S.D.); (•) human (n = 3); (○) rat (n = 6) and (□) in rat plasma (mean, n = 2).

blood prepared to contain drug by in vitro spiking or in vivo infusion. This finding suggested that (1) erythrocyte partitioning of ISDN was essentially instantaneous and (2) loss of ISDN in incubated whole blood was due to physical and/or biotransformation processes rather than time-dependent accumulation by erythrocytes. The data suggested that degradation was linear, at least over the concentration range monitored (ca. 200 down to 25 ng/ml).

If it is assumed that plasma-to-erythrocyte partitioning of ISDN is instantaneous, the apparent partition coefficient can be quantitated without determination of actual corpuscle concentrations. Using the measured initial plasma concentration, the theoretical initial whole blood concentration, and the hematocrit, the erythrocyte-to-plasma apparent partition coefficient (K_p) was estimated at 0.22 and 0.13 for rat and human blood, respectively. The reason(s) for this relatively low erythrocyte partitioning of ISDN is (are) not clear at present. Using radiolabelled ISDN, Sisenwine and Ruelius [33] reported negligible radioactivity in dog erythrocytes for 2 h after in vivo ISDN administration. Their finding is consistent with the present results which indicated minimal erythrocyte partitioning of ISDN in blood obtained from the rat and human species.

Wu et al. [20] and Sokoloski et al. [22] recently concluded that the mechanism responsible for the loss of nitroglycerin after incubation in human blood was physical in nature, since they could not detect the presence of any denitrated metabolites concomitant with nitroglycerin loss. On the other hand, Johnson et al. [17] and Noonan and Benet [21] reported the formation of denitrated metabolites following whole blood incubation of organic nitrates. In the present study, significant concentrations of mononitrate metabolites were observed when ISDN was incubated in blood (Fig. 5). During ISDN degradation in human whole blood, plasma mononitrate metabolite levels increased during the first 2 h and then remained relatively constant thereafter (Fig. 5). The 5-ISMN isomer was the major metabolite formed (approximately 6:1, 5-ISMN:2-ISMN). This ratio of mononitrates formed from ISDN in vitro was similar to the ratio observed after in vivo dosing of ISDN in man [34] and animals [26, 33,35].

Assuming that the calculated partition coefficient of ISDN is constant throughout the 4-h period studied, the total amount of ISDN lost in whole

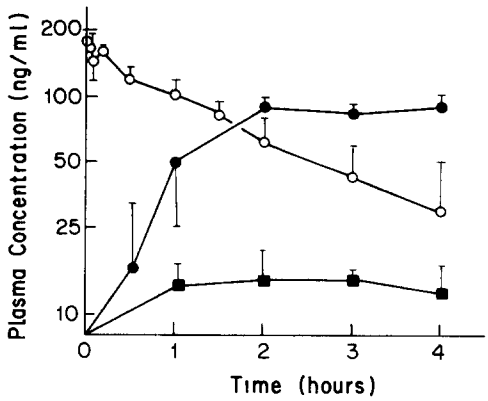

Fig. 5. Plasma concentration (mean ± S.D.) of ISDN (○) and its mononitrate metabolites 2-ISMN (▪) and 5-ISMN (●) observed after incubation of ISDN in human whole blood (37°C).

blood can be quantitated and compared with the amount of denitrated metabolites formed in plasma. For the first 2 h, 96% of the total molar amount of ISDN that was lost (0.28 nmol/ml) was accounted for by summation of the mononitrate amounts produced in the plasma (2-ISMN = 0.04 nmol/ml; 5-ISMN = 0.23 nmol/ml). This metabolite mass balance steadily decreased to about 50% at 4 h of incubation, presumably due to further denitration of the mononitrates themselves or slow accumulation of these mononitrates into erythrocytes. The quantitative production of mononitrate metabolites in the present study indicated that biotransformation via denitration, rather than physical loss, was the operative mechanism responsible for the loss of ISDN in whole blood. In addition, the fact that 96% of ISDN lost from whole blood was accounted for by the amounts of mononitrates formed in plasma suggested that, within the first 2 h at least, erythrocyte partitioning of these metabolites was also minimal.

Results of these in vitro stability experiments can also be used in furthering our understanding of in vivo pharmacokinetic data of ISDN. The contribution of blood degradation itself to the total body clearance of ISDN can be estimated using the product of the in vitro disappearance rate constant (ca. 0.007 min^{-1}) and the physiologic blood volume in these species (rat, 65 ml/kg [36] and man, 70 ml/kg [37]). This estimation produces a blood clearance value of 0.46 ml/min/kg in the rat and 0.49 ml/min/kg in man, respectively. This value represents less than 1% of the systemic clearance for ISDN in either species. Thus, denitration by whole blood itself does not appear to contribute significantly to the high systemic clearance and the extensive extrahepatic metabolism of ISDN suggested in rats [26] and cardiac patients [23].

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