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SEX-RELATED DIFFERENCE IN THE METABOLISM OF ISOSORBIDE DINITRATE FOLLOWING INCUBATION IN HUMAN BLOOD*†

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Abstract—Isosorbide dinitrate (ISDN) (at a concentration of 100 ng/ml) was incubated aerobically at 37° in whole blood from five male and five female normal volunteers. Following incubation of the blood samples for 0, 30, 60, 120, 240 and 360 min, the samples were centrifuged and the plasma was assayed for ISDN. A linear relationship was observed between the logarithm of the concentration of ISDN remaining and incubation time, and there was a significant difference between the $T_{\frac{1}{2}}$ of ISDN in blood from males (90.6 min) and females (161.4 min). Very little ISDN metabolism was observed when ISDN was incubated with plasma rather than whole blood. When erythrocytes, resuspended in saline, were incubated with ISDN, there was a time-dependent loss of ISDN from the saline incubation medium. Investigation of the soluble fraction obtained after hemolysis of these erythrocytes also showed a time-dependent loss of ISDN. The saline incubation medium contained sufficient concentrations of the two major ISDN metabolites (isosorbide 2- and 5-mononitrate) to account for the observed disappearance of ISDN. The results indicate that ISDN is metabolized in the cellular compartment of blood and that the metabolic rate in males is greater than that in females.

Although the systemic bioavailability of isosorbide dinitrate (ISDN¶) after oral administration is less than 3% [1], the radiolabel after oral doses of [^{14}C]ISDN is almost completely absorbed from the gastrointestinal tract [2]. These observations indicate that extensive presystemic elimination of ISDN occurs and accounts for the need to use large oral doses of ISDN to overcome this first pass effect [3].

Presystemic biotransformation of ISDN occurs predominantly in the liver but may also take place in blood or in the gastrointestinal tract before or during absorption [4]. Isosorbide dinitrate and other organic nitrates are denitrated by the glutathione-dependent glutathione *S*-transferase enzymes (EC 2.5.1.18), formerly known as organic nitrate reductase (EC 1.8.6.1). These enzymes have been isolated and purified from human and rat liver [5, 6]. One species, glutathione *S*-transferase ρ, has been isolated and purified from human erythrocytes [7]; its role in the metabolism of organic nitrates at therapeutic concentrations remains to be clarified.

We have demonstrated previously the rapid disappearance of nitroglycerin (GTN) after incubation in human whole blood and erythrocytes ($T_{\frac{1}{2}}$ = 6.2 and 6.6 min respectively) [8]. We suggested that glutathione *S*-transferase ρ could be responsible for metabolism of GTN in human erythrocytes. A similar $T_{\frac{1}{2}}$ of approximately 7 min was reported by Wu *et al.* [9] after incubation of GTN in human erythrocytes, but these investigators could not detect metabolite formation in the incubation medium. They concluded that the loss of GTN from blood could not proceed via enzymatic metabolism, but could not offer an alternate mechanism. One possibility which could not be excluded in our previous study was that GTN was concentrated in the erythrocytes without being degraded.

Metabolism of ISDN has been studied in rat blood *in vitro*, and a $T_{\frac{1}{2}}$ of 10–15 min was determined [10]. However, the fate of ISDN in human blood *in vitro* at therapeutic concentrations has not been examined and thus the contribution of blood to the pharmacokinetics of ISDN is not known. The objectives of this *in vitro* study were to: (1) determine the rate of disappearance of ISDN in whole blood and plasma and compare the $T_{\frac{1}{2}}$ with that found previously with GTN; (2) determine the concentration of ISDN inside erythrocytes at various time intervals following incubation of ISDN with erythrocytes resuspended in saline; and (3) determine the rate of appearance of the major ISDN metabolites, isosorbide 5- and 2-mononitrate (5-ISMN and 2-ISMN), after incubation of ISDN with erythrocytes resuspended in saline.

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¶ Abbreviations: ISDN,isosorbide dinitrate; 2-ISMN, isosorbide 2-mononitrate; 5-ISMN, isosorbide 5-mononitrate; and GTN, glyceryl trinitrate.

METHODS

Drugs and solutions. Isosorbide dinitrate (25% in lactose powder), crystalline 5-ISMN and 2-ISMN were gifts from Wyeth Ltd., Toronto, Ontario. Stock solutions of ISDN were prepared by extraction of the lactose powder with acetone (U.S. Pharmacopeia XX, 1979). The concentration of ISDN in the acetone solution was determined by comparison with ISDN solution prepared from crystalline ISDN (gift of Ayerst Laboratories Inc., Rouses Point, NY) by the gas chromatographic method described below. Dilutions of ISDN in saline were prepared daily for use in the incubation experiments. The final concentration of acetone in the incubation samples was less than 0.002% (v/v).

Preparation of samples. Immediately prior to incubation experiments, 90–130 ml venous blood was obtained from normal volunteers and placed in heparinized tubes. The average age of the male volunteers was 26.2 yr (range 21–35 yr) and of the female volunteers was 26.5 yr (range 21–37 yr). For studies in whole blood, 7-ml aliquots of blood were transferred to 25-ml Erlenmeyer flasks. To obtain plasma, 7-ml aliquots of whole blood were centrifuged at 310 *g* and 5° for 15 min. The plasma was decanted, and 3-ml aliquots were added to 25-ml Erlenmeyer flasks. Packed erythrocytes obtained from 7 ml of whole blood were suspended to a volume of 7 ml with isotonic saline. Following centrifugation at 310 *g* and 5° for 15 min, the saline supernatant fraction was withdrawn. This washing procedure was repeated three times and, following the fourth suspension, the 7-ml volume was transferred to 25-ml Erlenmeyer flasks.

Incubation conditions. The 25-ml Erlenmeyer flasks containing whole blood, plasma, or resuspended erythrocytes were incubated aerobically with gentle agitation in a Dubnoff metabolic incubator at 37°. Following a 15-min equilibration period, 200 μl of an ISDN solution in saline was added to each flask to produce a final concentration of 100 ng/ml ISDN. Two samples to which 200 μl saline was added served as controls. Duplicate flasks were used for each time period with the exception of the 0-min time period where four flasks were used. An additional incubation study with resuspended erythrocytes was performed at 0° to determine the effect of temperature on ISDN disappearance.

Incubation of ISDN with whole blood and plasma. Whole blood samples were incubated with ISDN for 0, 30, 60, 120, 240, or 360 min. After incubation, the samples were immediately centrifuged at 310 *g* and 5° for 15 min. The plasma was decanted and frozen at −20° until assayed for ISDN.

The plasma samples were incubated with ISDN for 0, 120, 240, or 360 min. After incubation, the plasma was decanted and frozen at −20°. The glutathione concentration in plasma (4.5 μM) is low compared to that found in whole blood (1.09 mM) [11]. We wanted to determine if the low plasma glutathione content limited the rate of ISDN metabolism. Therefore, a separate experiment was performed in plasma with glutathione added to a final concentration of 1.09 mM. To control for possible loss of ISDN due to evaporation, an experiment was performed using saline as the incubation medium instead of plasma. In these control studies, no loss of ISDN was observed over a 6-hr incubation period.

Incubation of ISDN with resuspended erythrocytes. Resuspended erythrocytes were incubated with ISDN for 0, 60, or 120 min. After incubation, samples were immediately centrifuged at 310 *g* and 5° for 15 min. The saline supernatant fractions were stored at −20° until assayed for ISDN, 2-ISMN and 5-ISMN. At each time point, the erythrocytes collected by centrifugation were lysed by a modified method of Marcus *et al.* [7]. A 1:1 (erythrocyte:distilled water) suspension was made, shaken vigorously, and kept for 1 hr at 5° with occasional mixing. The suspension was then centrifuged at 9000 *g* and 5° for 1.5 hr, and the supernatant fraction was frozen at −20° until assayed for ISDN.

Extraction of ISDN. Samples from each incubation experiment were assayed for ISDN within 5 days. After thawing and mixing with a vortex mixer, aliquots (2-ml) were extracted twice with 5 ml of a hexane mixture for 5-min periods. Following evaporation of the solvent under a stream of nitrogen gas, the residue from each sample was reconstituted in 100 μl benzene and frozen at −20° until chromatographed the following day. A standard curve was prepared by adding 100-μl aliquots of ISDN in saline to 2-ml aliquots of distilled water to produce final concentrations of 0–100 ng/ml. The standards were extracted by the procedure outlined above. Fresh aliquots of ISDN were prepared each week and used for the weekly preparation of standard curves. The use of aqueous ISDN standards for the measurement of ISDN in plasma was validated in a separate experiment comparing ISDN standard curves (0–100 ng/ml) prepared in distilled water and plasma. Both standard curves were prepared on the same day, and the slopes of the curves were determined by linear regression analysis. The slopes were compared using a modified *t*-test [12] and were found to be parallel ($P \leq 0.01$).

Extraction of 5-ISMN and 2-ISMN. Isosorbide 5- and 2-mononitrate were extracted by a method similar to that described previously for glyceryl 1,2-dinitrate [13]. The 2-ml samples previously extracted with hexane were extracted once more with 5 ml hexane to remove any remaining ISDN. Ethyl ether (5 ml), freshly distilled over potassium hydroxide pellets, was added, and the samples were shaken for 5 min and centrifuged at 194 *g* for 2 min. The supernatant fraction was removed, and the ether extraction repeated. Magnesium sulfate was added to the ether extracts, and the mixture was shaken for 20 min. Following centrifugation at 194 *g* for 2 min, the ether was removed and evaporated to dryness over a stream of nitrogen gas. The residue from each sample was reconstituted in 100 μl benzene and frozen at −20° until chromatographed. A standard curve was prepared by adding 100-μl aliquots of 5-ISMN and 2-ISMN in saline to 2-ml aliquots of saline to produce final concentrations of 0–100 ng/ml. The samples were extracted three times with 5 ml hexane and then extracted with ether as described above.

Chromatography. Chromatographic analysis was performed with a Hewlett–Packard 5700 series

gas–liquid chromatograph with a ^{63}Ni radioactive source in the electron capture detector. The operating conditions were as previously described [13], and the retention times for ISDN, 5-ISMN and 2-ISMN were 12.5, 7.0 and 4.3 min respectively. There was negligible background noise at these retention times. In the incubation studies with whole blood and plasma, the mean concentration of ISDN at each time point was plotted, and linear regression analysis was performed to determine the relationship between the logarithm of the concentration of ISDN remaining and incubation time. The significance of the regression analysis was tested using a Fisher F-test, and the $T_{\frac{1}{2}}$ of ISDN in whole blood and plasma was calculated.

RESULTS

ISDN, 2-ISMN and 5-ISMN analysis. The concentrations of ISDN, 2-ISMN and 5-ISMN were determined using peak height measurements. The recovery of ISDN from plasma was 91% [coefficient of variation (CV) 3.4%, N = 6] at 50 ng/ml. The recovery of ISDN from distilled water was 95% (CV 10.4%, N = 6) at 20 ng/ml and 92% (CV 11.1%, N = 6) at 80 ng/ml. The recovery of 2-ISMN and 5-ISMN from saline at 10 ng/ml was 52.5% (CV 9.6%, N = 5) and 61.5% (CV 5.5%, N = 5) respectively. The precision of the extraction method, defined as the CV of the mean of replicate assays, for ISDN, 2-ISMN and 5-ISMN at 50 ng/ml was 7.1% (N = 6), 9.0% (N = 5) and 10.1% (N = 5) respectively. The assays were linear over the concentration range used (5–100 ng/ml). The average concentration of ISDN at the 0-min time point in the plasma from whole blood samples was 97 ng/ml ± 14 (S.D., N = 40).

Incubation of ISDN in whole blood and plasma. The first objective of this study was to determine the $T_{\frac{1}{2}}$ of ISDN in human blood following incubation at 37°. The data from the five male and five female volunteers are shown in Fig. 1. A linear relationship was found between the logarithm of the concentration of ISDN remaining and incubation time (males and females: $r = 0.99$, $P \leq 0.001$). An unpaired *t*-test was performed on the mean $T_{\frac{1}{2}}$ and there was a significant difference between males and females ($P \leq 0.01$).

In contrast to the results found in whole blood, very little ISDN was metabolized by plasma alone,

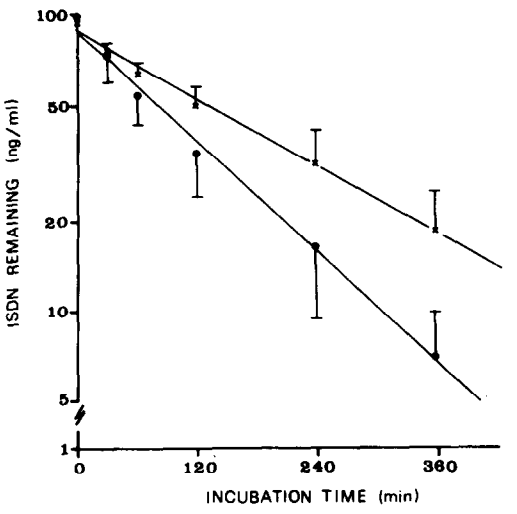

Fig. 1. Semilogarithmic plot of the mean concentration of ISDN remaining in male (●) and female (×) blood versus incubation time. The average concentrations (ng/ml) of ISDN ± S.D. remaining in blood after incubation for 0, 30, 60, 120, 240 and 360 min were males (N = 5), 99.0 ± 17.3, 74.4 ± 14.6, 54.4 ± 11.5, 34.2 ± 9.7, 16.5 ± 6.9 and 7.1 ± 2.9; females (N = 5), 95.8 ± 6.5, 75.6 ± 4.4, 64.0 ± 6.1, 50.1 ± 8.0, 31.7 ± 9.3, and 18.9 ± 6.7. A linear relationship was determined by regression analysis (males and females: $R = 0.99$, $P \leq 0.001$), and the $T_{\frac{1}{2}}$ for ISDN disappearance was 90.6 min for males and 161.4 min for females.

and the $T_{\frac{1}{2}}$ was too long to be accurately determined within the 6-hr period of study. When the glutathione concentration in plasma (4.5 μM) was adjusted to the concentration found in whole blood (1.09 mM) [11], no change in the rate of degradation of ISDN was observed.

Incubation of ISDN with erythrocytes resuspended in saline. The second objective was to determine whether ISDN was taken up into erythrocytes and concentrated there without being degraded. As seen in Table 1, the average concentration of ISDN at 0 min in the hemolysate supernatant fraction was lower than that found in the saline incubation medium (44.0 and 80.1 ng/ml respectively), and there was a time-dependent loss of ISDN from both the hemolysate supernatant fraction and the saline incubation medium. When the experiment was repeated at 0°, no loss of ISDN was observed with up to 360 min of incubation.

Table 1. Concentration of ISDN remaining in the saline incubation medium and hemolysate supernatant fraction following incubation of ISDN (100 ng/ml) with erythrocytes resuspended in saline

	Incubation time (min)	ISDN* (ng/ml)
Saline incubation medium	0	80.1 ± 15.1
	60	51.9 ± 10.5
	120	32.3 ± 10.6
Hemolysate supernatant fraction	0	44.0 ± 14.0
	60	32.6 ± 10.0
	120	22.1 ± 10.7

* Average concentration of ISDN in samples from nine subjects (four males, five females). Values are means ± S.D.

Table 2. Concentrations of ISDN, 2-ISMN and 5-ISMN in the saline incubation medium following incubation of ISDN (100 ng/ml) with erythrocytes resuspended in saline

Incubation time (min)	ISDN* (ng/ml)	2-ISMN* (ng/ml)	5-ISMN* (ng/ml)
0†	83.8 ± 13.2	0	5.3 ± 0.1
60‡	56.0 ± 7.0	9.7 ± 3.9	45.8 ± 17.5
120‡	33.6 ± 12.2	15.0 ± 4.0	75.5 ± 27.2

* Values are means ± S.D.

† Values represent the average concentration in twenty-four samples from six subjects (two male, four female).

‡ Values represent the average concentration in twelve samples from the same six subjects.

The third objective was to measure the appearance of ISDN metabolites after incubation of ISDN in erythrocytes resuspended in saline. Extracts of the saline samples were assayed for 2- and 5-ISMN, and there was a time-dependent increase in the concentration of both metabolites (Table 2, Fig. 2). The formation of 5-ISMN predominated and the ratio of 2-ISMN to 5-ISMN was approximately 1:5. Several of the 0-min hemolysate supernatant samples were analysed for 2-ISMN and 5-ISMN. The average concentration of 2-ISMN and 5-ISMN in twelve samples from three subjects was 5.9 ng/ml ± 5.1 (S.D.) and 29.5 ng/ml ± 11.3 (S.D.) respectively.

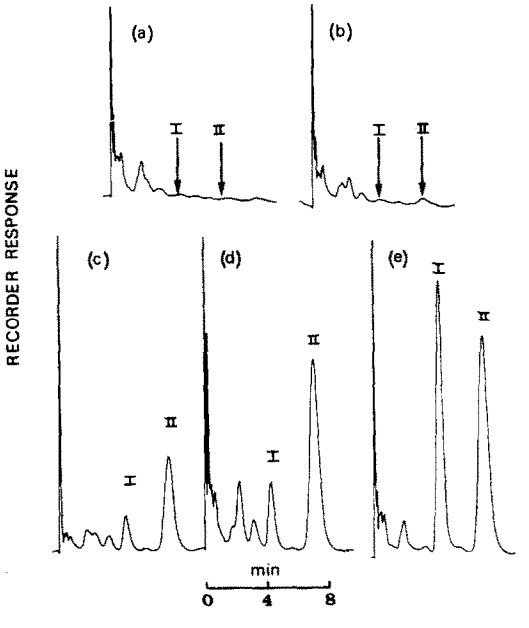

Fig. 2. Sample chromatograms of ether extracts of the saline incubation medium following incubation of ISDN with erythrocytes resuspended in saline. The samples were from the same subject and were extracted with hexane to remove intact ISDN prior to extraction with ether. Key: (I) retention time for 2-ISMN; (II) retention time for 5-ISMN; (a) saline incubation medium with no ISDN added, (b) saline incubation medium immediately following the addition of 100 ng/ml ISDN, (c) after incubation for 60 min, (d) after incubation for 120 min, and (e) saline spiked with 50 ng/ml of 2-ISMN and 5-ISMN.

DISCUSSION

Our first objective was to compare the fate of ISDN in plasma and whole blood *in vitro* with that found previously with GTN. It has been reported recently that ISDN has a $T_{\frac{1}{2}}$ in plasma of 55 hr [14]. In accord with this finding, very little degradation of ISDN in plasma was observed over the 6-hr period of the study. When the glutathione concentration in plasma was adjusted to the concentration found in whole blood, no change in the rate of degradation of ISDN was observed. This result confirmed that the low glutathione content of plasma did not limit the degradation of ISDN, a result similar to that seen for GTN in plasma [8, 15].

The $T_{\frac{1}{2}}$ of ISDN after incubation in whole blood (90.6 min in males, 161.4 min in females) was considerably longer than that observed with GTN (6.2 min; five males, one female) under the same experimental conditions [8]. This difference is in accord with the marked difference in the rate of disappearance of ISDN and GTN *in vivo*. The $T_{\frac{1}{2}}$ of GTN after intravenous infusion in humans is only 1.9 min [16] compared to 18 min for ISDN [17]. Organic nitrates are metabolized in the liver by the glutathione *S*-transferase enzymes and these enzymes have been reported to degrade GTN much more rapidly than ISDN [18].

A significant difference was found between the $T_{\frac{1}{2}}$ of ISDN in male and female blood. It is of interest that, in male rats, the $T_{\frac{1}{2}}$ of GTN is about 60% of that for females [19] and that the activity of rat glutathione *S*-transferase is greater in males than in females [20]. Sex-related differences in human drug metabolism have been demonstrated for relatively few drugs; some examples are: diazepam, lithium, aspirin and certain antibiotics [21, 22]. These sex-related differences have been attributed mainly to differences in the activity of certain enzymes in the liver, plasma and erythrocytes. Thus, the $T_{\frac{1}{2}}$ of aspirin is shorter in blood from males due to a higher aspirin esterase activity in plasma [23] and in erythrocytes [24]. The observed sex difference in ISDN metabolism could be due to hormonal or genetic differences or to differences in male and female hematocrits. For example, hexobarbital sleeping time is shorter in male rats due to a faster rate of metabolism by the hepatic mixed function oxidase system [25]. Hexobarbital sleeping time in estradiol-treated male rats approaches that found in control females, and

the sleeping time of testosterone-treated females is similar to that of control males. It will be of interest to determine whether the metabolism of ISDN by glutathione *S*-transferases is also sensitive to sex hormones. Since ISDN metabolism in blood is limited to the cellular compartment, subjects with higher hematocrits would be expected to metabolize ISDN at a faster rate. On the average, male hematocrits are 10–15% higher than females. However, in this study the $T_{\frac{1}{2}}$ for ISDN was 72% faster in males and thus the difference in hematocrits is not large enough to account for the results obtained.

The loss of ISDN from the saline of resuspended erythrocytes (Table 1) was similar to that from the plasma of whole blood incubations (Fig. 1), except that the concentration of ISDN at 0 min was lower in the saline than in the plasma (80.1 and 97.0 ng/ml; $P \leq 0.001$, unpaired *t*-test). It would thus appear that some component of plasma is affecting the distribution of ISDN between erythrocytes and plasma. Plasma protein binding of ISDN could explain this observation since ISDN is approximately 30% bound to plasma proteins [14].

Our second objective was to determine whether ISDN disappearance from blood could be explained by uptake and concentration of ISDN into erythrocytes without concomitant degradation. The results in Table 1 are not in accord with this explanation since the concentration of ISDN found in the hemolysate supernatant fraction decreased with respect to time and after 120 min was 50% of that found in 0 min. A complicating factor in this study was the finding that the mean concentration of ISDN at 0 min was less in the hemolysate supernatant fraction than in the saline incubation medium (44.0 and 80.1 ng/ml respectively). The low concentration of ISDN seen at 0 min in the hemolysate supernatant fraction could not be attributed to metabolism of ISDN during the 2.5-hr period of hemolysis and centrifugation at low temperature. This conclusion follows from the observation that no loss of ISDN occurred in both the hemolysate supernatant fraction and the saline incubation medium when resuspended erythrocytes were incubated with ISDN at 0° for up to 360 min. Due to the lipophilic nature of ISDN, it is possible that the low value resulted from an uneven distribution of ISDN between the hemolysate supernatant fraction and pellet, with the pellet containing a higher concentration of ISDN.

Our third objective was to determine if the disappearance of ISDN was due to conversion to 2-ISMN and 5-ISMN. The appearance of these metabolites was measured at 60 and 120 min in the saline incubation medium, and the results clearly indicate that conversion of ISDN to 2- and 5-ISMN occurs (Table 2, Fig. 2). It is likely that this is an enzymatic process, and further work is required to determine the nature of the erythrocyte enzyme(s) involved.

In addition to the metabolite formation detected in the saline incubation medium, metabolites were detected in the 0-min hemolysate supernatant samples. Although the hemolysis and centrifugation of the erythrocyte samples was performed at 5°, the extraction procedure was performed at room temperature and this could have allowed time for metabolism to occur. This could not occur while saline was being extracted since the enzyme is obviously not present in saline. Thus, the low concentration of ISDN in the 0-min hemolysate supernatant fraction was probably due to a combination of metabolism during the extraction procedure and uneven partitioning between the hemolysate supernatant fraction and pellet.

In this study, the ratio of 5-ISMN to 2-ISMN formed was about 5:1. The predominant formation of 5-ISMN from ISDN has also been demonstrated following oral administration of ISDN to dogs [26] and humans [3, 27]. This is thought to be due to the relative ease of denitration of the 2-exo nitrate group as compared to the more sterically hindered 5-endo nitrate group [26].

This study shows that ISDN disappears from blood with a $T_{\frac{1}{2}}$ of 90.6 min for males and 161.4 min for females. The $T_{\frac{1}{2}}$ is longer than that for GTN but, like GTN, the cellular compartment is important and little metabolism occurs in plasma. On further investigation we have determined that metabolites of ISDN are formed, eliminating the possibility that the disappearance of ISDN was merely due to concentration in erythrocytes. A related compound, pentaerythritol tetranitrate, has been found to undergo conversion to metabolites when incubated with human erythrocytes [28]. While we can offer no explanation for the lack of GTN metabolite formation reported by Wu *et al.* [9], a recent report by Noonan and Benet [29] involving incubation of blood with tritiated GTN has provided convincing evidence that metabolism of GTN does, in fact, occur.

Since the volume of distribution of ISDN is approximately 100 l [17], and the rate of ISDN metabolism in blood is low compared to the rate *in vivo* ($T_{\frac{1}{2}}$ = 18 min [17]), it appears that blood plays only a minor role in the overall metabolism of ISDN. However, because of the metabolism of ISDN in blood, it is important during pharmacokinetic studies to separate plasma and erythrocytes rapidly after withdrawal of the blood samples in order to prevent degradation of ISDN prior to analysis.

We have found that blood from males metabolizes ISDN at a faster rate than that from females. The reason for this finding is unclear but it may be related to hormonal or genetic differences. Whether a similar difference in ISDN metabolism occurs *in vivo* at therapeutic concentrations or in other tissues besides blood requires further study. If a sex difference in ISDN metabolism is a general phenomenon, there may be implications with respect to the selection of appropriate doses of this drug in male and female patients.

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