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Metabolism of glyceryl trinitrate

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Metabolism of Glyceryl Trinitrate -- Relationship to Its Pharmacological Effects

by

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DISSERTATION

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Dedication

This thesis is dedicated to my parents,

Mr. & Mrs. Chu-Shing Lau,

whose constant love, support, and prayers

make this work possible

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Abstract

The pharmacokinetics of glyceryl trinitrate (GTN) and its dinitrate metabolites (GDNs) were studied in rats. Upon GTN infusions (2.0 μg/min) into the left femoral vein, left jugular vein, left femoral artery and hepatic portal vein, different steady-state concentrations of GDNs were obtained, with GDN ratios (1,2-GDN/1,3-GDN) of 4.02±0.85, 3.41±0.62, 2.59±0.30, and 0.73±0.29, respectively. GTN extraction ratios for the liver, leg, and veins were 0.817, 0.625 and 0.209, respectively. GDNs exhibited similar pharmacokinetics when infused individually. No dose-dependent changes in GDN pharmacokinetics were found, and no pharmacokinetic interaction was observed when the GDNs were co-administered.

GTN metabolism, studied in cytosolic and microsomal fractions of rabbit liver, was more rapid in the cytosol, with 1,2-GDN as the predominant metabolite, although GTN concentration-dependence of the formation of this metabolite was observed. The microsomal fraction yielded 1,3-GDN as the major metabolite, while exhibiting linear kinetics. Various inhibitors of glutathione S-transferase (GST) decreased the rate of GTN degradation in the cytosol, and some altered the ratio of GDN formation as well.

The involvement of GST in GTN metabolism was investigated in bovine coronary arteries (BCA), where GTN was primarily metabolized by the cytosol. Upon addition of sulfobromophthalein (SBP) and ethacrynic acid (ECA), inhibitors of GSTs, GTN metabolic rate was decreased. In rabbit aorta (RA), all three classes of GSTs, alpha, mu, and pi, were found. The effects of SBP and ECA on GTN metabolism and GTN-induced vasorelaxation were studied using RA strips as a model. ECA, but not SBP, attenuated the response to GTN and reduced the extent of GTN metabolism in these tissues. Attenuations of GTN metabolism and GTN effects by ECA were well-correlated, suggesting that the crucial enzyme essential for nitrate metabolism and action may be a GST exhibiting activities towards ECA.

The concentration-response relationships for GTN, GDNs and glyceryl mononitrates (GMNs) were compared in RA strips. The ratios of EC_{50}'s for GTN versus its 1,2-GDN, 1,3-GDN, 1-GMN, 2-GMN metabolites were found to be 1:44:40:7400:59000, respectively. It is likely that GDNs, but not GMNs, can contribute significantly to the effects observed following GTN administration.

Glossary

1,2-GDN	1,2-Glyceryl dinitrate
1,3-GDN	1,3-Glyceryl dinitrate
1-GMN	1-Glyceryl mononitrate
2-GMN	2-Glyceryl mononitrate
ANOVA	Analysis of variance
AUC	Area under the concentration curve
AUMC	Area under the moment curve
BW	Body weight
C	Blood concentration
C_{ss}	Steady-state blood concentration
CDNB	1-Chloro-2,4-dinitrobenzene
cGMP	Cyclic 3',5'-guanosine monophosphate
CL	Apparent blood clearance
$CL_{(Area)}$	Apparent blood clearance (calculated from AUC)
$CL_{(ss)}$	Apparent blood clearance (calculated from Css)
$CL_{f,m}$	Apparent formation clearance of metabolite
CL_m	Apparent clearance of metabolite
CV	Coefficient of variation
E_{max}	Maximum effect elicited by the drug
EC_{50}	Concentration required to induce 50% of E_{max}
ECA	Ethacrynic acid
ED_{50}	Dose required to induce 50% of E_{max}
F	Availability of the drug through an organ
GC-ECD	Gas chromatography with electron capture detection
GDNs	Glyceryl dinitrates
GDN ratio	Ratio of dinitrate metabolites (1,2-GDN/1,3-GDN)
GMNs	Glyceryl mononitrates
GSH	Reduced glutathione
GST	Glutathione S-transferase
GTN	Glyceryl trinitrate, nitroglycerin
HPV	Hepatic portal vein infusion
IDM	Iodomethane
ISDN	Isosorbide dinitrate

ISMN	Isosorbide mononitrate
k_{el}	First-order elimination rate constant
K_m	Michaelis-Menten constant
LFA	Left femoral artery infusion
LFV	Left femoral vein infusion
LJV	Left jugular vein infusion
MRT	Mean residence time
MW	Molecular weight
NC	Nitrocellulose
NS	Not statistically significant
PAGE	Polyacrylamide gel electrophoresis
PE	Phenylephrine
p-NBC	p-Nitrobenzyl chloride
R_{inf}	Rate of infusion
RAS	Rabbit aortic strips
SBP	Sulfobromophthalein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error of the mean
$t_{1/2}$	Half-life
T_{inf}	Time of infusion
V_{max}	Maximum velocity of enzymatic reaction
Vss	Volume of distribution at steady-state
γ	Hill coefficient

Preface

Many controversies exist with respect to both the disposition and the mechanism of action of glyceryl trinitrate (GTN) and other organic nitrates. A full description of GTN availability and the formation of its dinitrate metabolites (GDNs) following various routes of GTN administration is lacking. Elucidation of the metabolic profile of GTN is important, especially since both GDN metabolites have been shown to possess pharmacologic activities, and thus may contribute to the overall effects of the parent drug. Even in a single organ, multiple pathways for GTN metabolism may exist. The previous belief that hepatic GTN metabolism is mediated by a single enzyme is probably inadequate, especially in light of the dose-nonlinearities in the pharmacokinetics of GTN and its metabolites following oral GTN administration. The enzyme responsible for the metabolism of GTN has not been identified in vascular tissues -- the site of action of this class of drugs. Elucidation of the mechanism by which GTN is metabolized in these tissues is essential as numerous reports have depicted the tightly-coupled relationship between vascular metabolism of organic nitrates and generation of pharmacologic effects. We propose that glutathione S-transferase (GST) may play a role in vascular GTN metabolism and subsequent generation of the pharmacologic effects of the drug. If GST is indeed involved in the mechanism of action of nitrates, a decrease in GST activity after prolonged contact with the drug may be associated with the development of tolerance to the drug.

The following will be investigated in subsequent chapters of this thesis which will describe the results of studies designed to:

(a) examine the role of different organs as sites for GTN metabolism and the preferential formation of individual GDNs (Chapter 3);

(b) explore if multiple enzymatic pathways are present for GTN metabolism in subcellular fractions of the liver (Chapter 4);

(c) study the role of GST in the metabolism of GTN in homogenates of blood vessels (Chapter 5);

(d) investigate the relationship between GST-mediated GTN metabolism and nitrate-induced vasorelaxation in an isolated muscle bath system (Chapter 6); and

(e) compare the relative potencies of the dinitrate and mononitrate metabolites of GTN with the parent drug, as well as the cross-tolerance among these agents, in an isolated muscle bath system (Chapter 7).

CHAPTER ONE

Pharmacokinetics and Pharmacodynamics of Glyceryl Trinitrate and Its Metabolites

Glyceryl trinitrate (GTN), more commonly known as nitroglycerin, is a frequently prescribed agent for patients suffering from anginal attacks. GTN was first synthesized by Sobrero in 1846, and was first used for treating angina in 1879 (Murrell, 1879). Although GTN has been used for over a century, there are still a lot of controversies with regard to both the disposition of the drug and its mechanism of action.

1. Physical and chemical properties of glyceryl trinitrate

The structure of GTN is shown in Fig. I-1. It is a simple ester of glycerol and nitric acid. It exists in a liquid form at room temperature, and crystalizes at 2.8 °C and 13.5 °C to form two different types of crystals. Upon heating, GTN starts to decompose at 50 °C and rapidly vaporizes. GTN is also an explosive. Explosion can occur spontaneously above 218 °C (DiCarlo, 1975).

GTN is chemically stable in neutral solutions; however, in acidic and alkaline conditions, GTN is susceptible to both hydrolytic and reductive reactions. In extremely alkaline conditions, the nitrate groups on GTN can be displaced by the hydroxyl moiety (OH^-) from the medium, either by directly attacking the carbon atom or via a concerted mechanism (DiCarlo, 1975). Acidic hydrolysis of GTN was shown to occur in the presence of 4N hydrochloric acid to yield its denitrated products, as described by Crew and DiCarlo (1968). Both of the above reactions were occurred at extremely unphysiological

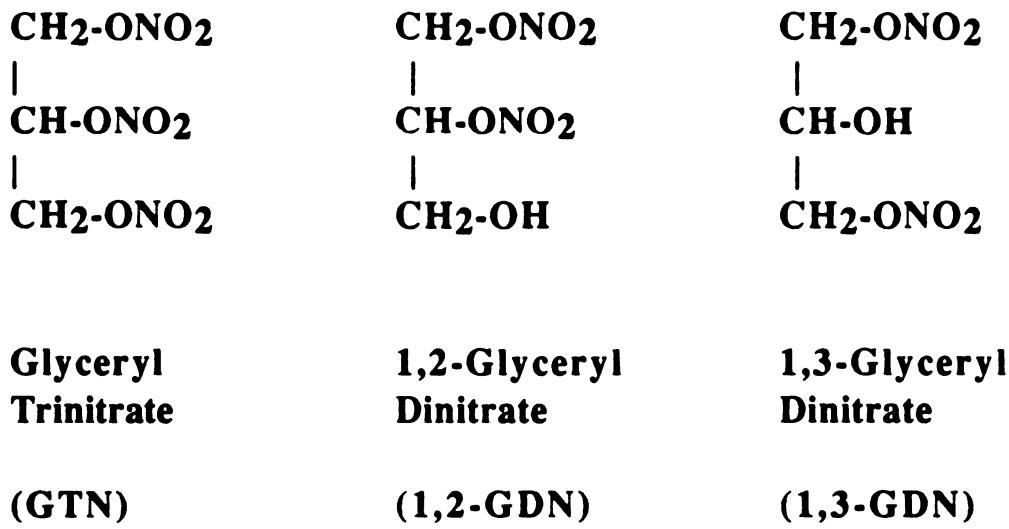


Fig. I-1. Chemical structures of glyceryl trinitrate (GTN) and its dinitrate metabolites (1,2-GDN & 1,3-GDN)

conditions, and hence, should not contribute significantly to the metabolism of the drug in vivo.

Reductive reactions with GTN have been studied by Heppel and Hilmoe (1950), who found that in the presence of reduced glutathione, GTN can be degraded to yield inorganic nitrite ions, accompanied by the formation of oxidized glutathione. The rate of this non-enzymatic reaction increased tremendously as the pH increased from 7.0 to 9.0. An equation describing the above reaction was proposed subsequently (Needleman and Hunter, 1965) :

$$C_3H_5(ONO_2)_3 + 2GSH \text{ ----> } C_3H_5(ONO_2)_2 + GSSG + HNO_2$$

It is possible that this reductive reaction, in the presence of sulfhydryl moieties, such as glutathione and cysteine, can occur at an appreciable rate at physiologic pH. At pH 7.2, all organic nitrates tested underwent reductive degradation, as demonstrated by the consumption of sulfhydryl compounds and the appearance of inorganic nitrite (Yeates et al., 1985). Interestingly, the disappearance of sulfhydryl compounds exceeded the rate of formation of inorganic nitrite. It was postulated that a thionitrate ($-S-NO_2$) may exist as an intermediate.

2. Pharmacokinetics of GTN

Although GTN has been used as a therapeutic agent for more than a century, the disposition of the drug in the body had not been studied in detail until the past two decades, when more advanced analytical methodologies for assaying the drug and its metabolites became available. GTN exhibits unusual pharmacokinetic behavior, which is characterized by an extremely high clearance and a large volume of distribution, the presence of a marked

arterial-venous concentration gradient, and extremely low bioavailability following oral administration.

2.1 Metabolism and Elimination

The plasma half-life of GTN in humans is reported to range from 1-5 minutes (Fung, 1983). A wide range of clearance values for GTN following iv administration are found in the literature. It is believed that estimates of clearance depends on the mode of administration (bolus vs. slow infusion), sampling site (arterial vs. venous), and health status of the subjects (normal vs. cardiac heart failure patients). When compared to cardiac output in man (5 L/min), the venous clearance of GTN is found to be about ten-fold higher (McNiff et al., 1981; Armstrong et al., 1982). Clearance values obtained following arterial sampling are lower, but still comparable to, or in some cases exceeding the cardiac output (Armstrong et al., 1982). This suggests that GTN is rapidly and extensively eliminated via different organs in the body.

It was also reported that GTN clearance is nonlinear (Noonan et al., 1985). Upon infusion of 10 μg/min GTN and increasing up to 50 μg/min in human volunteers, steady-state concentrations of GTN increased disproportionately by more than 25 fold. When the infusion rate was returned again to 10 μg/min, GTN concentrations did not return to the original level, but instead, remained approximately 5 fold higher than the original level. These results suggest that GTN may exhibit dose- and time-dependent clearance.

Most metabolism studies of GTN have been performed in animals, most frequently in rats, rabbits, and sheep. It is believed that the rapid and extensive elimination of GTN to form 1,2- and 1,3- glyceryl dinitrates (1,2-GDN and 1,3-GDN, Fig. I-1) accounts for the extraordinarily high clearance, since very little unchanged drug is detected in urine (DiCarlo et al., 1968; Hodgson and Lee, 1975). The metabolic scheme of GTN was examined in detail using ^{14}C-labeled GTN, and the profile is shown in Fig. I-2. Following administration, GTN is metabolized to the GDNs, which are subsequently denitrated to

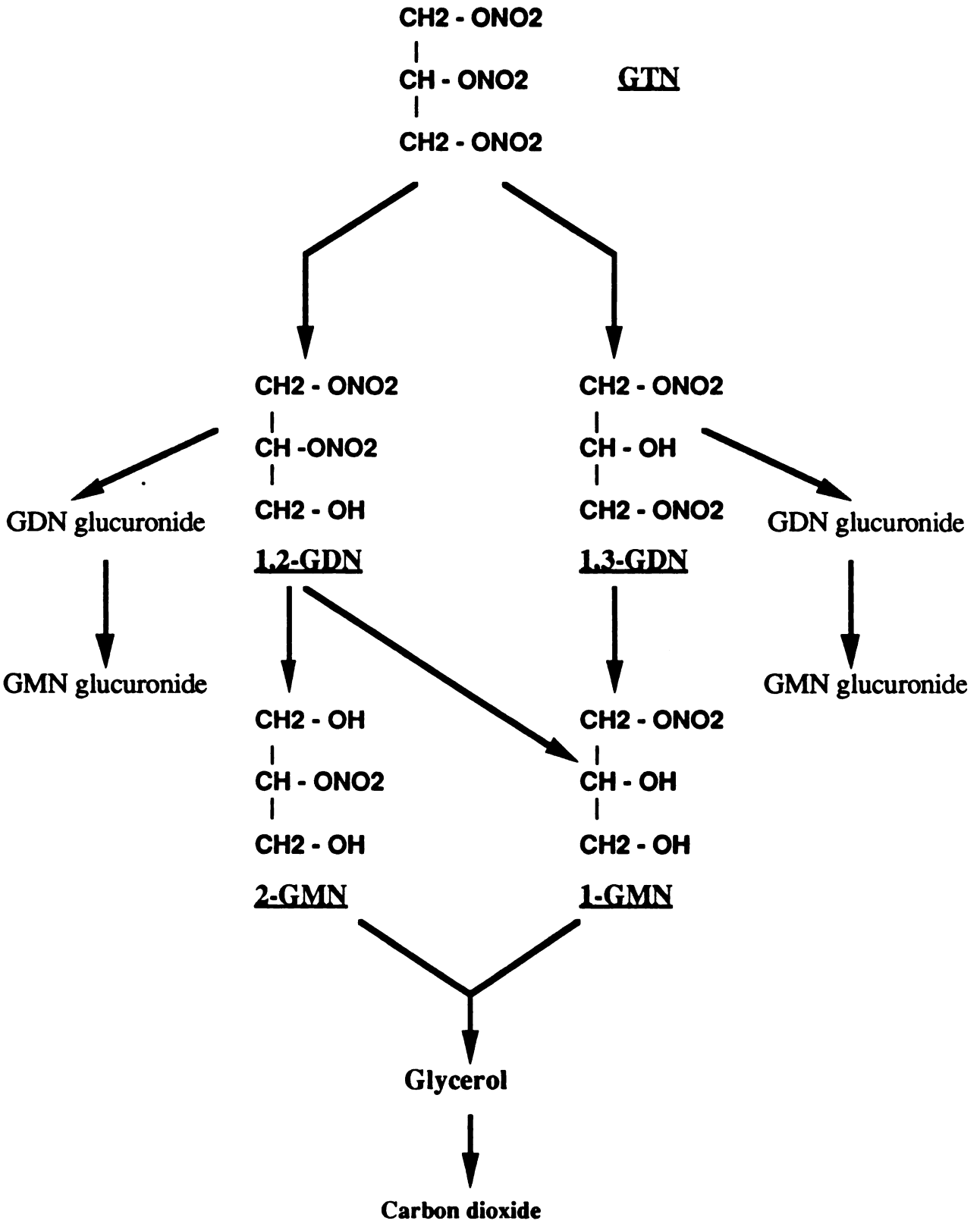


Fig. I-2 Metabolic scheme of glyceryl trinitrate (GTN)

form the glyceryl mononitrates (GMNs), and then further degraded to glycerol and carbon dioxide (Needleman et al., 1971). Glucuronides of both the GDNs and the GMNs were detected in the urine (DiCarlo et al., 1968). However, administration of the GMNs did not result in formation of GMN-glucuronides, suggesting that GMN-glucuronides which appear following GTN administration result from denitration of GDN-glucuronides. Isomers of GMN glucuronides can be formed by denitration of GDN-glucuronides, or alternatively, sequential metabolism of GTN or the GDNs to GMNs within the hepatocyte followed by glucuronidation prior to exit from the liver. The chemical structures of these glucuronides have not been characterized. It appears that denitration, followed by subsequential glucuronidation of its metabolites, represent the two major metabolic pathways of GTN in vivo.

Various organs have been shown able to metabolize GTN. In vitro incubations of GTN with homogenates of liver, lung, muscle and blood vessels in both sheep (Cossum and Roberts, 1985a) and rats (Fung et al., 1984a) led to formation of denitrated metabolites. In addition, metabolism of GTN occurs in blood itself (Noonan and Benet, 1982; Cossum and Roberts, 1985b). It is interesting to note that different organs can metabolize GTN not only at different rates, but also, can produce different patterns of dinitrate metabolite formation. For example, a study in which GTN metabolism was examined in isolated tissue homogenates, 1,2-GDN was the predominant product in liver and lung homogenates. In blood vessel homogenates, however, 1,3-GDN is the slightly preferred product (Cossum and Roberts, 1985a). In addition, the pattern of metabolite formation may also be species-dependent. For example, in the rat liver, 1,3-GDN is the predominant metabolite of GTN metabolism, whereas in the liver of other species (rabbit, dog, cat), 1,2-GDN is preferentially formed at a ratio of at least 2:1(Lee, 1973).

2.2 Distribution

In humans, the volume of distribution of GTN is about 3 L/kg, indicating that the drug distributes extensively into extravascular sites. The distribution of intact GTN in humans has not been studied in detail. However, the distribution of the ^{14}C label after oral administration of ^{14}C-GTN to rats, was studied by DiCarlo et al. (1968). The liver and the carcass took up the majority of the ^{14}C label, with the heart, lung, kidney, and spleen containing traces of the label as well. However, no distinction has been made on the identity of the ^{14}C label, which can represent both the drug and its metabolites.

Recently, tissue levels of GTN in rats following subcutaneous administration of the drug were studied using a specific gas chromatographic assay (Torfgård et al., 1989). The highest concentration was found in abdominal adipose tissue, yielding a tissue to plasma ratio of 40-180 : 1. It is believed that the high lipophilicity of GTN may facilitate its partition between these tissues. Adipose tissues may act as a reservoir of GTN, especially during sustained transdermal delivery of the drug. In the study of Torfgard et al. (1989), concentrations of GTN in the brain, the heart, and the aorta were found to yield tissue to plasma ratios greater than 2 : 1.

The uptake of GTN into blood vessels is important, especially in light of the fact that these tissues represent the effector site for organic nitrates which are potent vasodilators. Following intravascular injections in rats, GTN levels could be detected in various blood vessel segments in the vasculature (Fung et al., 1984a). The tissue to plasma ratios were found to vary with time, as well as the location of the blood vessels in relation to the site of injection, and can reach 40 : 1. In addition, GTN was found to partition better into veins than arteries. This may be an important determinant of the relatively higher potencies of GTN on the venous, as compared to the arterial, circulation.

2.3 Arterial-venous gradient

The extensive uptake of GTN into various organ beds, coupled with the high metabolism rate of the drug, results in a large gradient between the arterial and the venous

concentrations of GTN. Brymer et al. (1979) reported that following sublingual administration of nitroglycerin to human subjects, concentrations in samples drawn from the superior vena cava, the brachial artery, and a peripheral vein, were different. When steady-state conditions were achieved via administering GTN infusions, the arterio-venous gradient remained. The whole body extraction ratio was estimated to be approximately 60% across the arterial-venous bed (Armstrong et al., 1982). The large circulatory gradient for GTN suggests that the drug is extensively eliminated from various organ beds; since in this steady-state study, gradients due to transient distribution into and out of the organ beds would have dissipated after reaching equilibrium. A similar GTN arterial-venous gradient was observed in sheep catheterized at various vascular beds (Cossum et al., 1986). Extraction via different organ beds was calculated. The liver (50%-83%), the lung (28%-47%), the mesentery bed (61%-80%), and even the leg (61%-88%) all extracted GTN extensively.

It is interesting to note that a significant portion of the arterial-venous gradient may be due to vascular uptake followed by metabolism of the drug in the tissues. In dogs, which have developed tolerance to GTN, the arterial-venous gradient disappeared, indicating possible linkages between vascular GTN uptake, metabolism and effects (Armstrong-Moffat et al., 1981). The relationship between vascular metabolism and the pharmacologic effects of GTN will be discussed in detail in the next chapter.

2.4 Bioavailability

Although clearance by the liver was shown to be of relatively minor importance following intravenous administration of GTN (Blei et al., 1984a), as suggested by a clearance that greatly exceeds hepatic blood flow, the liver is the major organ for GTN metabolism when GTN is dosed orally. The availability of GTN through the sheep liver is estimated to be less than 20% (Cossum et al., 1986). In organ homogenates, the liver exhibited the highest GTN metabolic activity (Fung et al., 1984a; Cossum and Roberts,

1985a). In addition, Tam et al. (1988) reported that GTN degradation also occurs in the gastrointestinal tract, thus contributing to presystemic degradation of oral GTN preparations.

Following oral GTN administration to humans, little or no GTN was found in plasma (Noonan and Benet, 1986). As a result of the low GTN bioavailability observed, a lot of controversy with regard to the efficacy of oral GTN had been raised. Needleman et al. (1972) stated that oral dosing of GTN would be of no clinical benefit to the patient. However, other investigators have consistently observed clinical effectiveness of oral GTN (Krantz and Leake, 1975; Winsor and Berger, 1975). Some investigators suggested that saturable hepatic metabolism may occur (Bashir et al., 1982). It is our belief that oral GTN preparations are active, with most of the activity not due to GTN concentrations but rather resulting from the high levels of GDN metabolites which result from GTN metabolism.

3. Glyceryl Dinitrates (GDNs) -- Pharmacokinetics and Pharmacodynamics

The pharmacologic activities of the GDNs were first evaluated in the late 1960's. Using blood pressure depression as the measure of pharmacologic activity, Needleman et al. (1969) observed in dogs that 1,2-GDN and 1,3-GDN were respectively 10 and 14 times less potent than GTN. In another study using guinea pigs, the two metabolites were 40 and 53 times less active respectively than the parent drug (Bogaert et al., 1968). It should be noted, however, that in these studies the pharmacologic effects were measured at a single time-point. Due to differences in the pharmacokinetics of GTN and GDN metabolites, integrated measures of pharmacologic activity, such as the area under the blood pressure curve, may be more appropriate comparative indicators.

The GDNs exhibit relatively longer half-lives when compared to GTN. In dogs, GTN exhibited a half-life of 4 min, the half-lives of 1,2- and 1,3-GDN were 43 and 46 min respectively (Lee et al., 1990). Similarly, half-lives of the GDNs were much longer than

that of GTN in human subjects (Noonan et al., 1985). The longer half-lives suggest that although the GDNs may be less active than GTN, the metabolites can reside in the body much longer and thus, may significantly contribute to the overall effect observed following GTN administration. The effect due to the GDNs may be more important in cases where GDNs concentrations are many fold higher than that for GTN. The bioavailabilities of 1,2- and 1,3- GDNs were estimated to be 62% and 68%, respectively, in dogs (Lee et al., 1990), compared to low or no bioavailability of GTN. These results suggest that the pharmacokinetic differences between the GDNs and GTN may compensate for differences in "potencies" and that the contribution of GDNs to the overall effect should not be ignored.

Recently, the two GDNs were administered orally to healthy volunteers (Gumbleton and Benet, 1991), at doses which would yield similar plasma concentrations of GDNs as compared to those observed following a 6.5 mg GTN solution dose. Significant blood pressure-lowering effects were observed, further suggesting the possible contribution of GDNs to the overall GTN activity, and the potential of these active metabolites as therapeutic agents.

4. Regioselectivity in Denitration of Glyceryl Nitrates

The regioselectivity of the formation of the dinitrate metabolites from GTN presents an interesting aspect of GTN disposition. Theoretically, one might expect the ratio of 1,2-GDN to 1,3-GDN to be 2:1, simply because there are two C-1 terminal nitrate groups to be removed, thus forming 1,2-GDN, versus only one C-2 nitrate, which upon denitration yields 1,3-GDN. This expectation assumes no effect of steric factors on denitration. However, it is highly likely that steric factors would influence the pattern of metabolite formation, especially in the case of enzymatic reactions, where the active site of the enzyme may accommodate the substrate at a preferred orientation, facilitating regioselective

denitration. In vitro incubation experiments have demonstrated that GDN formation is organ-dependent (Cossum et al., 1986), as well as species-dependent (Lee, 1973).

Recently, Noonan et al. (1987) reported that variable GDN formation resulted from dosing GTN via different routes of administration. In that study, GTN was administered intravenously, sublingually, transdermally and orally. The resultant GDN ratios, measured as the ratio of the steady-state concentrations or areas under the curve of the metabolites, were different following various routes of administration. Intravenous infusion yielded the highest GDN ratio (1,2-/1,3-GDN, ratio used hereafter) at 7:1, indicating a fairly specific 1,2-GDN formation. The ratios were lower for transdermal and sublingual routes, approximately 4:1 in each case. Oral GTN dosing resulted in the lowest GDN ratio -- a 2:1 ratio of 1,2- to 1,3-GDN. These data suggest that different tissues may represent different site-specific "first pass organs" for GTN metabolism and that the various organs may contain enzymes which possess different regioselectivities for GDN formation. Metabolism of GTN was initially proposed to be carried out by one single enzyme -- organic nitrate ester reductase (Needleman and Hunter, 1965). The observation of route-dependent GDN formation, together with the in vitro organ-dependent differences in GDN formation, suggest that there is more than a single enzyme which can metabolize GTN in the body.

However, a confounding variable exists in the previous study of Noonan and Benet (1987). Since GTN was given at different dosages when administered via different routes, it is possible that the observed differences in GDN ratios may be dose-related. To address this possibility, GDN ratios were measured in healthy volunteers for a single route of GTN dosing, oral administration, with solution doses ranging from 0.4 mg to 13 mg (Nakashima et al., 1990). A gradual decrease in the 1,2-/1,3- GDN ratio (from 3.64 to 1.87) was observed as the dose was increased from 0.4 mg to 13 mg. These data suggest the presence of a dose-dependent metabolic pathway that preferentially forms 1,2-GDN may be present in the liver. It is probable that nonlinearities may also exist in other

metabolizing systems throughout the body, yielding GDN ratios which change with substrate concentration.

The regioselectivity of the formation of GMNs from GDNs has not been studied in detail. Recently, the formation of GMNs following dosing of the GDNs was examined in a preliminary study in 2 dogs, dosed intravenously with either 1,2- and 1,3- GDN on separate occasions (Carlin et al., 1989). 1,3-GDN administration resulted in formation of 1-GMN, the only possible metabolite. Surprisingly, although 1,2-GDN can theoretically generate 1-GMN or 2-GMN, only 2-GMN formation was observed. The source of this extreme regiospecificity of mononitrate formation is unknown, but it is conceivable that it may be related to the enzymatic pathways which are responsible for the C-1 denitration from GTN to form 1,2-GDN.

5. Glyceryl Mononitrates -- Pharmacokinetics and Pharmacodynamics

In the past, it was believed that the GMNs were inactive metabolites (Needleman et al., 1969). However, Leitold et al. (1986a and 1986b) reported recently that both glyceryl mononitrates (1-GMN and 2-GMN) can yield hemodynamic effects qualitatively similar to those seen for GTN. In rats, the ED_{50}'s for 1-GMN and 2-GMN with regard to T-wave suppression, supposedly a measurement of antianginal activity, were 1,200x and 5,900x, respectively, higher than the ED_{50} of GTN. Interestingly, 1-GMN was shown to be almost five times as potent as 2-GMN (Leitold et al., 1986b).

The bioavailability of 2-GMN has been reported to be 100% (Leitold et al., 1986a). With significantly lower clearances than GTN, the half-lives of the mononitrates were also shown to be much higher than that of GTN (Laufen et al., 1986; Leitold et al., 1986a), suggesting that it is possible that high doses of GMNs can give sustained pharmacologic effects. However, the contribution of the GMNs to the overall pharmacologic effects observed following GTN administration are probably minor, since the effective

concentrations of the GMNs required in these studies were much higher than those observed after GTN administration.

6. Pharmacokinetic-pharmacodynamic relationship of GTN

The pharmacokinetic-pharmacodynamic relationship for GTN is complicated and controversial (Thadani and Whitsett, 1988). Although some investigators claimed success in finding a correlation between plasma GTN concentrations and effect (Wei and Reid, 1981), most other investigators have found no evidence of such correlations (Armstrong, 1987). The presence of large inter- and intra- individual variability in both the pharmacokinetics and the pharmacodynamics of GTN certainly makes the relationship more difficult to obtain. Moreover, it is believed that there are two other major factors which will hinder attempts to establish the relationship -- the presence of active metabolites and the development of tolerance.

The pharmacokinetics and activities of the denitrated products, GDNs and GMNs, have been discussed previously. These metabolites, especially the GDNs, can contribute to the overall activity observed following GTN administration, thus making it difficult to obtain a concentration-effect relationship for the parent drug. On the other hand, one would predict that a concentration-effect relationship would be easier to obtain when these metabolites were administered and their levels were measured. In fact, a recent human study showed that upon oral administration of 1,3-GDN, the E-max model can be fitted nicely to 1,3-GDN plasma concentrations and the resultant systolic pressure depression (Gumbleton et al., 1991). In conclusion, the presence of pharmacologically active metabolites can hinder the development of concentration-response relationships for GTN.

Tolerance development is a serious clinical problem with respect to GTN therapy. It is especially apparent when GTN is administered at a fixed rate of delivery, e.g., intravenous infusion (Dupuis et al., 1990) and transdermal delivery (Abrams, 1989). The

hemodynamic effects of GTN can be lost in a few days. As a result, a change in the dosing regimen or the use of an alternative drug may be necessary. The mechanism of tolerance will be discussed in detail in the next chapter.

CHAPTER TWO

Pharmacology and Mechanism of Action of Glyceryl Trinitrate and Other Organic Nitrates

1. General therapeutic use of glyceryl trinitrate and other organic nitrates

GTN belongs to the class of drugs known as the organic nitrate esters, many of which are potent vasodilators. Table II-1 shows the various members of this class of agents currently available on the market (Murad, 1990). Among these agents, GTN and isosorbide dinitrate (ISDN) are the most commonly prescribed. In addition to the list, the 5-mononitrate metabolite of ISDN has recently been approved for use in Europe. These agents are available for different routes of administration -- intravenous, oral, transdermal, and sublingual. Preparations for acute relief of angina include the sublingual tablets of both GTN and ISDN. Controlled release preparations for oral and transdermal administration have been formulated and extensively used, mainly for the prophylactic treatment of angina.

Besides its main therapeutic use as an antianginal agent, GTN is also used in other cardiovascular diseases such as congestive heart failure (Cohn, 1985) and acute myocardial infarction (Flaherty et al., 1975; McGregor, 1983) based on its vasodilating properties. The dilatory effects of GTN on smooth muscles other than the vasculature may also be of use therapeutically. Bronchial, gastrointestinal, and uterine smooth muscles can be relaxed with GTN, though these effects may be transient and spasm of these tissues frequently follow (Needleman et al., 1985). It has been shown that GTN can be used to improve esophageal emptying in patients with achalasia (Wong et al., 1987).

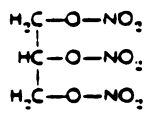
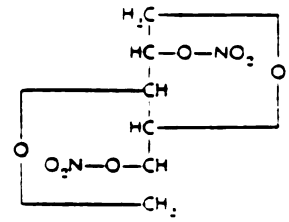
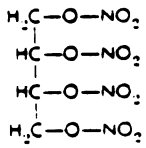
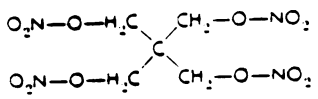
Drug	Chemical structure	Dosage Form	Dose	Dosing interval
Glyceryl trinitrate (nitrobid, nitrostat, nitro-derm, etc.)		Sublingual tablet Oral SR capsule Buccal tablet Ointment Transdermal patch Intravenous solution	0.15-0.6 mg 2.5-9 mg 1-2 mg 1.25-5 cm 2.5-15 mg 5 μg/min	-- 8-12 hr 3-8 hr 4-8 hr 24 hr --
Isosorbide dinitrate (Isordil, Sorbitrate, etc.)		Sublingual tablet Chewable tablet Oral-tablet Oral-SR capsule	2.5-10 mg 5-10 mg 5-30 mg 40 mg	4-6 hr 2-3 hr 6 hr 6-12 hr
Erythritol tetranitrate (Cardilate)		Sublingual tablet Chewable tablet	5-10 mg 10 mg	8 hr 8 hr
Pentaerythritol tetranitrate (Pentritol, Peritrate, etc.)		Oral-tablet Oral-SR capsule	10-40 mg 30-80 mg	6 hr 12 hr

Table II-1. A list of the organic nitrates currently available for clinical use (adapted from Murad, 1990)

GTN is also used to relax the contractile tone of the smooth muscles of the biliary tract in order to relieve the elevation in pressure produced by obstruction in the biliary tract (Needleman et al., 1985; Sharma et al., 1990). In patients receiving general anesthesia, GTN can be used to control intraocular pressure elevation, and therefore, may be useful to administer to patients undergoing eye surgery (Mahajan et al., 1988). Recently, GTN has been shown to increase penile blood flow (Owen et al., 1989) and could represent a new therapy for patients suffering from impotence. In addition to their vasodilating properties, these agents have been observed to inhibit platelet aggregation (Schafer et al., 1980). Thus organic nitrates could possibly be used as potential antiplatelet aggregating agents.

2. Hemodynamic effects of organic nitrates -- Mechanism of anti-anginal activities

Although GTN has been suggested for use in angina treatment for over a century, controversy remains as to which of the many hemodynamic effects of GTN (Sorkin et al., 1984) and other organic nitrates is most crucial to their antianginal activities. Understanding the pathogenesis of angina is necessary if the mechanism of action of these drugs is to be elucidated.

Angina pectoris is a common symptom of ischemic heart diseases which are usually caused by the presence of atherosclerotic plaques in the coronary vasculature (McGregor, 1983). These obstructions block blood flow to certain areas of the myocardium and cause an insufficient supply of oxygen to those regions. An imbalance between the supply and demand of oxygen therefore develops, producing ischemic conditions and causing the release of substances such as lactic acid, histamines, and kinins. Accumulation of these substances then leads to the sensation of pain in the chest area (Guyton, 1982).

Patients suffering from typical (stable, exercise-induced) angina usually encounter anginal attacks during heavy exercise, while experiencing strong emotions, or while eating, when the oxygen demand of the myocardium increases sharply. However, a second form

of angina occurs in some individuals, who may or may not have atherosclerotic plaques in their coronary circulation. This variant (Prinzmetal's) form of angina is believed to be a result of vasospasm of coronary vessels. The abnormal spasm of these vessels will lead to local ischemia in the myocardium which is similar to that in exercise-induced angina, even when the individual is at rest (Prinzmetal et al., 1959; Masseri et al., 1977). GTN and other organic nitrates are used for the management of both stable and variant angina, although in the latter case, combination therapy with calcium channel blockers, such as nifedipine and verapamil, or with beta-blockers such as propranolol, has been suggested (Conti et al., 1983).

Since angina is caused primarily by an imbalance between the demand and supply of oxygen in some regions of the myocardium, relief of angina can be obtained by either a reduction in oxygen demand on, or an increase in oxygen supply to, the myocardium. GTN and other organic nitrates probably operate via both of the above mechanisms and can be characterized within the following categories of pharmacologic actions (McGregor, 1983) : (a) indirect action via systemic venodilation, (b) indirect action via systemic arteriodilation, and (c) direct venodilatory action on the coronary circulation.

Dilating blood vessels in the systemic venous tree results in a decrease in venous return to the heart. Consequently, the left ventricular end diastolic pressure and volume are both decreased, leading to a reduction in the "pre-load" of the heart. The myocardial oxygen demand is therefore decreased. This mechanism was previously believed to be the main mechanism of nitrate action (Abrams, 1980). However, Flaherty et al. (1975) reported that a reduction in arterial pressure was also observed following nitrate administration. Strohm et al. (1983) also showed that following sublingual GTN dosing, the diameters of both abdominal arteries and veins were enlarged, as detected by ultrasound tomography. Thus, as a result of the dilation of various peripheral arterial beds, the mean arterial pressure decreases following nitrate administration. In most studies, a substantial reduction in systolic blood pressure is observed, whereas the diastolic blood pressure

remains relatively unchanged. In any case, arteriodilation following nitrate dosing constitutes a reduction in the "after-load", which helps to further reduce myocardial oxygen demand.

Although the primary hemodynamic effects of nitrates are believed to be due to their indirect actions on the venous circulation, it has been demonstrated that the coronary blood supply may also be affected following nitrate administration. Sublingual GTN administration was found to result in a dilation of conductive coronary arteries with an average 26% increase in the diameter (Feldman et al., 1980). Moreover, coronary collaterals can also be dilated by nitrates (Cohen et al., 1973). However, other investigators have observed that there is no change in the total coronary blood flow (Sethna et al., 1982). In some cases, a surprising decrease in regional coronary blood flow, such as to the left ventricles, was observed (Strauer and Scherpe, 1978), presumably due to a decreased demand for blood and oxygen because of a much-reduced left-ventricular filling pressure ("pre-load"). Therefore, it appears that GTN and other organic nitrates do not dilate all coronary blood vessels to increase oxygen delivery to all regions of the myocardium, but instead, cause a redistribution of coronary blood flow, which can selectively benefit the ischemic or stenotic regions and increase local oxygen supply to those areas.

It is extremely difficult to pinpoint the most significant hemodynamic effect of nitrates which result in benefit to angina patients. The primary action of nitrates in a certain individual may depend on the pathogenesis of angina (i.e., the type of angina) and other cardiovascular abnormalities from which the patient suffers (e.g., congestive heart failure), which affect the baseline values of the parameters upon which nitrates act.

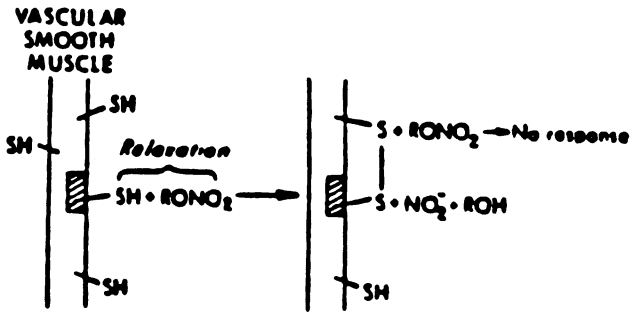
3. Molecular mechanism of action of GTN and other organic nitrates

As shown by the rapid and dramatic pharmacologic response to organic nitrates in the relief of angina, these drugs are fast-acting vasodilators. The primary mechanism of vasodilation has not been generally agreed upon, although two major hypotheses have been proposed. The major difference between the two hypotheses is that the first suggests that receptors responsible for the recognition and binding of organic nitrates exist in vascular smooth muscles (the nitrate receptor hypothesis); whereas the second hypothesis postulates that organic nitrates are in fact prodrugs, which undergo metabolism to form a pharmacologically active species which then elicits vasorelaxation via the stimulation of guanylate cyclase (the S-nitrosothiol hypothesis).

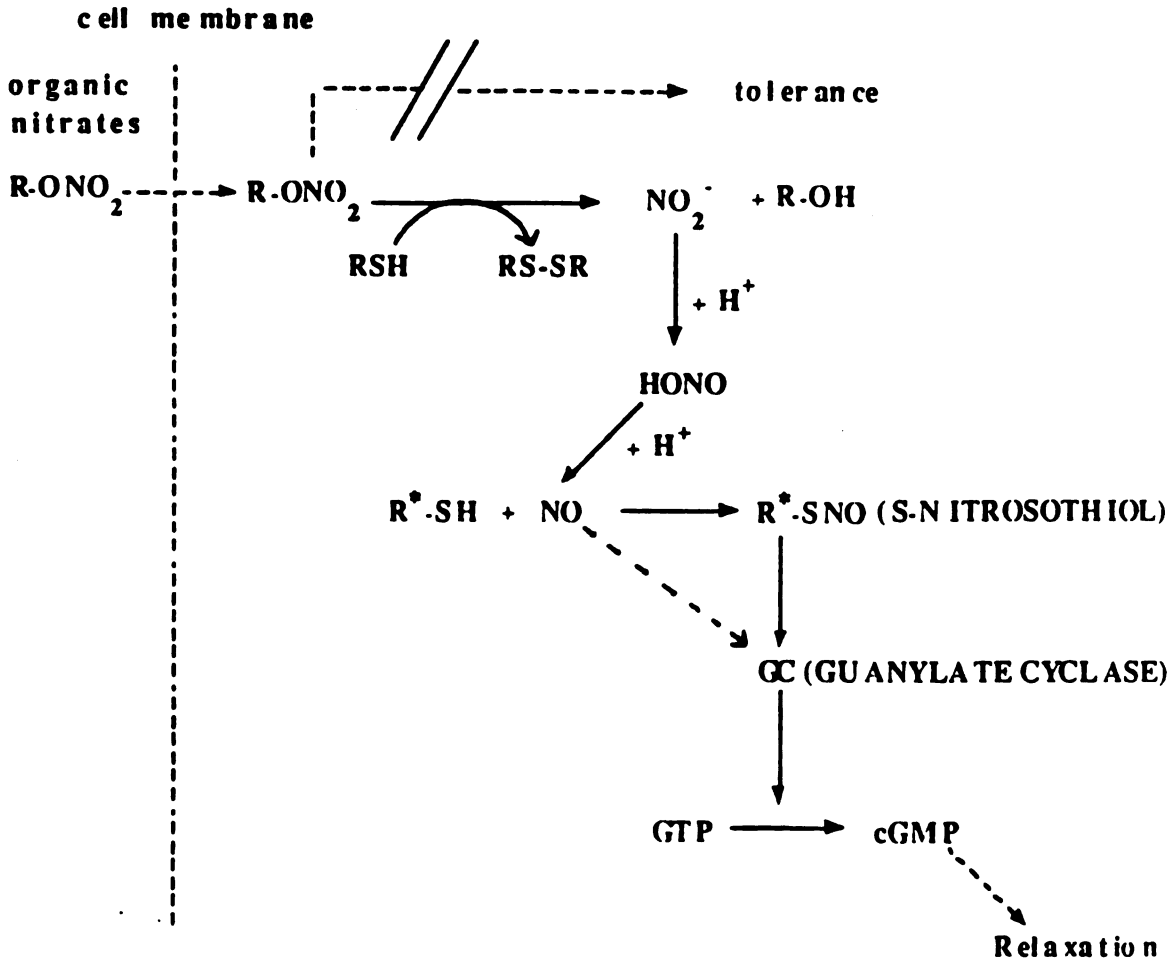
3.1 Nitrate receptor hypothesis

In 1973, Needleman and Johnson (1973) suggested that tissue sulfhydryl groups were of significance in the mechanism of action of organic nitrates. The principal evidence in favor of this hypothesis is the fact that when blood vessels were induced to develop GTN tolerance in vitro, there was a significant decrease in the total tissue sulfhydryl content. Moreover, the loss of effect was reversed when dithiothreitol, a strong reducing agent, was added to the incubation. Therefore, Needleman and Johnson (1973) proposed the existence of a receptor possessing a critical sulfhydryl residue on vascular smooth muscles that was responsible for the binding of organic nitrates (Fig. II-1a). When an organic nitrate binds to the receptor, the drug molecule is reduced to release an inorganic nitrite molecule, accompanied by oxidation of the receptor to an inactive disulfide form. Upon reduction by dithiothreitol, the receptor returns to its active form.

The major weakness of this hypothesis is that until now, attempts to isolate the proposed receptor, using the traditional method of radioligand binding, have not been successful (Ahlner et al., 1986a; Kawamoto et al., 1988). In addition, no specific antagonists for the receptor have been identified. Despite the fact that further additional evidence supports the importance of sulfhydryl compounds in the action of nitrates, the role



(a)



(b)

Fig. II-1. **Proposed mechanisms of vasodilation by organic nitrates: (a) nitrate receptor hypothesis (Needleman and Johnson, 1973) and (b) S-nitrosothiol hypothesis (Ignarro et al., 1981).**

of these compounds may be different from that suggested by this hypothesis.

3.2 S-nitrosothiol hypothesis

In 1981, Ignarro and his co-workers proposed the S-nitrosothiol hypothesis (Ignarro et al., 1981), suggesting that metabolism of organic nitrates in vascular smooth muscles leads to the formation of an active species causing vasodilation -- the S-nitrosothiol. According to the hypothesis (Fig. II-1b), the highly lipophilic organic nitrates readily enter vascular smooth muscle cells. Within the cell, sulfhydryl groups react with the drug, probably via an enzymatic process, to form denitrated product(s), e.g., GDNs form from GTN, and an inorganic nitrite ion (NO_2^-). Various investigators have reported the importance of sulfhydryl containing molecules in the metabolism of organic nitrates (Needleman and Hunter, 1965; Ignarro and Gruetter, 1980). The possible relationship of these sulfhydryl containing compounds to the generation of the vasodilating effects of nitrates has also been proposed (Needleman et al., 1969; Needleman and Johnson, 1973). The inorganic nitrite ion produced then reacts with protons in the cellular medium to generate nitrous acid (HNO_2), and subsequently nitric oxide (NO). The nitric oxide formed is then postulated to react with a second group of sulfhydryl compounds to generate the S-nitrosothiols (RSNO). S-nitrosothiols have been synthesized chemically, and were found to elicit vasodilation (Ignarro et al., 1981). As depicted in Fig. II-1b, the production of S-nitrosothiols leads to the stimulation of guanylate cyclase, an enzyme responsible for converting guanosine triphosphate (GTP) to cyclic 3'-5' guanosine monophosphate (cGMP). As a result, an accumulation of cGMP will occur in the vascular smooth muscle cells. An increase in cGMP is known to be associated with an attenuation of free cytosolic calcium concentrations (Lincoln, 1983), which will in turn lead to inhibition of calcium-dependent phosphorylase *a* activities (Johnson and Lincoln, 1985). The decrease in the activity of phosphorylase *a* causes an attenuation in myosin light chain

phosphorylation, an important step in the development of smooth muscle tension. Vasorelaxation therefore occurs as a result of this reaction cascade.

Much evidence in support of cGMP involvement in the mechanism of action of organic nitrates has been reported. In vitro, relaxation of blood vessels is preceded by an elevation in cGMP (Kawamoto et al., 1990). In addition, inhibition of guanylate cyclase activity by methylene blue was found to inhibit GTN relaxation (Gruetter et al., 1981; Martin et al., 1985). Furthermore, stabilization of intracellular cGMP levels by inhibiting phosphodiesterase, the enzyme responsible for degrading cGMP, potentiates the effects of GTN (Kukovetz et al., 1979). Therefore, it is now generally accepted that the action of organic nitrates is mediated via the stimulation of guanylate cyclase, which in turn leads to the accumulation of cGMP. There is also evidence (Brien et al., 1988) demonstrating a relationship between nitrate metabolism and cGMP elevation, in which the increase in cGMP levels was found to correlate with the extent of GTN metabolism, as measured by the formation of GDN metabolites, in rabbit aortic strips.

However, the intermediate steps between GTN metabolism and inhibition of guanylate cyclase activity have not been clarified. Two major questions exist in this part of the scheme. Firstly, controversy exists as to whether S-nitrosothiols are the true vasoactive species responsible for activating guanylate cyclase. Recently, investigators have discovered that nitric oxide is a very potent smooth muscle relaxant. In fact, nitric oxide is postulated to be (Ignarro et al., 1988), or very closely associated with (Moncada et al., 1988; Shikano et al., 1988) endothelium-derived relaxation factor (EDRF), an endogenous substance believed to be responsible for maintaining the baseline vascular tone as well as for mediating a number of other cellular processes. Nitric oxide has also been shown to stimulate guanylate cyclase (Ignarro, 1989). After incubation of GTN with blood vessel preparations, nitric oxide can be detected (Feelisch and Noack, 1987). Therefore, nitric oxide may represent the "active species" generated during organic nitrate metabolism. These observations have led to doubts as to the necessity for the generation of S-

nitrosothiols as precursors for the expression of vasodilatory effects. Secondly, the identity of the metabolizing or activating enzyme for organic nitrates has yet to be unambigiously characterized. Candidates for this crucial enzyme have been proposed, as discussed in Section 4 below.

3.3 Other mechanisms

Other reports have suggested that the effects of nitrate on vasoactive prostaglandins (Metha et al., 1983), oxidative phosphorylation in mitochondria (Needleman and Hunter, 1966), and inhibition of ATPase activity (Krantz et al., 1951) may contribute to the mechanism of vasodilation. All of these biochemical effects can modulate intracellular calcium homeostasis in vascular smooth muscle cells. However, none of these mechanisms can explain the tight relationship between GTN metabolism and effects; therefore, these effects probably represent pharmacologically active pathways of minor importance.

4. Identity of the crucial enzyme involved in vascular GTN metabolism

Chung and Fung (1990) observed that the activity of nitric oxide liberation following incubation of blood vessel preparations was associated with the plasma membrane fraction, suggesting that the crucial enzyme for the generation of pharmacologically active species may be located on the plasma membrane. However, the reported time profile for nitric oxide liberation was relatively slow, when compared to the rapid rate of GTN metabolism. Moreover, other investigators have demonstrated that activation of guanylate cyclase can occur solely in the presence of the cytosolic fraction from blood vessels (Ignarro and Gruetter, 1980; Gruetter et al., 1981), suggesting that GTN metabolism by the crucial enzyme may occur in the cytoplasm of vascular smooth muscle cells.

Another membrane fraction associated enzyme postulated as a candidate for the crucial enzyme is cytochrome P-450. Servent et al. (1989) first observed nitric oxide liberation in microsomal fractions of the liver. Recently it was shown that a cytochrome P-450 inhibitor, SKF-525A, can affect hepatic GTN metabolism under anaerobic conditions (McDonald and Bennett, 1990). Bennett et al. (1990) have shown that stimulation of guanylate cyclase was enhanced in rabbit aorta homogenates which were reconstituted with microsomal fractions of liver homogenates. The reaction mechanism proposed involves a reduction of the nitrate moiety on the parent drug to a nitrite, which is subsequently cleaved to yield nitric oxide. However, the exact mechanism has not been proven, especially for an oxidizing enzyme which would be required to carry out reductive reactions in a richly oxygenated environment. Furthermore, similar to the argument against the proposed plasma membrane enzyme as discussed above, cytochrome P-450 is known not to exist in the cytoplasm.

The crucial enzyme has also been suggested to belong to the class of hemoproteins. Various investigators have observed that GTN and other organic nitrates can be degraded by myoglobin and hemoglobin (Bennett et al., 1986). Interestingly, only the deoxy-forms of these hemoproteins react with organic nitrates. The proposed reaction involves an oxidation of the heme molecule from its ferrous state (Fe^{2+}) to its ferric state (Fe^{3+}) by organic nitrate (Marks, 1987). An inorganic nitrite will then be released, which then undergoes further metabolism as suggested by the S-nitrosothiol hypothesis. Interestingly, guanylate cyclase is also a heme-containing enzyme. According to the above reaction scheme, direct interaction between the organic nitrate and the enzyme may be possible. However, until now no attempts to purify the hemoproteins from vascular smooth muscles have been reported so as to demonstrate that organic nitrates can be metabolized by these proteins.

Glutathione S-transferase (GST) has been shown to be involved in the metabolism of GTN in the liver (Kamisaka et al., 1975). Since this enzyme is present in many

different organs in the body, it is possible that the enzyme can be located in vascular smooth muscles and be responsible for the metabolism of organic nitrates. The sulfhydryl-dependent nature of the metabolism of organic nitrates also supports the involvement of an enzyme which utilizes sulfhydryl compounds as co-factors. The majority of GSTs exist in the cytosol, although a microsomal form of the enzyme has also been recently isolated and characterized (Friedberg et al., 1979; Morgenstern et al., 1980; Morgenstern et al., 1982). Cytosolic GST exists as dimers (Jakoby et al., 1984) comprised of two identical or different subunits, resulting in the formation of different isozymes. The various isozymes for GSTs exist in different tissues (Corrigall and Kirsch, 1988), and are known to exist in different subcellular locations (Morgenstern et al., 1984). GSTs react with a wide spectrum of electrophilic and hydrophobic substrates. Different isozymes of GST possess selective, although usually not exclusively specific, substrate preferences (Habig et al., 1974). These isozymes are classified into three major classes: the α -, μ-, and π- GSTs, according to their similarity in gene constituents and cross immunoreactivities. The potential significance of GSTs in GTN metabolism and GTN-induced vasorelaxation will be further investigated in Chapters 4, 5, and 6 of this thesis.

5. Tolerance to organic nitrates and the mechanism of its development

Tolerance to organic nitrates is a common clinical problem encountered in organic nitrate therapy. When transdermal nitrate patches were first used in early 1980's, many cases of attenuated or complete loss of the drug's activity following daily application of GTN patches were reported (Parker et al., 1984; Nabel et al., 1989). Development of tolerance is also common during continuous intravenous nitrate therapy for the treatment of stable and unstable angina (Elkayam et al., 1987; Horowitz et al., 1988) and congestive heart failure (Packer et al., 1987). Tolerance development in other formulations has also been reported (Parker et al., 1985; Parker et al., 1987a). It is believed that nitrate tolerance

is associated with prolonged constant contact between organic nitrates and blood vessels, leading to attenuation of the vessel responsiveness to this class of drugs.

There are many different proposed mechanisms for nitrate tolerance development, as reviewed recently by Fung (1989) and Katz (1990). These hypotheses can be classified into 3 major categories of alterations which can occur upon prolonged administration of nitrates : pharmacokinetic, physiologic, and biochemical alterations.

5.1 Pharmacokinetic alteration

One could rationalize the development of nitrate tolerance as resulting from changes in the pharmacokinetics of organic nitrates, which could lead to a lower concentration of the parent drug in the vascular tissues, via decreased absorption, decreased tissue uptake, or increased elimination of the drug. Yet, Parker et al. (1984) observed an increase in ISDN and its 5-mononitrate plasma concentrations in patients who had developed tolerance to transdermal ISDN administration. Axelsson and Ahlner (1987) also examined whether GTN elimination via metabolism may have been altered in nitrate-tolerant animals. No evidence of altered biotransformation was found. In any case, from a practical standpoint, inter-individual variability in the pharmacokinetics of nitrates are usually quite extensive so that any pharmacokinetic alteration may not be readily detectable.

5.2 Physiologic alteration

An indirect mechanism for organic nitrate tolerance may be due to an elevation of the body's own neurohormonal response which counteracts the action of the nitrates upon prolonged administration. Packer et al. (1987) observed an increase in renin activity during a 48-hr GTN infusion, when clinical nitrate tolerance was observed. This alteration was reversible when the renin activity was measured again 24 hours after nitrate withdrawal. In another study (Dupuis et al., 1990), elevation of plasma epinephrine levels was observed upon nitrate tolerance development. This epinephrine elevation was accompanied by a

significant decrease in atrial natriuretic peptide levels. Changes in neurohormonal factors may cause salt and water retention in the body. In fact, in these studies, a decrease in hematocrit (Dupuis et al., 1990), as well as increases in total blood volume (Dupuis et al., 1990) and body weight (Packer et al., 1987), were observed. These alterations constitute a scenario of 'hemodilution', which lead to an apparent attenuation on the effects of organic nitrates. However, although good correlations exist between these physiologic changes and nitrate tolerance, the exact cause and effect relationship has yet to be determined.

5.3 Biochemical alteration

Biochemical alterations of vascular smooth muscles following development of tolerance to nitrates has been studied most extensively. One phenomenon that is generally accepted is that upon tolerance development, vascular biotransformation of organic nitrates is attenuated. Although the principal enzyme responsible for the biotransformation of organic nitrates has not been identified, various investigators have observed that in nitrate-tolerant tissues (Slack et al., 1989), homogenates (Fung and Poliszczuk, 1986), and cells (Bennett et al., 1989), a reduction in the extent of nitrate metabolism occurred. It is not clear whether the observed phenomenon is caused by a decrease in enzyme levels, enzyme activities, or cofactor levels. However, it is interesting to note that in addition to the attenuation in the extent of metabolism, there is also an alteration with respect to the regioselectivity of the denitration step. Fung and Poliszczuk (1986) first found that in rat arterial homogenates, the preference for 1,2-GDN formation during GTN degradation dissipated when incubations of GTN were performed with tolerant tissue homogenates. Similar results were reported in other in vitro experimental systems (Bennett et al., 1989; Slack et al., 1989). These investigators suggest that the pharmacologically active pathway of GTN's effect may result from 1,2-GDN formation. According to the S-nitrosothiol hypothesis, denitration of GTN from either C-1 or C-2 should result in the same inorganic nitrite ion, which is subsequently converted to the same vasoactive species. The rationale

of the proposed relationship between metabolic specificity and formation of active species requires further investigation.

The cause of the decrease in nitrate metabolism in tolerant tissues has been suggested to be a result of depletion of tissue sulfhydryl groups, which act as cofactors for the denitration reaction. Needleman and Johnson (1973) observed that tolerant tissues were associated with a decrease of tissue sulfhydryl groups. Moreover, clinical evidence has shown that administration of sulfhydryl compounds such as N-acetylcysteine (Torresi et al., 1985; May et al., 1987) and methionine (Levy et al., 1991) can reverse GTN tolerance. However, these results were not reproduced in all reported in vitro (Fung et al., 1988) and in vivo (Parker et al., 1987b; Hogan et al., 1989; Münzel et al., 1989) experiments. It was demonstrated that the beneficial effects of sulfhydryl compound co-administration may arise from a protein-catalyzed (Chong and Fung, 1990), extracellular (Fung et al., 1988) reaction between these compounds and the organic nitrates, leading to formation of S-nitrosothiols, which can then penetrate the cell membrane and activate guanylate cyclase. These data suggested that sulfhydryl depletion may not be necessary to cause nitrate tolerance.

Another biochemical change in nitrate-tolerant tissues is reported to be associated with an attenuated sensitivity of guanylate cyclase to stimulation by the pharmacologically active species from organic nitrates. In some studies of nitrate-tolerant tissue preparations, the responses to nitroprusside and nitric oxide, vasodilators which do not need metabolic activation, were unexpectedly attenuated. For instance, Waldman et al. (1986) purified guanylate cyclase from tolerant tissues via monoclonal antibodies immunoprecipitation. It was found that these purified enzymes exhibited decreased activities towards GTN, nitroprusside, and nitric oxide. Waldman et al. (1986) proposed that there is a stable alteration of the guanylate cyclase upon prolonged contact with GTN. Similar results have been reported by other groups (Axelsson and Karlsson, 1984; Ahlner et al., 1986b; Romanin and Kukovetz, 1989). In addition, an elevated activity of phosphodiesterase, the

enzyme responsible for the degradation of cGMP, was observed. As a result, an increase in cGMP turnover occurred, which may also contribute to nitrate tolerance development. However, data in conflict with this hypothesis also exist. For example, Kowaluk and Fung (1990) observed that although cGMP accumulation following GTN and nitric oxide treatment were both diminished in nitrate-tolerant tissues, the relaxation response to nitric oxide was not attenuated. These data suggest that despite the change in guanylate cyclase activity, there may be a dissociation between the resultant relaxation and cGMP response. Therefore, the observed decrease in guanylate cyclase activity may not be relevant for tolerance development in vivo. Mülsch et al. (1989) reported that the decrease in guanylate cyclase activity only occurred in homogenized preparations, and that no decrease in cGMP accumulation in intact tissues was observed -- suggesting that desensitization of guanylate cyclase should not be regarded as the basis of nitrate tolerance development.

Of all the above mechanisms, the biochemical mechanisms are generally believed to be the most crucial factors to cause nitrate tolerance. Attenuation in the metabolism of nitrates in tolerant tissues have been observed. Conflicting data on the role of desensitization of guanylate cyclase exist in the literature. Neurohormonal alterations upon repeated nitrate dosing have been observed in clinical studies. The actual development of tolerance is probably the result of a combination of all the above factors.

CHAPTER THREE

Intravenous Infusions of GTN, 1,2-GDN, and 1,3-GDN in Rats

1. Introduction

GTN has been reported to possess an unusually high clearance that approximates, or even exceeds the cardiac output (McNiff et al., 1981; Fung et al., 1984b; Noonan et al., 1985). The drug has also been shown to be metabolized rapidly and extensively by different organ homogenates (Fung et al., 1984a; Cossum and Roberts, 1985a), as well as in blood (Noonan and Benet, 1982; Cossum and Roberts, 1985b). Furthermore, it has been demonstrated that GTN can be extensively taken up by vascular tissues (Fung et al., 1984a; Cossum et al., 1986). Biotransformation of GTN can then proceed in vascular tissues, which according to the generally recognized scheme of action of organic nitrates, is essential for the generation of vasodilating effects (Ignarro et al., 1981). Following administration, GTN is rapidly converted to its denitrated metabolites : 1,2-GDN and 1,3-GDN. Recently, results from our laboratory demonstrated that the pattern of GDN formation may be a function of the route of GTN administration (Noonan and Benet, 1987), indicating that various organs may have different specificities towards GTN denitration. However, when GTN is administered via various routes of administration, different doses are generally applied. Therefore, in order to investigate whether site-dependent GTN metabolism exists, a fixed dose of GTN should be applied to various sites in the body.

The glyceryl dinitrate metabolites, 1,2-GDN and 1,3- GDN, are considered to be relatively inactive when compared to the parent drug, as evaluated previously in 'single

time-point' hemodynamic studies (Bogaert et al., 1968; Needleman et al., 1969). However, based on the differences in the pharmacokinetic behavior between GTN and the GDNs, it is possible that these metabolites can significantly contribute to the overall effects observed following GTN administration, principally because of the much prolonged residence times of the GDNs in the body (Lee et al., 1990).

2. Objectives

The objectives of the studies described in this chapter were :

a. to elucidate the pharmacokinetic properties of GTN, 1,2-GDN and 1,3-GDN in rats following intravascular infusions of these compounds;

b. to investigate the effects of varying the site of GTN infusion on the disposition of GTN and the GDNs;

c. to examine the extraction efficiency of different organs with regard to GTN; and

d. to depict the pattern of preferential GDN formation in selected organs which have been shown to be important for GTN metabolism.

3. Materials and Methods

3.1 Animals

Male Sprague-Dawley rats (250-280g) were purchased from Bantin-Kingman (Fremont, CA), and were housed in the Animal Care Facility of the University of California at San Francisco for at least two days prior to the experiment. The rats were kept in facilities maintained at twelve hour cycles of alternating light and dark periods. The animals were allowed free access to water and laboratory Purina Chow (Ralston Purina Co., Richmond, IN) until the day of experimentation.

3.2 Chemicals

GTN was purchased as 10-ml vials of Tridil (5 mg/ml) solution from DuPont Pharmaceuticals (Wilmington, DE). 1,2-GDN and 1,3-GDN (> 99% purity) were graciously supplied by Marion Laboratories (Kansas City, MO), and were used without further purification. The organic solvents used in the extraction procedure, i.e., pentane, methyl-*t*-butyl ether, and butyl acetate, were purchased at the highest grade obtainable from EM Sciences (Cherry Hill, NJ).

3.3 Surgical and Blood Sampling Procedures

3.3.1. GDN infusion studies (Rats A1-A24)

For induction of anesthesia, the rats received a 60 mg/kg i.p. injection of pentobarbital sodium (Sigma Chemical Co., St. Louis, MO), administered as a 1.5% w/v solution in saline. Subsequent maintenance doses of 6 mg/kg i.p. were given throughout the duration of the pharmacokinetic studies. A sufficient depth of anesthesia was judged to have been attained when the corneal reflex and response to hind-limb pinch were no longer elicitable.

The left femoral artery of each animal was exposed and catheterized with PE-50 polyethylene tubing (Clay Adams, Parsippany,NJ) to enable arterial blood sampling. The right femoral vein of the animal was then exposed, and catheterized with PE-10 polyethylene tubing (Clay Adams) which was directly connected to a silanized glass syringe held in place in a Harvard slow infusion pump. The catheters were kept patent with heparin sodium (20 IU/ml; Elkins-Sim Inc., Cherry Hill, NJ). All surgical wounds were covered with gauze, and kept moist with normal saline to minimize tissue fluid loss. Rectal temperatures were monitored and maintained at 38±1 ^{o}C using an incandescent

lamp. As soon as the surgical procedure was complete, the pharmacokinetic study began with the collection of a zero-time blood sample, followed by the initiation of the GDN infusion.

1,2-GDN and/or 1,3- GDN were dissolved in normal saline, and the dosing solution was infused at a constant rate over a 70 min period into the right femoral vein. The GDN infusion rates for each experimental group were as follows :

Group 1 (A1-A4)	:	1,2-GDN infusion at 0.25 μg/min
Group 2 (A5-A8)	:	1,2-GDN infusion at 2.0 μg/min
Group 3 (A9-A12)	:	Co-infusion of 1,2-GDN at 0.25 μg/min and 1,3-GDN at 2.0 μg/min
Group 4 (A13-A16)	:	1,3-GDN infusion at 0.25 μg/min
Group 5 (A17-A20)	:	1,3-GDN infusion at 2.0 μg/min
Group 6 (A21-A24)	:	Co-infusion of 1,3-GDN at 0.25 μg/min and 1,2-GDN at 2.0 μg/min

For all infusions, the flow rate was maintained at 0.0136 ml/min. Left femoral arterial blood samples (200 μl) were collected at 0, 10, 20, 30, 40, 50, 60, 70, 72, 75, 80, 85, 90, and 100 min after the initiation of infusion. An aliquot of each blood sample was placed into a silanized glass tube and immediately frozen by placing the tube into a mixture of dry ice and methanol. Blood samples were stored at -80 ^{o}C until analysis.

3.3.2 GTN infusion studies (Rats B1-B20)

The procedures for the induction and maintenance of anesthesia in the animals were similar to those of the GDN infusion studies. The right femoral artery of each rat was exposed and catheterized with PE-50 polyethylene tubing for blood sampling. For the purpose of infusing GTN, a piece of PE-10 tubing was placed in one of the following locations: the left femoral vein (for LFV infusions, B1-B5), the left external jugular vein, towards the direction of the heart (for LJV infusions, B6-B10), the left femoral artery (for

LFA infusions, B11-B15), with the cannula placed in the direction away from the descending aorta, and the hepatic portal vein (for HPV infusions, B16-B20). A scheme representing the sites of GTN administration and blood sampling used is depicted in Fig. III-1. GTN infusions were administered at a rate of 2.0 μg/min, using a flow rate maintained at 0.0136 ml/min for 60 min. Femoral arterial blood samples (100 μl) were collected at 30, 40, 50, 60, 62, 65, 70, 75, and 80 min after the initiation of GTN infusion. The blood samples were immediately frozen by pipeting into silanized glass tubes that were placed upon a mixture of dry ice and methanol. Blood samples were stored at -80 ^{o}C until analysis.

3.4 Analytical Methodology

The blood concentrations of GTN and/or the GDNs following both GTN and GDNs infusions were determined simultaneously by an improved gas chromatographic assay (Watari et al., 1991). The procedure was modified from previous methods used in our laboratory (Noonan et al., 1984; Lee et al., 1988). The procedure has the following advantages: 1. No purification of solvents is needed. 2. No interference peaks are observed, which leads to higher sensitivity of detection in particular for the GDNs. 3. The use of 2,6-dinitrotoluene and o-iodobenzyl alcohol as the internal standards makes the assay applicable in both blood/plasma samples as well as aqueous solutions, e.g., buffer samples from in vitro studies. Details of the assay are described by Watari et al. (1991), and will only be briefly discussed in this chapter.

3.4.1. Extraction

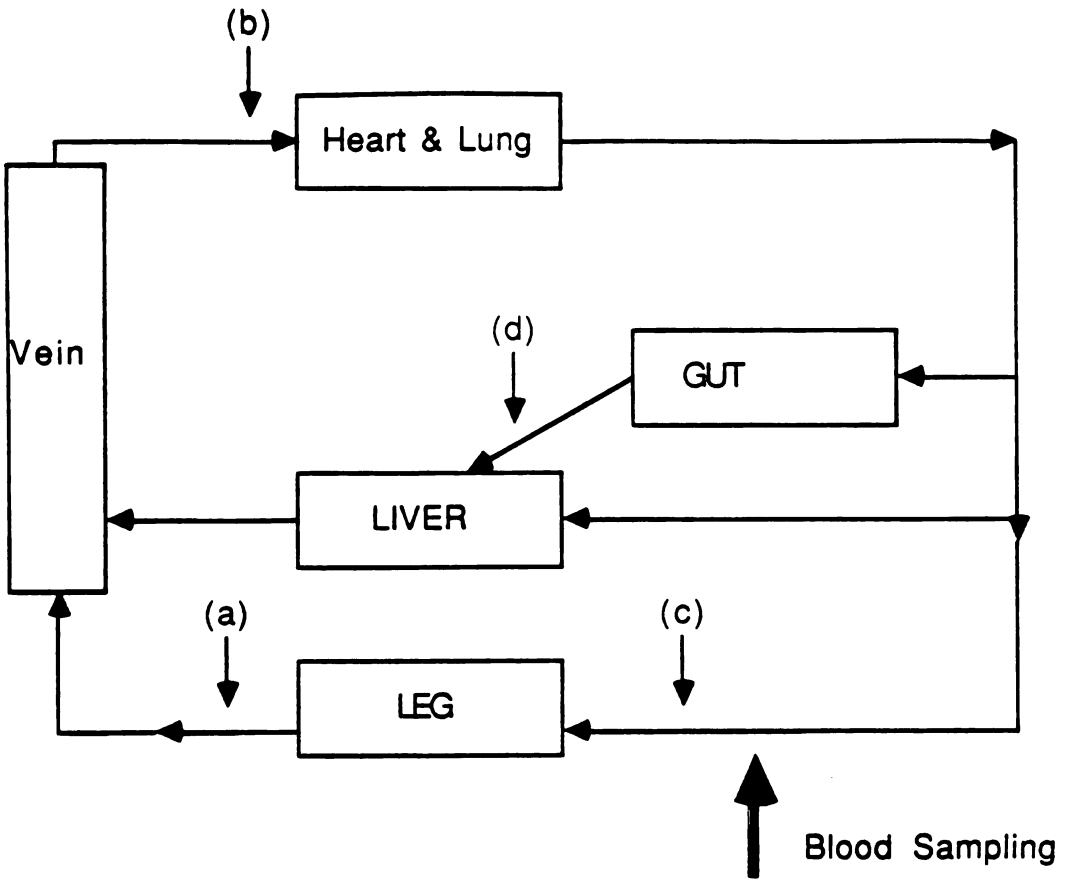


Fig. III-1. **A simplified scheme of the sites of GTN infusion and blood sampling: (a) left femoral vein infusion, (b) left jugular vein infusion,(c) left femoral artery infusion, (d) hepatic portal vein infusion. Blood samples were consistently drawn from the right femoral artery for all infusion sites.**

The physical properties of GTN and the GDNs are so different that the use of a single organic solvent to extract the three chemical entities with a reasonable recovery is extremely difficult. However, using a mixture of pentane/methyl-*t*-butyl ether, two solvents that possess low boiling points and do not require any clean-up procedures before use, GTN and the GDNs can be extracted simultaneously. The methyl-*t*-butyl ether provides some polarity to the solvent mixture, and thus the relatively polar GDNs can be extracted extensively. However, many impurities in the samples may also be extracted by methyl-*t*-butyl ether. Therefore, the optimum ratio for the two organic nitrates was examined. As shown in Fig. III-2, extracting the samples three times using a 4:1 v/v mixture of pentane and methyl-*t*-butyl ether gave the highest recovery for both GTN and GDNs; therefore, this composition was selected for subsequent extractions.

The blood samples (100 µl) were spiked with 10 ng of 2,6-dinitrotoluene as the internal standard, and mixed thoroughly with a 10-ml mixture of pentane and methyl-*t*-butyl ether (4:1 v/v) for five minutes using a Sepco Tube Rotator (Scientific Equipment Products, Baltimore, MD). Each sample was then centrifuged for 10 min at 3000 rpm, and the organic layer was collected. These procedures were repeated two more times. The organic layers from the three extractions were pooled, and placed under a stream of nitrogen for evaporation to a final volume of approximately 1 ml. This solution was then transferred to a 1-ml reaction vial and further evaporated to complete dryness. 50 µl of n-butyl acetate was immediately added to reconstitute the sample. The samples were vortexed thoroughly and stored at -20 °C until analysis.

3.4.2. Instrumentation

Varian 6000 and 6500 gas chromatographs (Varian Associates, Los Altos, CA), equipped with ^{63}Ni electron capture detectors, were used for the analysis of GTN and GDNs. Manual on-column injections were performed, which required modifying the injector port with an on-column injector accessory (J & W Scientific Inc., Rancho

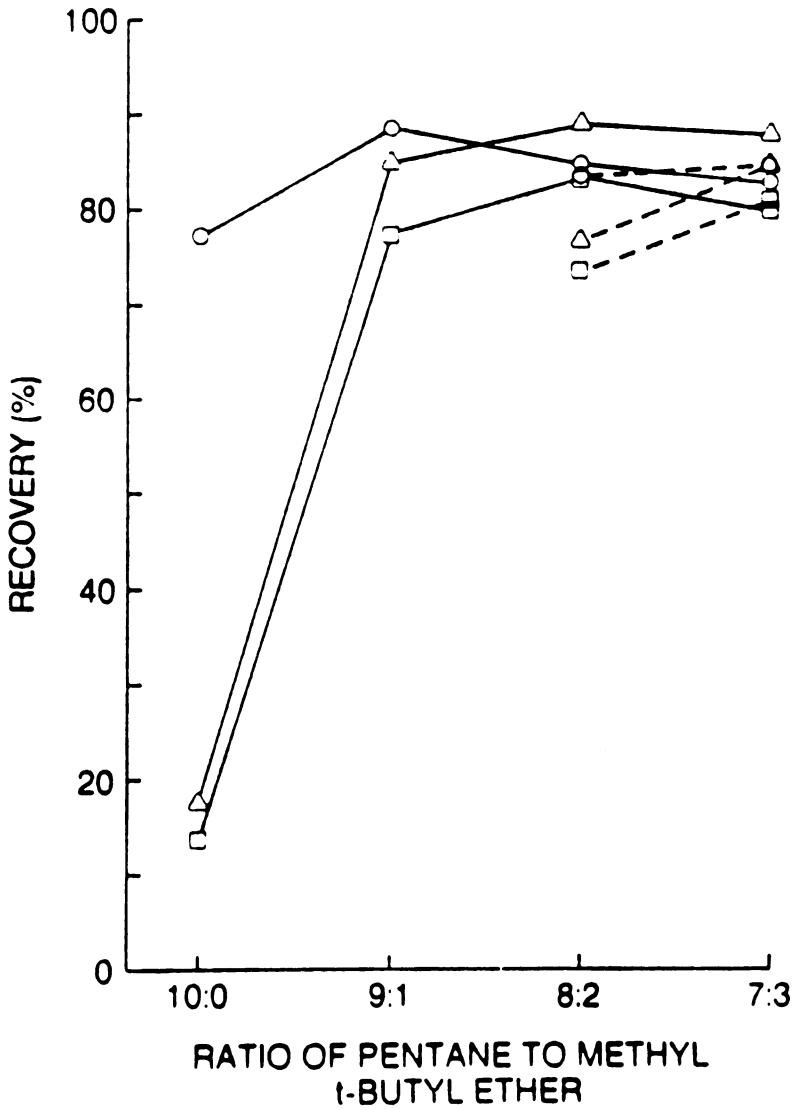


Fig. III-2. Recovery of GTN and GDNs from 1 ml of human plasma spiked with 1 ng of each compound using 10 ml extractions with various ratios of plasma and methyl-t-butyl ether. Adapted from Watari et al., 1991. (GTN -- (o), 1,2-GDN -- (□), 1,3-GDN -- (Δ). Solid lines -- 3 extractions, dotted lines -- 2 extractions)

Cordova, CA). The column found to be most suitable for the separation of the components was the HP-1 column (0.32 mm i.d. x 25 m), which contains a 1.05 μm thickness film of crosslinked methyl silicone gum as the stationary phase (Hewlett-Packard, Palo Alto, CA). A 1 m pre-column, connected to the main column via a zero-volume connector (J & W Scientific Inc.), was used to remove nonvolatile residues which would otherwise reside in the main column. The pre-columns were replaced daily. Hydrogen was used as the carrier gas through the column and the flow rate was maintained at 1.5 ml/min. Nitrogen was used for the flow into the electron capture detector as well as serving as the make-up gas; flow rates were kept at 5 and 25 ml/min, respectively.

The gas chromatographs were equipped with automatic temperature programming. The oven was kept at 95 °C for 10 min, and programmed to increase to 120 °C at a rate of 3 °C/min. This program was then followed by a second temperature ramp (50 °C/min to 280 °C) in order to burn off any residues on the column, and the oven was held at 280 °C for 5 min. Each sample run-time was approximately 30 min. The chromatograms were recorded using HP-3392 integrators (Hewlett-Packard). The peak-height ratios between the sample peaks and the internal standard peak were calculated.

3.4.3. Chromatograms

n-Butyl acetate-reconstituted samples (0.3 μl) were injected onto the gas chromatograph via a 10-μl gas-tight syringe modified to hold a fused-silica capillary needle (0.21 x 190 mm). Sample chromatograms of 1,3-GDN, 1,2-GDN, and GTN spiked into water and rat blood are shown in Fig. III-3. The three nitrates are separated cleanly; the retention times are indicated by arrows. Standard curves of GTN and GDNs for known amounts over the range of 0.5-10 ng/sample are shown in Fig. III-4. Data were expressed in peak height ratios and fitted by linear regression analysis. A positive intercept was observed for all three compounds. Linearity is observed within this sample range, but curving of the standard curves may occur beyond this range. Dilution of samples to the

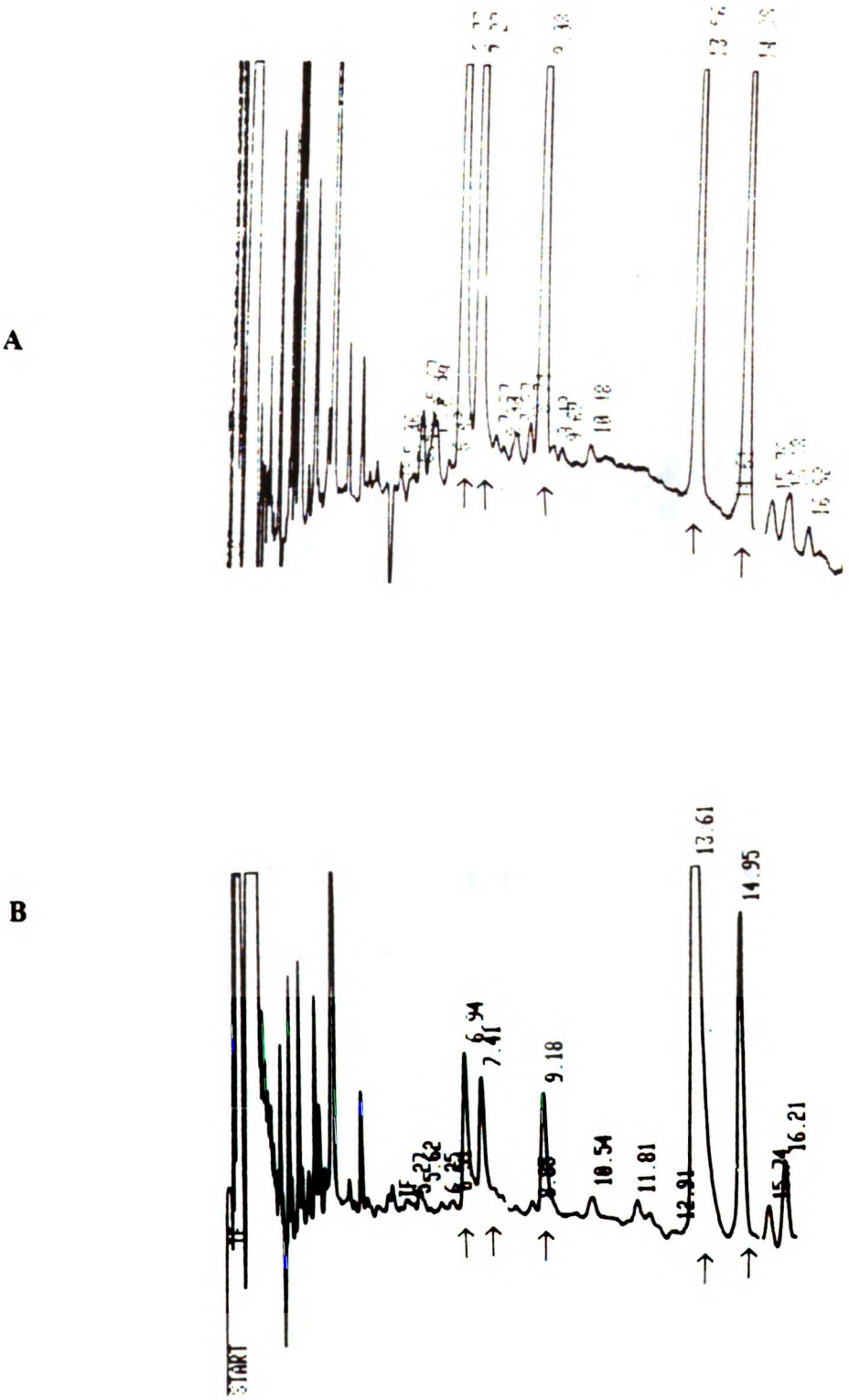


Fig. III-3. Chromatograms of GTN and GDNs in (A) a water sample and (B) rat blood. Retention times of the relevant compounds are indicated by arrows. Order of appearance : 1,3-GDN, 1,2-GDN, GTN, iodobenzyl alcohol (internal standard 1) and 2,6-dinitrotolunene (internal standard 2)

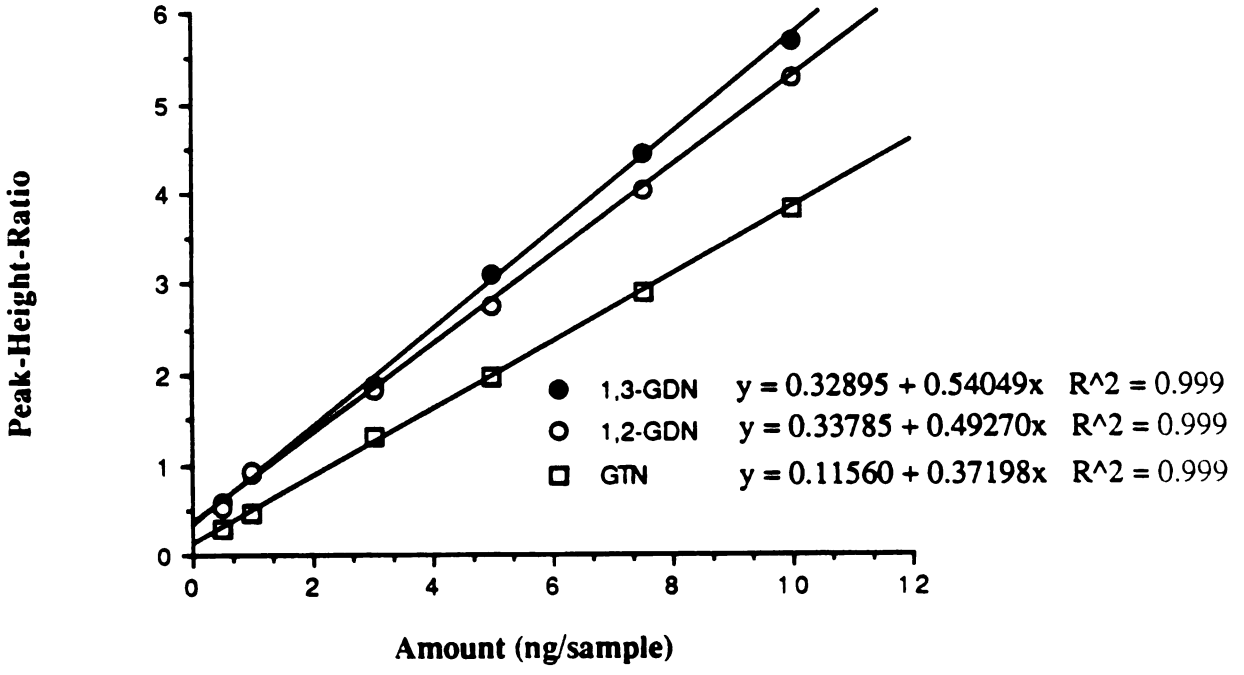


Fig. III-4. An example of the standard curves of 1,3-GDN, 1,2-GDN, and GTN in rat blood samples.

linear range is then necessary. The precision and the accuracy of the assay were examined, and found to be within 10%. Details of the methodologies involved in the validation of the assay are described in the original manuscript (Watari et al., 1991).

3.5 Pharmacokinetic Analysis

For the GDN infusions, the pharmacokinetic parameters for 1,2-GDN and 1,3-GDN were calculated using standard non-compartmental techniques (Nakashima and Benet, 1988; Watari and Benet, 1989), with the area under the plasma concentration-time curve (AUC) and the area under the moment-time curve (AUMC) calculated using log trapezoidal and trapezoidal methods, respectively. The elimination rate constant (k_{el}), and subsequently, the half-life ($t_{1/2}$), were determined from monoexponential fitting of the final 6 points during the post-infusion phase of the concentration-time profile. The steady-state blood concentrations of GDNs ($C_{ss,GDN}$) were determined as an average of the concentrations at 50, 60, and 70 min. The apparent clearances of the GDNs (CL_{GDN}) were calculated as:

$$CL_{GDN} = R_{GDN} / C_{ss,GDN} \quad \text{(ia)}$$

and

$$CL_{GDN} = Dose_{GDN}/AUC_{GDN} \quad \text{(ib)}$$

where R_{GDN} and $Dose_{GDN}$ were the rate of infusion and the dose of the GDNs. The mean residence times (MRT) and the volumes of distribution at steady-state (V_{ss}) of the GDNs were calculated as follows:

$$MRT = (AUMC / AUC) - T_{inf} / 2 \quad \text{(ii)}$$

$$V_{ss} = MRT \cdot CL_{GDN} \quad \text{(iii)}$$

where T_{inf} represents the infusion time.

For the GTN infusion studies, the apparent clearances (CL_{GTN}) of GTN for the various routes of administration are calculated by dividing the rate of GTN infusion (R_{GTN}) by the steady-state concentrations ($C_{ss,GTN}$) of GTN (determined as the mean of the 40, 50, and 60 min GTN measurements). For example, for the left femoral vein infusions:

$$CL_{GTN,LFV} = R_{GTN} / C_{ss,GTN,LFV} \quad \text{(iv)}$$

Similarly, the apparent GTN clearances for the other three infusion sites can be calculated. The availabilities of GTN through different organs (F_{organ}) can be calculated as a ratio of the apparent clearances, which are equivalent to the ratio of steady-state concentrations of GTN following different routes of administration :

$$F_{LEG} = CL_{LFV} / CL_{LFA} = C_{ss,GTN,LFA} / C_{ss,GTN,LFV} \quad \text{(v.a)}$$

$$F_{LIVER} = CL_{LFV} / CL_{HPV} = C_{ss,GTN,HPV} / C_{ss,GTN,LFV} \quad \text{(v.b)}$$

$$F_{VEIN} = CL_{LJV} / CL_{LFV} = C_{ss,GTN,LFV} / C_{ss,GTN,LJV} \quad \text{(v.c)}$$

Based on the mass balance principle, which assumes the amount of metabolite formed must equal the amount of metabolite that is eliminated, the formation clearances of each GDN from GTN are estimated by the following equations :

$$CL_{f,1,2\text{-GDN}} \times C_{ss,GTN} / MW_{GTN} = CL_{1,2\text{-GDN}} \times C_{ss,1,2\text{-GDN}} / MW_{1,2\text{-GDN}} \quad \text{(vi.a)}$$

$$CL_{f,1,3\text{-GDN}} \times C_{ss,GTN} / MW_{GTN} = CL_{1,3\text{-GDN}} \times C_{ss,1,3\text{-GDN}} / MW_{1,3\text{-GDN}} \quad \text{(vi.b)}$$

where the CL_f's represent the formation clearances of the individual GDNs. $CL_{1,2\text{-GDN}}$ and $CL_{1,3\text{-GDN}}$ are the mean clearances (elimination) of the GDNs obtained from the GDNs infusion studies. $C_{ss,1,2\text{-GDN}}$ and $C_{ss,1,3\text{-GDN}}$ are measured (average of 50 and 60 min

GDN concentration values). MW_{GTN} and MW_{GDN} are the molecular weights of GTN (227 g/mol) and the GDNs (182 g/mol), respectively.

The percentage of GTN clearance that can be accounted for by GDN formation (% recovery) is calculated as follows :

$$\% \text{ recovery} = (CL_{f,1,2\text{-GDN}} + CL_{f,1,3\text{-GDN}}) / CL_{GTN} \quad \text{(vii)}$$

where CL_{GTN} is the apparent clearance of GTN for any particular route of administration.

3.6 Statistical Analysis

For both GTN and GDNs studies, the different experimental groups were compared using either the unpaired t-test or the one-way analysis of variance. The Newman-Keuls test was performed for the purpose of multiple comparisons between individual groups. Statistical significance is achieved at $p < 0.05$.

4. Results

Figures III-5 & 6 depict the concentration-time profiles following GDN infusions of 1,2- and 1,3- GDNs for Groups 1-3 and 4-6, respectively. Steady-state concentrations were generally achieved by these different infusions within 50 min. The resulting C_{ss}, k_{el}, $t_{1/2}$, AUC, and AUMC values following the various infusions are shown in Tables III-1 and III-2 for animals receiving 1,2-GDN and 1,3-GDN, respectively. No statistical difference was found within the 3 groups for each individual GDN dosing. Although considerable variability are noted for C_{ss}, AUC and AUMC calculations, the elimination parameters (kel, t1/2) for the GDNs appear to be less variable and similar for the two GDNs.

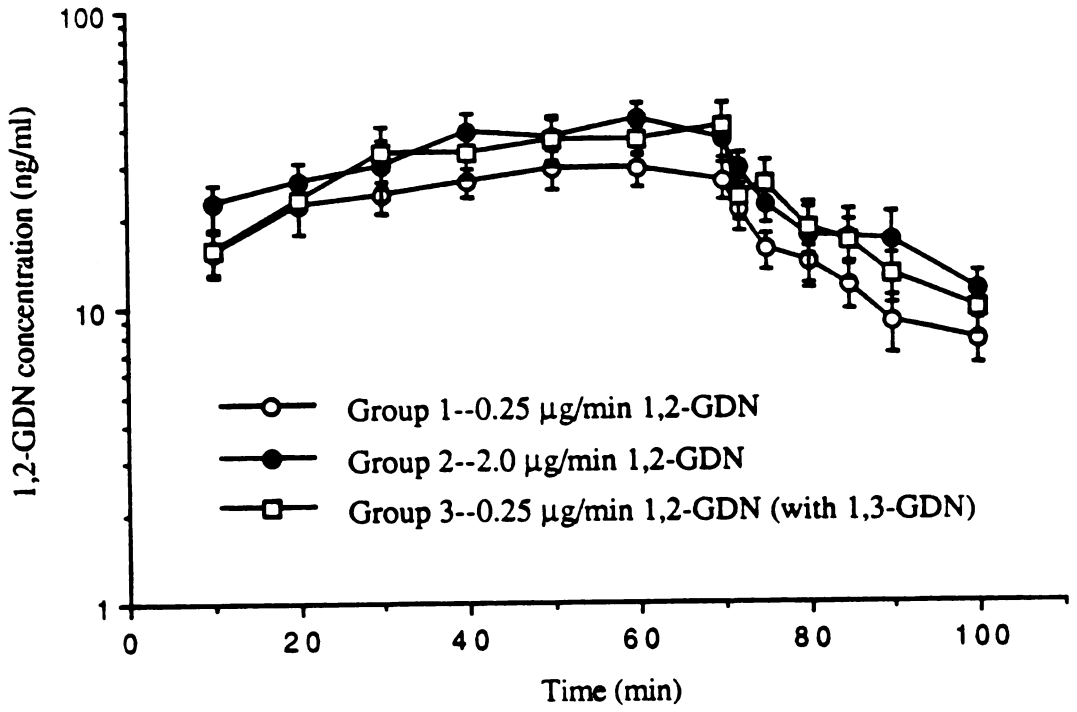


Fig. III-5. Blood concentration of 1,2-GDN following its administration to rats in Groups 1-3. Concentrations for Group 2, i.e., animals receiving the higher infusion rate, are normalized to the lower infusion rate. (mean±SE, n=4)

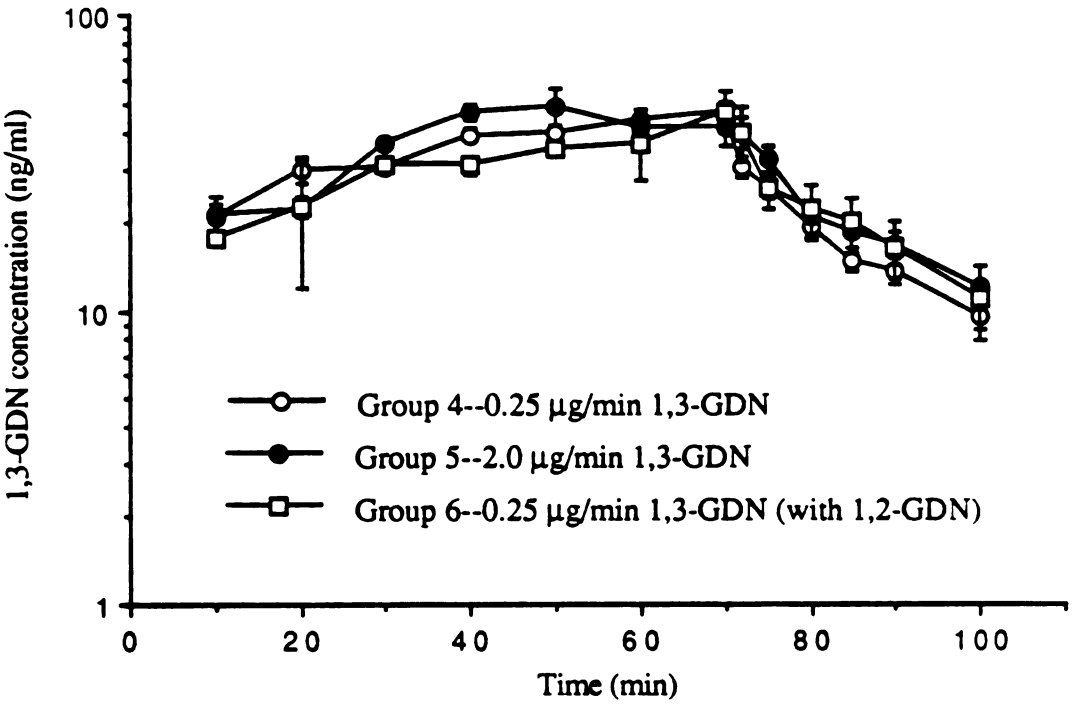


Fig. III-6. Blood concentration of 1,3-GDN following its administration to rats for Groups 4-6. Concentrations for Group 5, i.e., animals receiving the higher infusion rate, are normalized to the lower infusion rate. (mean±SE, n=4)

Infusion	Animal I.D.	Css * (ng/ml)	k el (min -1)	t 1/2 (min)	AUC * (µg/ml•min)	AUMC * (µg/ml•min^2)
1,2-GDN (0.25 µg/min)	A1	29.4	0.0315	22.0	2.246	138.4
	A2	28.0	0.0389	17.8	2.267	126.5
	A3	18.7	0.0392	17.7	1.442	76.4
	A4	37.9	0.0344	20.2	2.836	163.4
	mean±SD	28.5±7.9	0.0360±0.0037	19.4±2.1	2.198±0.573	126.2±36.6
	%CV	27.6%	10.3%	10.6%	26.1%	29.0%
1,2-GDN (2.0 µg/min)	A5	24.7	0.0336	20.6	1.802	109.3
	A6	48.8	0.0319	21.7	3.681	219.9
	A7	40.6	0.0332	20.9	3.462	189.4
	A8	46.7	0.0271	25.6	3.883	233.6
	mean±SD	40.2±10.9	0.0315±0.0030	22.2±2.3	3.207±0.952	188.1±55.6
	%CV	27.1%	9.5%	10.4%	29.7%	29.6%
1,2-GDN (0.25 µg/min; + 2.0 µg/min 1,3-GDN)	A9	48.8	0.0381	18.2	3.845	225.8
	A10	30.1	0.0435	15.9	1.930	112.6
	A11	48.1	0.0394	17.6	3.831	216.1
	A12	25.5	0.0355	19.5	2.027	117.1
	mean±SD	38.1±12.1	0.0391±0.0033	17.8±1.5	2.908±1.07	167.9±61.4
	%CV	31.6%	8.5%	8.4%	36.9%	36.6%
STATISTICS : One-way ANOVA		NS	NS	NS	NS	NS

** Values for A5-A8 have been normalized to the low dose*

Table III-1. Pharmacokinetics of 1,2-GDN following its intravenous infusion

Infusion	Animal I.D.	Css * (ng/ml)	k el (min -1)	t 1/2 (min)	AUC * (µg•min/ml)	AUMC * (µg•min^2/ml)
1,3-GDN	A13	37.8	0.0369	18.8	2.670	152.5
(0.25 µg/min)	A14	41.1	0.0466	14.9	3.091	162.1
	A15	45.6	0.0357	19.4	3.183	194.1
	A16	49.8	0.0459	15.1	3.220	182.3
	mean±SD	43.6±5.2	0.0413±0.0058	17.0±2.4	3.041±0.253	172.7±18.9
	%CV	12.0%	14.0%	14.0%	8.3%	10.9%
1,3-GDN	A17	40.2	0.0395	17.6	3.149	182.4
(2.0 µg/min)	A18	36.9	0.0382	18.2	3.061	181.2
	A19	N.C.	N.C.	N.C.	N.C.	N.C.
	A20	55.0	0.0449	15.4	4.022	229.1
	mean±SD	44.1±9.7	0.0409±0.0036	17.0±1.4	3.410±0.531	197.6±27.3
	%CV	17.3%	8.7%	8.4%	15.6%	13.8%
1,3-GDN	A21	47.8	0.0458	15.1	3.154	197.9
(0.25 µg/min; +	A22	29.2	0.0204	34.0	3.049	209.8
2.0 µg/min	A23	33.5	0.0592	11.7	2.507	125.3
1,2-GDN)	A24	44.8	0.0269	25.8	3.691	239.4
	mean±SD	38.8±8.9	0.0381±0.018	21.7±10.2	3.100±0.486	193.1±48.5
	%CV	16.5%	46.6%	47.0%	15.7%	25.1%
STATISTICS : One-way ANOVA		NS	NS	NS	NS	NS

** Values for A17-A20 have been normalized to the low dose*
N.C. A19 was dropped from the calculation because of huge GDN concentrations in blood

Table III-2. Pharmacokinetics of 1,3-GDN following its intravenous infusion

Tables III-3 and III-4 allow comparison of the clearance (CL), volume of distribution at steady state (V_{ss}), as well as the mean residence time (MRT) of the GDNs in various groups receiving 1,2-GDN and 1,3-GDN, respectively. Weight-normalized CL values were determined utilizing both the steady-state GDN concentrations (Eq. 1a) and the area under the curve (Eq. 1b). The MRT and the weight-normalized V_{ss} of GDNs were calculated according to equations (ii) and (iii) as described in section 3.5. Although the variability of these parameters is at times relatively high, there were be no significant differences among the three different groups receiving each GDN. These results suggest the absence of dose-dependent pharmacokinetics over this range of GDN concentrations in the circulation, as well as a lack of pharmacokinetic interactions between the two GDNs; although comparing Groups 1 (rats A1-4) and 4 (rats A13-16), it appears that 1,2-GDN possesses slightly higher CL (p=0.09)and Vss (p=0.06)when compared to 1,3-GDN. When the pharmacokinetic parameters obtained from all animals in the three experimental groups for each GDN administration are pooled together, as shown in Table III-5, the clearance of 1,2-GDN is slightly, but significantly, higher than that of 1,3-GDN, independent of how the clearance values are derived. However, no significant difference is found for the other parameters. Most importantly, the $t_{1/2}$ and MRT are not different between the two GDNs. Therefore, it is safe to assume that the ratio of GDNs upon GTN administration reflects the formation differences of the GDNs from GTN.

The concentrations of GTN and GDNs following the four different vascular routes of GTN infusion at 2.0 μg/min are depicted in Fig. III-7. It is evident that there were differences in the steady-state concentrations of GTN, indicating significant GTN extraction through various organ beds. Figure III-8 depicts the post-infusion GTN concentration profile following the four routes of infusions. GTN concentration rapidly decreased below the detection limit. Interestingly, the post-infusion decrease in the concentration of GTN for the LFA group appears to be much slower than for the other three routes of infusion. The LJV infusions yield a biphasic GTN degradation profile, but

Infusion	Animal I.D.	CL (area) per kg BW (ml/min/kg) *	CL (ss) per kg BW (ml/min/kg) **	MRT (min)	Vss per kg BW (ml/kg)***
1,2-GDN	A1	29.7	32.4	26.6	792
(0.25 µg/min)	A2	30.8	35.6	20.8	639
	A3	44.5	49.1	18.0	801
	A4	24.2	25.9	22.6	502
	mean±SD	32.3±8.6	35.8±9.8	22.0±3.6	695±123
	%CV	26.7%	27.3%	16.4%	17.7%
1,2-GDN	A5	39.5	41.2	25.7	1013
(2.0 µg/min)	A6	19.6	21.1	24.7	484
	A7	20.3	24.8	19.7	400
	A8	18.3	21.8	25.2	461
	mean±SD	24.4±10.1	27.2±9.5	23.8±2.8	589±284
	%CV	41.3%	34.8%	11.6%	48.2%
1,2-GDN	A9	17.3	19.6	23.7	412
(0.25 µg/min; +	A10	35.6	32.5	23.3	830
2.0 µg/min	A11	17.9	20.4	21.4	384
1,3-GDN)	A12	34.1	38.7	22.8	777
	mean±SD	26.2±10.0	27.8±9.4	22.8±1.0	600±236
	%CV	37.9%	33.8%	4.4%	39.3%
STATISTICS :					
One-way ANOVA		NS	NS	NS	NS

** CL(area) = Clearance determined by Dose/AUC*
*** CL(ss) = Clearance determined by Rate of Infusion/Css*
**** Vss calculated using CL(area)*

Table III-3. Clearance, mean residence time, and volume of distribution at steady-state of 1,2-GDN following its intravenous infusion

Infusion	Animal I.D.	CL (area) per kg BW (ml/min/kg) *	CL (ss) per kg BW ml/min/kg) **	MRT (min)	Vss per kg BW (ml/kg)***
1,3-GDN	A13	22.7	22.9	22.1	502
(0.25 µg/min)	A14	19.1	20.5	17.4	333
	A15	19.0	18.9	26.0	493
	A16	22.6	20.8	21.6	487
	mean±SD	20.8±2.	20.8±1.6	21.8±3.5	453±80
	%CV	9.9%	7.9%	16.4%	17.7%
1,3-GDN	A17	22.8	25.5	22.9	523
(2.0 µg/min)	A18	24.0	28.5	24.2	581
	A19	N.C.	N.C.	N.C.	N.C.
	A20	18.0	18.5	21.5	385
	mean±SD	21.6±3.2	24.1±5.1	22.9±1.4	496±101
	%CV	14.9%	21.3%	6.0%	20.4%
1,3-GDN	A21	20.7	19.5	27.8	575
(0.25 µg/min; +	A22	22.0	32.8	33.8	744
2.0 µg/min	A23	27.6	29.5	15.0	414
1,2-GDN)	A24	19.4	22.8	29.9	578
	mean±SD	22.4±3.6	26.1±6.1	26.6±8.1	577±135
	%CV	16.1%	23.2%	30.6%	23.4%
STATISTICS :					
One-way ANOVA		NS	NS	NS	NS

** CL(area) = Clearance determined by Dose/AUC*

*** CL(ss) = Clearance determined by Rate of Infusion/Css*

**** Vss calculated using CL(area)*

N.C. A19 was dropped from the calculation because of extraordinarily high GDN concentrations in blood

Table III-4. Clearance, mean residence time, and volume of distribution at steady-state of 1,3-GDN following its intravenous infusion

Pharmacokinetic Parameters	1,2-GDN (n=12)		1,3-GDN (n=11)		Significance Level (p)*
Css (ng/ml)	35.6 ±	10.8	42.0 ±	7.5	0.119
k el (min -1)	0.0355 ±	0.0045	0.0400 ±	0.0104	0.190
t 1/2 (min)	19.8 ±	2.6	18.7 ±	6.2	0.582
AUC (µg•min/ml)	2.771 ±	0.920	3.163 ±	0.415	0.209
AUMC (µg•min^2/ml)	160.7 ±	54.4	186.9 ±	33.0	0.182
CL(area)/BW (ml/min/kg	27.6 ±	9.3	21.6 ±	2.8	0.053
CL(ss)/BW (ml/min/kg)	30.2 ±	9.5	23.7 ±	4.8	0.052
MRT (min)	22.9 ±	2.6	23.8 ±	5.4	0.589
Vss (ml/kg)	625 ±	211	510 ±	112	0.125

** As determined by the unpaired t-test. Animals were grouped according to the GDN administered.*

Table III-5. Comparison of pharmacokinetic parameters for 1,2-GDN and 1,3-GDN

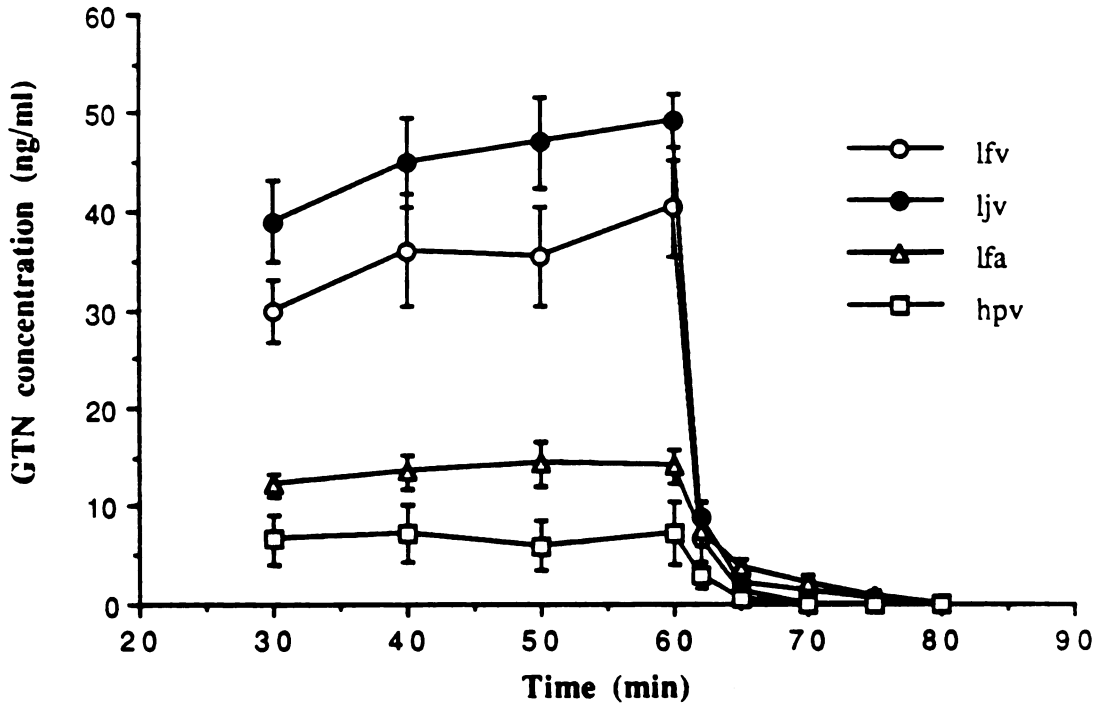


Fig. III-7 **Blood concentrations of GTN in right femoral arteries of rats during and after 60-min GTN infusions (2.0 μg/min) via left femoral vein (lfv), left jugular vein (ljv), left femoral artery (lfa), and hepatic portal vein (hpv) infusions. (mean±SE, n=5)**

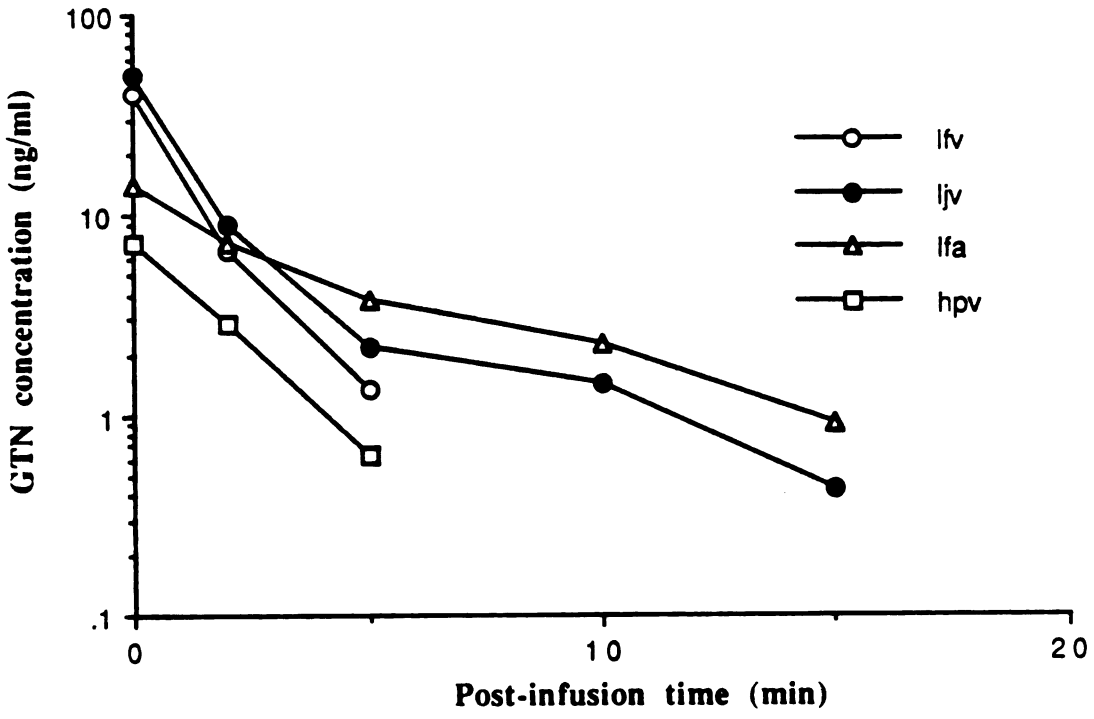


Fig. III-8. Post-infusion profile of mean GTN concentrations following infusions at various sites. (n=5)

the initial slope is similar to that for LFV and HPV infusions. In addition to the differences in GTN concentrations, differences in the ratio of resultant GDN metabolites were apparent. The concentration profiles of the GDNs following the four routes of infusion are shown in Fig. III-9. The results suggest that different organ beds which extract and metabolize GTN may exhibit different patterns of GDN formation. Table III-6 shows the steady-state concentrations of GTN (average of the 40, 50, and 60 min time-points) and GDNs (average of the 50 and 60 min time-points). LFV and LJV infusions result in significantly higher GTN steady-state concentrations than the LFA and HPV infusions. Furthermore, the 1,2-GDN concentrations resulting from the LFV and LJV infusions exceed those for LFA and HPV infusions. HPV infusions produced the highest 1,3-GDN concentrations, which were significantly higher than 1,3-GDN concentrations following LFV, LJV, and LFA infusions, all of which were comparable. The total concentration of nitrate (i.e., GTN + 1,2-GDN + 1,3-GDN) following LFA infusions was significantly lower than that following each of the other three routes of infusion. The GDN ratios, calculated as the ratios of the concentrations of 1,2-GDN to 1,3-GDN, resulting from the various GTN infusions are shown in Fig. III-10. LFV and LJV infusions produced significantly higher GDN ratios than LFA infusions. The selectivity of 1,2-GDN over 1,3-GDN formation was reversed when GTN was infused via the hepatic portal vein.

The apparent clearance values for GTN following infusion into various sites are determined as described by equation (iv), are listed in Table III-7. The apparent GTN clearances for the LFV and LJV infusions are not significantly different and are comparable to the cardiac output of the rat. On the other hand, the apparent GTN clearances of the LFA and HPV infusions are much higher than the cardiac output, indicating significant first-pass GTN extraction, presumably by the leg and liver, respectively. Using the GDNs elimination clearance values from Table III-5, the formation clearances of the GDNs from GTN are calculated as described by equations

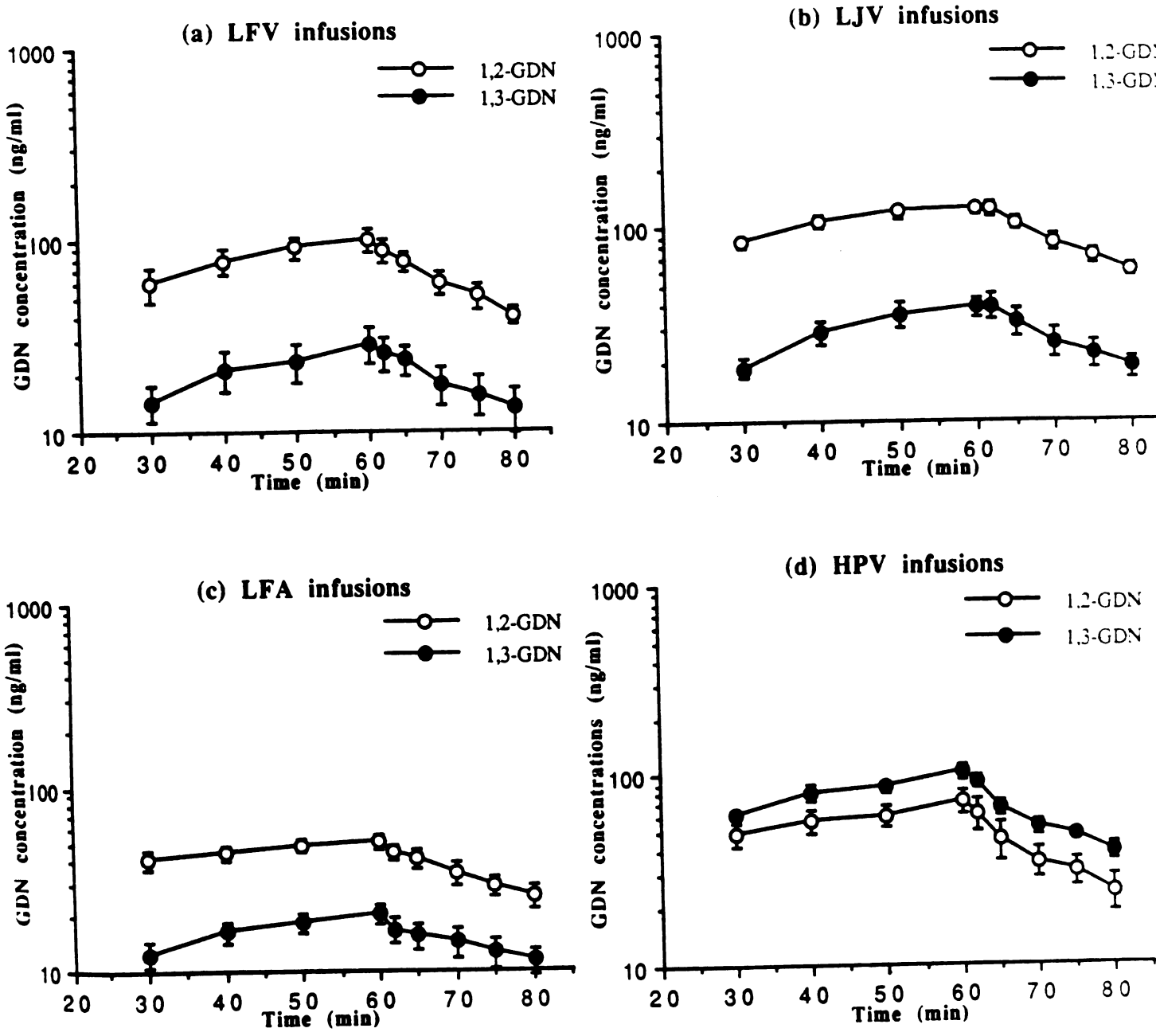


Fig. III-9 Blood concentrations of 1,2- and 1,3- GDN following various infusion routes. (mean±SE, n=5)

Site of Infusion	Animal I.D.	Css, GTN (ng/ml)	Css,1,2-GDN (ng/ml)	Css,1,3-GDN (ng/ml)	Total nitrate (nmol/ml)
LFV	B1	25.4	80.0	20.2	0.662
	B2	27.1	70.7	14.3	0.586
	B3	48.1	130.1	41.4	1.15
	B4	49.1	119.8	37.2	1.08
	B5	36.9	78.1	16.3	0.681
	mean±SD	37.3±11.2	95.7±27.2	25.9±12.5	0.832±0.260
	%CV	30.1%	28.4%	48.5%	31.6%
LJV	B6	51.0	124.7	37.6	1.12
	B7	58.6	126.8	49.4	1.23
	B8	41.3	114.9	32.4	0.991
	B9	36.2	97.7	22.7	0.821
	B10	48.8	141.3	42.5	1.22
	mean±SD	47.2±8.7	121.1±16.1	36.9±10.1	1.08±0.17
	%CV	18.5%	13.3%	27.4%	16.0%
LFA	B11	18.3	49.8	21.8	0.474
	B12	8.8	37.2	13.8	0.319
	B13	11.8	59.8	21.4	0.498
	B14	14.7	42.6	14.7	0.379
	B15	16.2	56.7	25.1	0.521
	mean±SD	14.0±3.8	49.2±9.4	19.4±4.9	0.438±0.086
	%CV	26.9%	19.2%	25.3%	19.6%
HPV	B16	0.0	49.9	88.8	0.762
	B17	6.4	59.5	96.9	0.887
	B18	2.4	48.9	118.7	0.931
	B19	10.3	77.5	73.7	0.876
	B20	15.1	96.2	96.0	1.12
	mean±SD	6.82±6.05	66.4±20.2	94.8±16.3	0.916±0.130
	%CV	88.6%	30.5%	17.2%	14.3%
STATISTICS :					
One-way ANOVA		p<0.0001	p<0.0001	p<0.0001	p<0.001
Multiple Comparison (p<0.05)		ljv=lfv>lfa=hpv	ljv=lfv>hpv=lfa	hpv>ljv>lfv=lfa	ljv=hpv=lfv>lfa

Table III-6. Steady-state concentrations of GTN, GDNs, and total nitrate following various intravascular GTN infusions

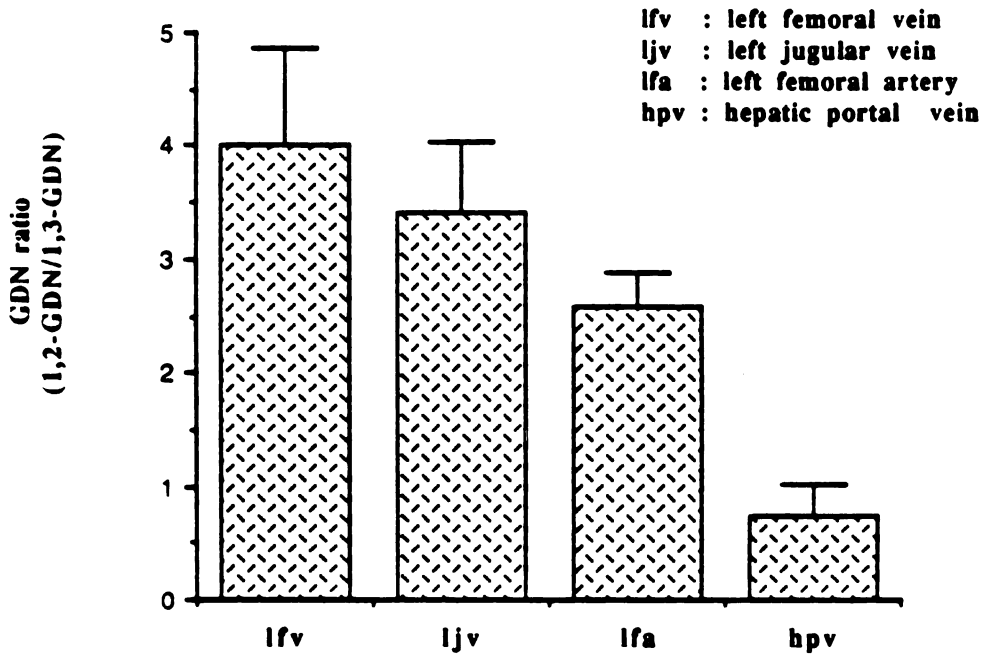


Fig. III-10. GDN concentration ratio (1,2-GDN/1,3-GDN) following GTN infusions into various sites in the rat. (mean±SE, n=5)

Site of Infusion	Animal I.D.	Body Weight (g)	CL GTN (ml/min)	CL GTN per kg BW (ml/min/kg)
LFV	B1	259	78.8	304
	B2	249	73.9	297
	B3	255	41.6	163
	B4	255	40.7	160
	B5	255	54.3	213
	mean±SD	255±4	57.9±17.8	227±70
	%CV	1.4%	30.8%	30.9%
LJV	B6	251	39.2	156
	B7	250	34.1	136
	B8	255	48.5	190
	B9	251	55.2	220
	B10	265	41.0	155
	mean±SD	254±6	43.60±8.29	172±33
	%CV	2.5%	19.0%	19.4%
LFA	B11	257	109	424
	B12	262	228	869
	B13	251	170	676
	B14	264	136	514
	B15	264	123	467
	mean±SD	260±6	153.1±47.3	590±183
	%CV	2.2%	30.9%	30.9%
HPV	B16	251	N.C.	N.C.
	B17	260	314	1208
	B18	258	835	3237
	B19	255	195	763
	B20	261	133	509
	mean±SD	257±4	369.2±320	1429±1240
	%CV	1.6%	86.6%	86.7%
STATISTICS :				
One-way ANOVA		NS	p<0.05	p<0.05
Multiple Comparison (p<0.05)		NS	hpv>lfa=lfv=ljv	hpv>lfa=lfv=ljv

N.C. -- not calculated due to undectable GTN concentration
NS -- not statistically significant

Table III-7. Apparent clearance of GTN (CL GTN) following intravascular infusions at various sites

(vi.a. & b.), as listed in Table III-8. The percentage of GTN clearance accounted for by measurable GDN formation for the LFV, LJV, and HPV infusions is about 60% (determined by equation (vii)), whereas the value for LFA infusions is approximately 30%. The GTN availabilities were calculated by equations (v.a, b & c.), yielding the following values: liver = 18.3%; leg (hind-limb) = 37.5%; veins = 79.1%. From the differences in the formation clearances, it appears that the rat liver preferentially forms 1,3-GDN, while the rest of the body forms 1,2-GDN predominantly.

5. Discussion

Although GTN has been used for the management of angina for over a hundred years, there has been little success in establishing a meaningful pharmacokinetic-pharmacodynamic relationship for the drug. For example, after GTN oral administration, physiologic effects may be observed for hours (Winsor and Berger, 1975), although no GTN can be detected in the bloodstream because of extensive first-pass metabolism. It is likely that the effects of GTN, particularly the observable sustained effects after GTN has disappeared completely from the blood stream, are at least partially mediated through the dinitrate metabolites. Previous pharmacological studies in guinea pigs and dogs have shown that the GDNs possess much less blood pressure-lowering potency than GTN (GDN < 10 % of GTN) (Bogaert et al., 1968; Needleman et al., 1969). However, recent dog studies have shown that when the blood pressure depression curve is integrated with respect to time, it can be shown that the GDNs are at least equipotent with their parent drug (Lee et al., 1990). Information on the pharmacokinetics and pharmacodynamics of GDNs upon their dosing are lacking, despite the significance that these agents may have upon GTN pharmacodynamics, and the potential of these agents as therapeutic entities in their own right. Recent work in our laboratory has been performed to elucidate the pharmacokinetics and pharmacodynamics of GDNs in dogs (Lee et al., 1990). The GDNs

Site of Infusion	Animal I.D.	CL f, 1,2-GDN (ml/min)	CL f, 1,3-GDN (ml/min))	% recovery (%) *
LFV	B1	28.1	5.54	42.7
	B2	22.4	3.54	35.1
	B3	23.7	5.91	71.4
	B4	21.4	5.19	65.3
	B5	18.6	3.03	39.9
	mean±SD	22.9±3.5	4.65±1.30	50.9±16.3
	%CV	15.3%	27.5%	32.1%
LJV	B6	21.1	4.97	66.6
	B7	18.6	5.67	71.2
	B8	24.4	5.39	61.5
	B9	23.3	4.24	49.9
	B10	26.4	6.21	79.6
	mean±SD	22.8±3.0	5.30±0.74	65.8±11.1
	%CV	13.3%	14.0%	16.9%
LFA	B11	24.0	8.23	29.6
	B12	38.1	11.1	21.6
	B13	43.8	12.3	33.1
	B14	26.3	7.07	24.6
	B15	31.8	11.0	34.7
	mean±SD	32.8±8.2	9.94±2.20	28.7±5.5
	%CV	25.0%	22.0%	19.3%
HPV	B16	N.C.	N.C.	N.C.
	B17	83.7	107	60.6
	B18	181	345	63.0
	B19	66.1	49.3	59.3
	B20	57.4	44.8	77.0
	mean±SD	97.1±57.2	136±142	65.0±8.1
	%CV	58.9%	103.9%	12.5%
STATISTICS :				
One-way ANOVA		p<0.01	p<0.001	p<0.001
Multiple Comparison (p<0.05)		hpv>lfa=lfv=ljv	hpv>lfa=ljv=lfv	ljv=hpv=lfv>lfa

* % recovery is defined as in eq. (vii)

N.C. -- not calculated due to undectable GTN concentration

Table III-8. Formation clearances of 1,2- and 1,3- GDNs from GTN following intravascular infusions at various sites

infusion studies of GDNs described in this chapter represent the first data of their pharmacokinetics in rats.

Arterial blood concentrations of the dinitrates were measured so that the clearance of these compounds can be compared to physiological flow parameters such as cardiac output. It has been demonstrated that GTN clearance exceeds both the hepatic blood flow and cardiac output of the rat (Fung et al., 1984b). The MRT of GTN, calculated as the CL divided by Vss values reported in that study approximates 6 to 11 minutes. The MRT values for the GDNs in the present study are approximately 2 to 4 fold larger, averaging 22 min. Clearance values for 1,2-GDN and 1,3-GDN as determined here (27.6 and 21.6 ml/min/kg respectively) are much lower than the cardiac output (228 ml/min/kg) and the hepato-splanchnic blood flow (60 ml/min/kg) in the pentobarbital-treated rat (Gumbleton et al., 1990). The volumes of distribution of the GDNs reported in the present study (0.6 and 0.5 L/kg) are approximately one-tenth of that for GTN, as reported to be 4-6 L/kg (Fung et al., 1984b). Based upon the greater lipophilicity of GTN relative to the GDNs, a higher partitioning of GTN into tissues would be expected. Inter-individual differences in the tissue distribution may account for the marked variability noted in the disposition of GTN.

Previously, studies of GDN disposition have been limited to the examination of metabolite kinetics following various routes of GTN administration (Noonan and Benet, 1987). In such studies, the half-lives for 1,2-GDN and 1,3-GDN were essentially the same. Here, the primary pharmacokinetic parameters of CL and Vss were examined, with the 1,2-GDN isomer observed to possess slightly larger values than the 1,3-GDN isomer (Groups 1 and 4), although the values for MRT are the same. These higher values for 1,2-GDN may be due to its higher lipophilicity, and hence, more extensive uptake into both eliminating and non-eliminating sites in the body.

Both dose-dependence (Noonan et al., 1985, Lee et al., 1990) and metabolite inhibition by GDNs (Cossum and Roberts, 1985a) have been reported in pharmacokinetic studies of GTN. The results in this study suggest that the pharmacokinetics of the GDNs

are linear over an 8-fold increase in infusion rates. Moreover, there is no pharmacokinetic interaction between the two GDNs. At present, no information is available regarding the influence of the mononitrate metabolites of the GDNs (i.e., 1-glyceryl mononitrate and 2-glyceryl mononitrate) may have on GDN disposition, although it has been reported that the mononitrate metabolites of isosorbide dinitrate can affect the pharmacokinetics of the parent drug in the rat (Sutton and Fung, 1984). Since the GDNs appear to exhibit linear disposition, the mononitrates probably exert little effect on GDN elimination.

If the GDNs are confirmed to possess significant pharmacologic activity, their administration via the oral route, rather than dosing the parent GTN may prove advantageous in terms of pharmacokinetic considerations. For instance, the improved oral bioavailability, longer residence time in the body, and smaller variability associated with their pharmacokinetics (Lee et al., 1990), may yield a more reproducible clinical response and hence, a better-defined therapeutic window, following administration of 1,2- or 1,3-GDN.

The pharmacokinetics of GTN following various intravascular infusions, and the corresponding patterns of GDN formation were also investigated here. The relationship between GTN metabolism and its mechanism of actions is intriguing. GTN is believed to exert its vasodilating actions by serving as a prodrug that can deliver the active species (an S-nitrosothiol, or nitric oxide) to vascular smooth muscle cells, which will then lead to stimulation of soluble guanylate cyclase and subsequent vasorelaxation (Ignarro et al., 1981). It is interesting to note that GTN metabolism, by which the active species are released, is essential for the generation of pharmacologic effects (Brien et al., 1988). Elucidation of the metabolic profile may be essential for understanding the disposition and effectiveness of GTN. Yet, only limited information on the selective metabolite formation by various organ beds under in vivo conditions is available. Although in vitro metabolic studies have been performed in homogenates, it has been demonstrated that organ

homogenates may give different metabolite profiles than those observed in intact organs (Marks et al., 1989a).

GTN metabolism and GDN metabolite formation in various organ beds of the rat were investigated. Since the blood samples were collected at a fixed location, i.e. the right femoral artery, in all four experimental groups, comparisons of GTN and GDN concentrations following various routes of infusion allow examination of GTN availability (or extraction) and the preference of dinitrate formation for different organs. For example, comparing LFA and LFV infusions provides estimates for the leg; similarly, comparisons of LJV with LFV infusions and HPV with LFV infusions gives information about the venous bed (mainly vena cava) and the liver, respectively. The dinitrate metabolite ratios were determined as the ratio of 1,2- to 1,3-GDN. In some animals, it appeared that the blood concentrations were still increasing slightly at the end of infusion. This is understandable because the infusion time was only slightly more than 3 times the half-lives of the GDNs. However, the half-lives of the GDN metabolites have been found to be similar in human studies (Noonan and Benet, 1987), as well as in the rat studies described above. Hence, although true steady-states for the GDNs may not have been reached in some experiments, the GDN ratios should still reflect the differences in the formation of the two GDNs, rather than their elimination.

The rank order of the GTN steady-state concentrations following infusions at the four sites are : LJV ≈ LFV > LFA ≈ HPV. It is evident that all three of the organ beds investigated here can extract and metabolize GTN, but are different with respect to both organ availability and the selective GDN formation. From comparisons between LFV and HPV, the availability of GTN through the liver is approximately 18 percent. It has been reported (Winsor and Berger, 1975; Noonan and Benet, 1986; Yu et al., 1988; Nakashima et al., 1990) that either negligible, or very low GTN plasma levels were observed following oral GTN doses in man. Fung and his co-workers (1984a) demonstrated that when higher oral doses of GTN were given to rats, the oral bioavailability was increased.

A previous GTN infusion study in sheep by Cossum et al. (1986) reported liver GTN availability measurements (0.17-0.24) similar to the values found here, at comparable GTN concentrations. It should be noted that in this study and that of Cossum et al. (1986), GTN was administered via the hepatic portal vein, and therefore may yield different, and probably higher availability values when compared to oral GTN dosing in that gastrointestinal GTN metabolism (Tam et al., 1988) is bypassed in the infusion studies.

It is also interesting to note that only the HPV infusions yield 1,3-GDN concentrations higher than 1,2-GDN. In an in vitro study of GTN metabolism, liver homogenates of various species were compared in terms of their GDN metabolite production patterns (Lee, 1973). The rat liver is unique when compared to livers of rabbits, cats, and dogs, producing 1,3-GDN as the predominant product, whereas all the other species preferentially form 1,2-GDN at ratios exceeding 2:1. The species-difference of dinitrate formation is an interesting phenomenon. Studies with subcellular fractions of rabbit livers suggest that multiple metabolic pathways for GTN metabolism may be present in the liver (Lau and Benet, 1989) and that different isozymes of glutathione S-transferase are capable of forming one GDN preferentially (Lau and Benet, 1990). Thus, the observed unique GDN formation ratio in rat livers may be due to a different distribution of various enzymes, or isozymes, when compared to other species. These in vitro experiments on hepatic GTN metabolism will be discussed in detail in the subsequent chapter.

In this study, the leg of the rat demonstrates significant GTN extraction and metabolism (F=0.375). Cossum et al. (1986) also reported significant uptake of GTN in the legs of sheep, with availability ranging from 0.12 to 0.29. When LFA infusions were administered, the GDN ratio obtained was significantly lower than the ratio obtained after LFV infusions. The difference in the GDN ratios between these infusions suggests that GTN metabolism in the leg yields a GDN ratio less than 4:1 (the resultant GDN ratio from LFV infusions). Moreover, the LFA infusion studies yielded the lowest percentage of GTN clearance that could be accounted for by the formation of GDNs. These results could

be due to high sequential metabolic activity, most probably to the mononitrate metabolites, for the GDNs in the leg. In addition, upon stopping the GTN infusion, the degradation profile of GTN was slower than with the other routes of infusion (Fig. III-8). This may be explained by the extensive GTN uptake by the hind-limb, where the drug is re-distributed to the bloodstream after the infusion ends.

Fung et al. (1984b) reported significant uptake of GTN by various blood vessels after bolus injections into the vasculature. Furthermore, the vascular GTN uptake is more extensive in venous tissues than in arterial tissues. Cossum et al. (1986) reported that the sheep aorta exhibited a very low GTN extraction ratio of 1 to 2 percent. However, no estimate of venous uptake in the sheep was determined, although it is generally believed that GTN exerts its action predominantly on the venous tissues (Kinadeter et al., 1979). In the studies reported here, the venous GTN uptake was examined by comparing the LFV and LJV infusions. The segment of the venous tree between these two infusion sites is mainly represented by the inferior vena cava. The extraction of GTN by the venous bed is approximately 20 percent, a higher value than that reported for the sheep aorta. Previously, Bennett et al. (1986) reported a very specific 1,2-GDN formation from GTN by hemoglobin and myoglobin, with ratios of 10:1 and 4:1, respectively. Therefore, the high specificity for 1,2-GDN formation for the intravenous infusions in this study may result from blood metabolism. Hence, blood metabolism may be more important than reported earlier, where metabolism in blood was estimated as only 1 % of the total body clearance of GTN (Noonan, 1982). On the other hand, Slack et al. (1989) reported a very specific 1,2-GDN formation (8 : 1) in rabbit aortic strips. In another rat study, homogenates of the arteries and veins generated GDN ratios of approximately 4 : 1 and 2 : 1, respectively (Fung and Poliszczuk, 1986). This indicates that blood vessels may also possess the capability to form 1,2-GDN preferentially. In addition, it has been reported that in tolerant tissues, the 1,2-GDN formation decreased more extensively than the 1,3-GDN formation (Fung and Poliszczuk, 1986; Bennett et al., 1989; Slack et al., 1989). This leads to the

suggestion that the 1,2-GDN formation pathway may in fact be the pharmacologically effective pathway which is responsible for generating vasoactive species. It is tempting to speculate that the enzymatic pathway which is responsible for the highly selective GDN formation in LFV and LJV infusions, may also represent the GTN-metabolizing pathway that is responsible for generating the effects of GTN on the venous tree.

It is important to mention that many of the pharmacokinetic calculations described in the GTN infusion studies assume linearity in the disposition of GTN and GDNs. The GDN infusion studies in rats show linearity in the pharmacokinetics of both 1,2-GDN and 1,3-GDN with increasing infusion rates at GDN concentrations similar to those observed in the multiple infusion site studies. Moreover, there was no noticeable interaction between the GDNs. Although 1,2-GDN seemed to possess a higher clearance value than 1,3-GDN, it also possesses a slightly higher volume of distribution. As a result, the resultant half-lives and mean residence times remained similar. Since the elimination half-lives of both GDNs are similar, it is possible to conclude that the observed GDN ratio is a reflection of the formation ratio, and not a function of differences in elimination kinetics for the GDNs.

Previously, Noonan and Benet (1987) reported variable GDN ratios as a function of GTN dosing route in healthy human volunteers. Intravenous infusions resulted in the highest GDN ratio (about 8 : 1). The transdermal and sublingual preparations yielded intermediate ratios (about 4 : 1 and 5 : 1, respectively), while oral dosing resulted in the lowest GDN ratio (2:1). The results for LFV and HPV infusions in this study with rats are consistent with the trend observed in the previous human study -- i.v. infusions yielding the highest GDN ratio, oral doses yielding the lowest ratio, although the values are different, probably due to inter-species differences. Therefore, this study supports the hypothesis that the differences in GDN ratios observed in the previous human study are a function of the route of administration.

6. Summary

In summary, the pharmacokinetics of GTN and GDNs following their intravascular infusions have been studied. The two GDNs were found to be similar with respect to their pharmacokinetic behavior. Furthermore, no dose-dependency and pharmacokinetic interactions between the GDNs were observed. Intravascular infusions of GTN at various sites - though at the same rate - produce significantly different GTN and GDN concentrations. The resulting ratios of GDN formation are also different. The liver, the leg, and the veins are all organs that can extract and metabolize GTN from the bloodstream, and they possess different patterns of dinitrate formation.

CHAPTER FOUR

Metabolism of GTN in Subcellular Fractions of Rabbit Liver

1. Introduction

The metabolism of GTN by the liver has been a subject of investigations for decades. Although it has been shown that the liver may not be an important organ for either the pharmacokinetics (Blei et al., 1984a) or the pharmacodynamics (Blei et al., 1984b) of GTN following systemic administration of the drug, the organ remains the most significant site for GTN metabolism following oral administration. In animal studies, the liver is shown to metabolize GTN extensively both in vivo (Cossum et al., 1986; Nakashima et al., 1991) and in vitro (Fung et al., 1984a; Cossum and Roberts, 1985a). It has also been reported that GTN elimination following oral dosing is apparently carried out via a saturable first-pass process. Moreover, the resulting dinitrate metabolite ratio (1,2-GDN/1,3-GDN) was also found to be dose-dependent in a human study recently carried out in our laboratory (Nakashima et al., 1990). The nature of these metabolic pathways, as well as the enzymes responsible for these processes, have not been clarified.

Needleman and Hunter (1965) reported the presence of a single, drug-class specific enzyme identified as "organic nitrate reductase", which is believed to be the enzyme responsible not only for GTN metabolism in the liver, but other organs in the body as well. It was reported subsequently that hepatic GTN metabolism may in fact be mediated by the enzyme glutathione S-transferase (GST) (Kamisaka et al., 1975), an enzyme which is responsible for many phase II conjugation reactions. GST is known to exist in various isozymic forms (Habig et al., 1974; Jakoby et al., 1984; Boyer, 1989), and to be present at

different subcellular locations within the cell (Morgenstern et al., 1982 & 1984). These isozymes, though similar in structure, exhibit differences in substrate specificities. It is conceivable that these various isozymes can constitute separate pathways which have different roles in carrying out GTN metabolism and GDN formation by the liver as a function of different incoming substrate concentrations.

2. Objectives

The objectives of the studies described in this chapter are :

a. to examine the differences in the rate of GTN metabolism, as well as the pattern of GDN formation, in the cytosolic and microsomal fractions of rabbit liver;

b. to characterize the source of the non-linearity in GTN metabolism and GDN formation observed when oral GTN doses were administered; and

c. with the use of various GST inhibitors, to investigate the role of GST isozymes in hepatic GTN metabolism.

3. Materials and Methods

3.1 Chemicals

GTN solutions were diluted from Tridil solutions (American Critical Care, McGaw Park, IL) used for intravenous GTN administration. GDNs were generously supplied by Marion Laboratories (Kansas City, MO). Tris hydrochloride, potassium chloride, reduced glutathione (GSH), sulfobromophthalein (SBP), p-nitrobenzyl chloride (p-NBC), ethacrynic acid (ECA), 1-chloro-2,4-dinitrobenzene (CDNB), and iodomethane (IDM) were all purchased from Sigma Co. (St. Louis, MO) at the highest grade of purity obtainable.

3.2 Preparation of subcellular fractions

New Zealand White rabbits (2-3 kg, male), obtained from Nitabell Rabbitry (Hayward, CA), were sacrificed by decapitation. The liver of each animal was immediately perfused with an ice-cold buffer of 0.05 M Tris HCl-0.15 M KCl buffer at pH 7.4 to remove any blood remaining in the organ. The organ was then minced and homogenized with two volumes of buffer using a Sorvall ® Omni-mixer (Ivan Sorvall Inc., Newtown, CT). The liver homogenates were centrifuged at 9000g and the supernatant layer was decanted and saved for further ultracentrifugation at 105,000g at 4° C. The 105,000g supernatant (cytosolic fraction) and pellet (microsomal fraction) were separated and re-centrifuged two more times to minimize contamination of the content of each with the other fraction. The fractions were stored at -70 °C prior to the incubation experiments. Similar methods were used for the preparation of the subcellular fractions from rat livers.

3.3 Incubation of subcellular fractions

3.3.1. 2-hour incubation studies

In each incubation, 2.0 mg/ml of protein from either subcellular fraction was used, with protein content determined by the method of Lowry et al. (1951). Glutathione (2 mM) was added to the buffered sample solutions (Tris HCl-0.15 M KCl, pH 7.4) to avoid co-factor depletion. GTN, at a starting concentration of 20 ng/ml, was added to the incubation, and 500 µl samples were drawn at 0.5, 5, 10, 15, 30, 60, 90, and 120 minutes. Further reaction was stopped by immersing the samples into a mixture of dry ice and methanol. In studies with SBP (at 17.6 and 88 µM), the inhibitor was pre-incubated for five minutes before GTN was added.

3.3.2 Single time-point incubation studies

Subcellular fractions from 4 rabbits were pooled for the incubation experiments. Protein concentrations of 1.5 and 2.0 mg/ml were used for incubations of cytosolic and microsomal fractions, respectively. GSH (2 mM) was added to the buffered incubation medium. Various inhibitors of GTN metabolism were added at 16.0 μM, a concentration two-hundred-fold greater than the lowest GTN concentration and sixteen-fold greater than the highest concentration utilized. Subcellular fractions, as 1.0 ml aliquots, were then pre-incubated for 5 min at 37°C. A 100 μl aqueous solution of GTN (containing one of the following amounts of drug: 20, 50, 100, 200, and 250 ng, resulting in starting GTN concentrations of 0.08, 0.2, 0.4, 0.8, 1.0 μM) was added to the incubate (volume of total incubation mixture is 1.10 ml) and a 500 μl sample was drawn from the incubation medium at 1.5 min and 2.0 min, respectively for cytosolic and microsomal incubations. The samples were frozen immediately in a mixture of dry ice and methanol.

3.4 Protein determination assay

Determination of the protein concentration in each subcellular fraction was adapted from the methods of Lowry et al. (1951). Samples from various subcellular fractions (100 μl) were diluted to 3 ml by the addition of a 0.1 M sodium hydroxide solution mixture which contained sodium carbonate (2%), sodium potassium tartrate (1%) and copper (II) sulfate (1%). The mixture was let stand for 15 min at room temperature. Then, 0.3 ml of 50%-diluted Folin and Ciocalteu's phenol reagent was added to the mixture (Sigma Co.). The reaction mixture was then allowed to stand for 30 min at room temperature, and the absorbance was measured at 550 nm using a Perkin-Elmer Spectrophotometer LC-55 (Oak Brook, IL). Known amounts of bovine serum albumin were prepared as standards over the range of 0-100 μg protein to construct a standard curve. A sample of the standard curve is shown in Fig. IV-1. Each point on the standard curve represents the mean of

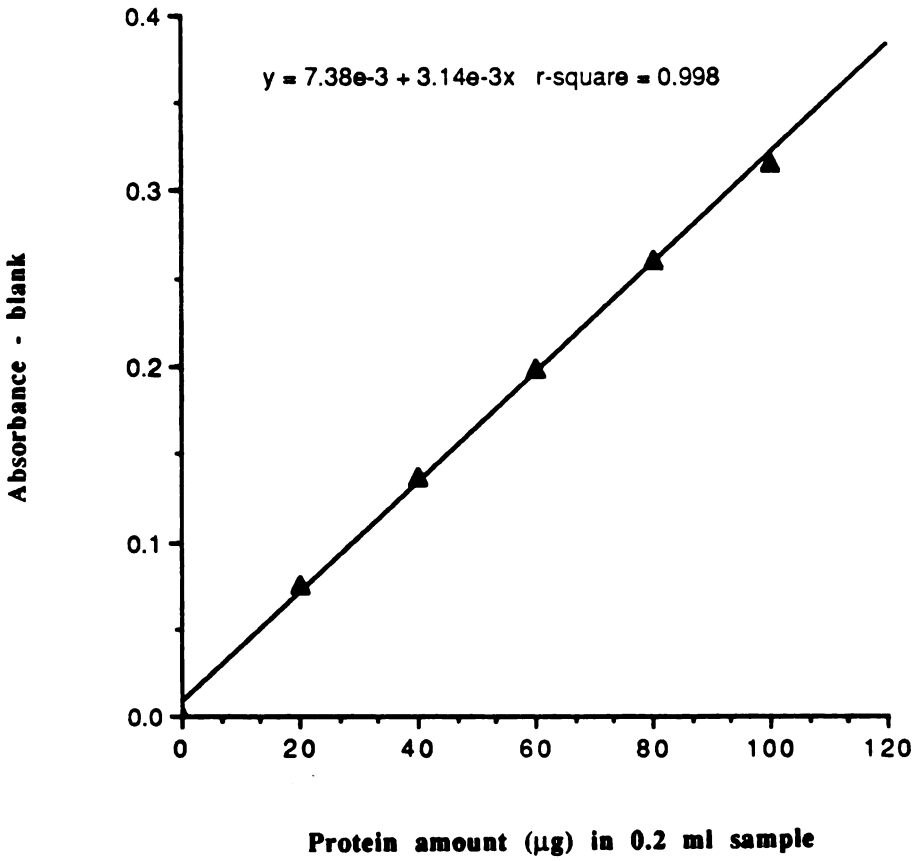


Fig. IV-1. An example of a standard curve for the determination of protein content according to Lowry et al. (1951).

duplicate samples; experimental samples from various subcellular fractions were run in triplicate. The absorbance values of the samples were converted to amount of protein by means of linear regression, and were multiplied by the dilution factor to obtain the actual protein concentration of the subcellular fractions.

3.5 Assay of GTN and GDN metabolites

The concentrations of GTN and GDNs in the samples were determined simultaneously by the capillary gas-chromatographic method described in Section 3.4 in Chapter III. Briefly, samples were extracted three times with 10-ml mixtures of pentane and methyl-*t*-butyl ether (8:2). The organic phase was almost evaporated to dryness and 50 µl of *n*-butyl acetate was used for reconstitution of samples. A 0.3 µl aliquot was injected onto Varian 6000 & 6500 GCs equipped with electron capture detectors. Iodobenzyl alcohol and 2,6-dinitrotoluene were used as the internal standards. A clean baseline was obtained with 1,3-GDN, 1,2-GDN and GTN clearly separated and eluted in that order. Separate standard curves were prepared using different subcellular fractions used in the incubation studies. Linearity was observed over the range between 0.25 ng/ml to 20 ng/ml. Samples at higher concentrations were diluted with blank subcellular fractions to concentrations within the linear range.

3.6 Statistical Analysis

One-way ANOVA was used to detect differences among various incubations. The Dunnett's test was used for comparison of a single group to the control. Data are expressed in mean ± SD. The Lineweaver-Burke analysis was performed to obtain the E_{max} and K_m of various kinetic processes.

4. Results

4.1 Conditions of incubation studies

Figure IV-2 demonstrates GTN metabolism in rabbit liver homogenates, resulting in the formation of dinitrate metabolites from 20 ng/ml GTN. GTN was metabolized rapidly in the homogenate, and the concentrations of the GDNs reached constant values after 10 min of incubation. The importance of pH in the incubation is demonstrated in Fig. IV-3. Incubations of 20 ng/ml GTN in liver homogenates from a rabbit were performed at different pH's, and the concentrations of GDNs formed at 120 min were measured. GTN metabolism occurred at the highest rate at pH 7.4. Under more alkaline (pH=7.8) or acidic (pH=7.0 or 6.6) conditions, formation of the GDNs was significantly reduced.

GTN metabolism was found to be GSH-dependent in both the cytosolic and the microsomal fractions of rabbit liver. Rates of GTN degradation with varying concentrations of GSH in the subcellular fractions are shown in Figs. IV-4a & b. Without GSH addition, the cytosolic fraction still exhibited GTN metabolism, although at a reduced rate (Fig. IV-4a). This is probably due to the presence of residual GSH in the cytosol of liver cells. When GSH was added, the rates of GTN degradation increased considerably . In the microsomal fraction, without GSH addition, no GTN metabolism was observed (Fig. IV-4b). These results suggest that the pathways by which GTN is metabolized in both subcellular fractions are glutathione-dependent.

Figure IV-5 demonstrates the effect of heat on GTN metabolism in the cytosol and the microsomes of rabbit liver, which were heated to boiling in a water bath before incubation. Negligible metabolism was found to occur with the subcellular fractions, even in the presence of 2 mM GSH. This suggests that the metabolic processes observed with GTN are predominantly enzymatic in nature. Figure IV-6 shows the effect of varying the protein concentrations on GTN degradation. The cytosolic and the microsomal fractions

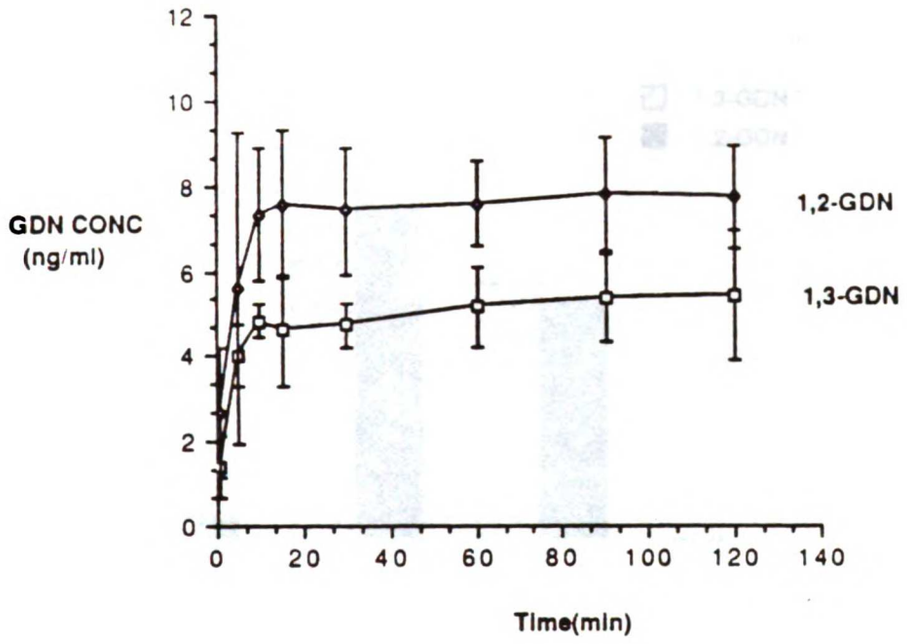


Fig. IV-2. GDN concentrations obtained from liver homogenate incubations of GTN in rabbits (n=5, mean±SD).

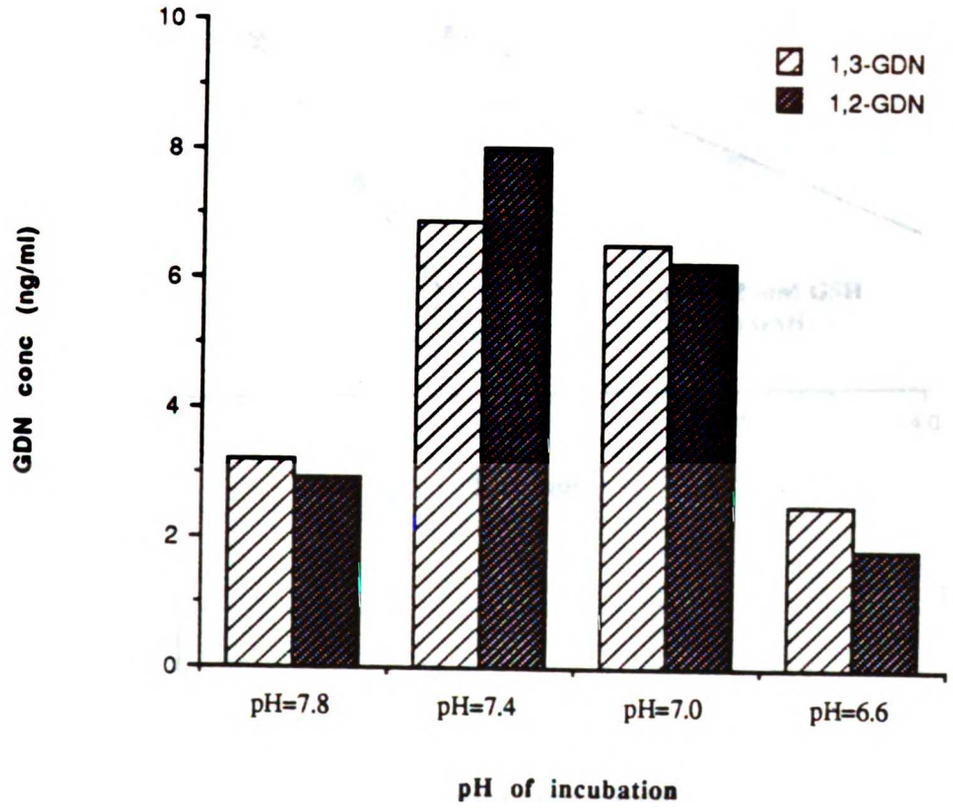


Fig. IV-3. Formation of GDN metabolites at 120 min, from 20 ng/ml GTN incubations in liver homogenates (from a single rabbit) adjusted to different pH's.

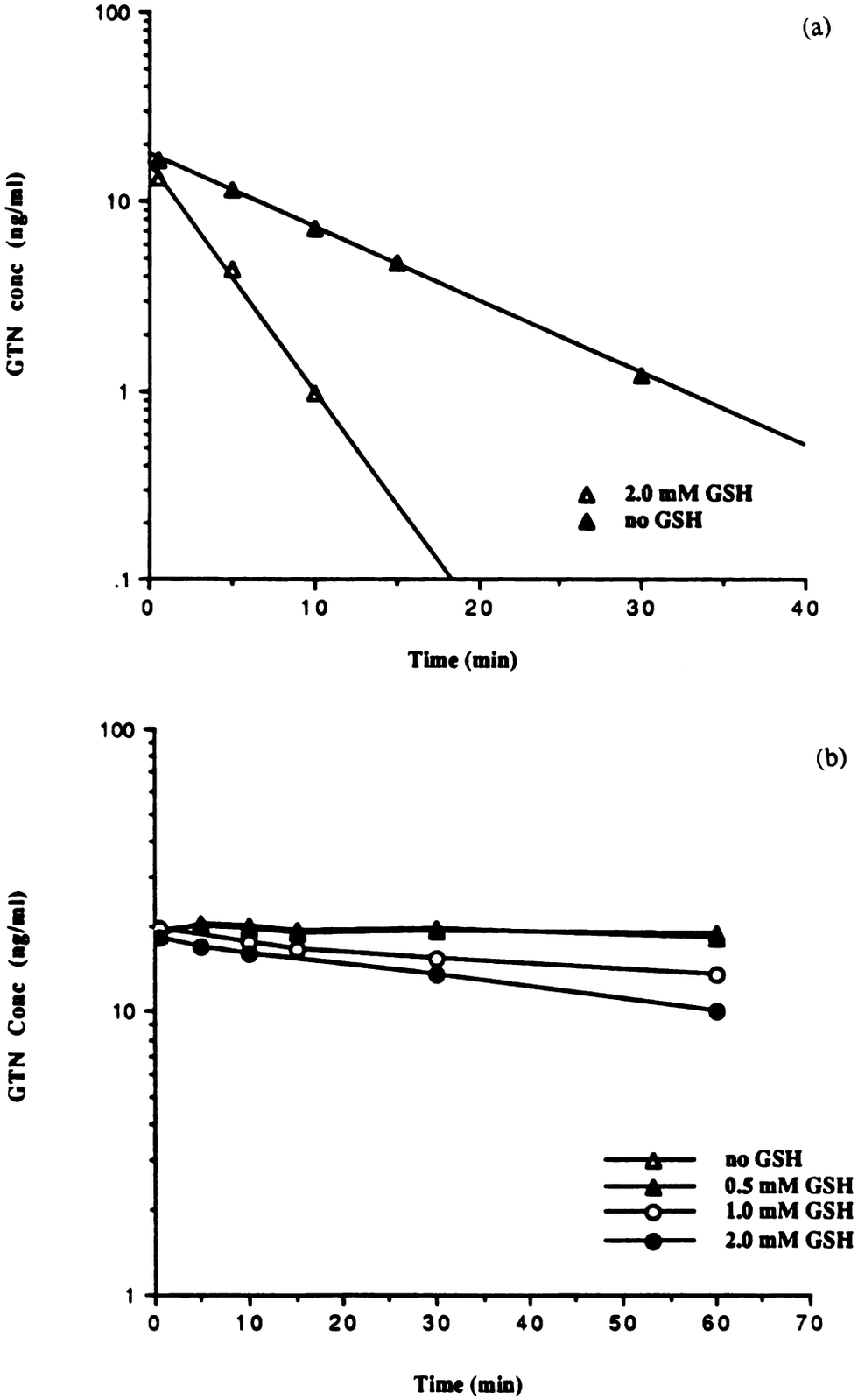


Fig. IV-4. GSH-dependence of GTN metabolism in (a) the cytosolic and (b) the microsomal fractions of a rabbit liver.

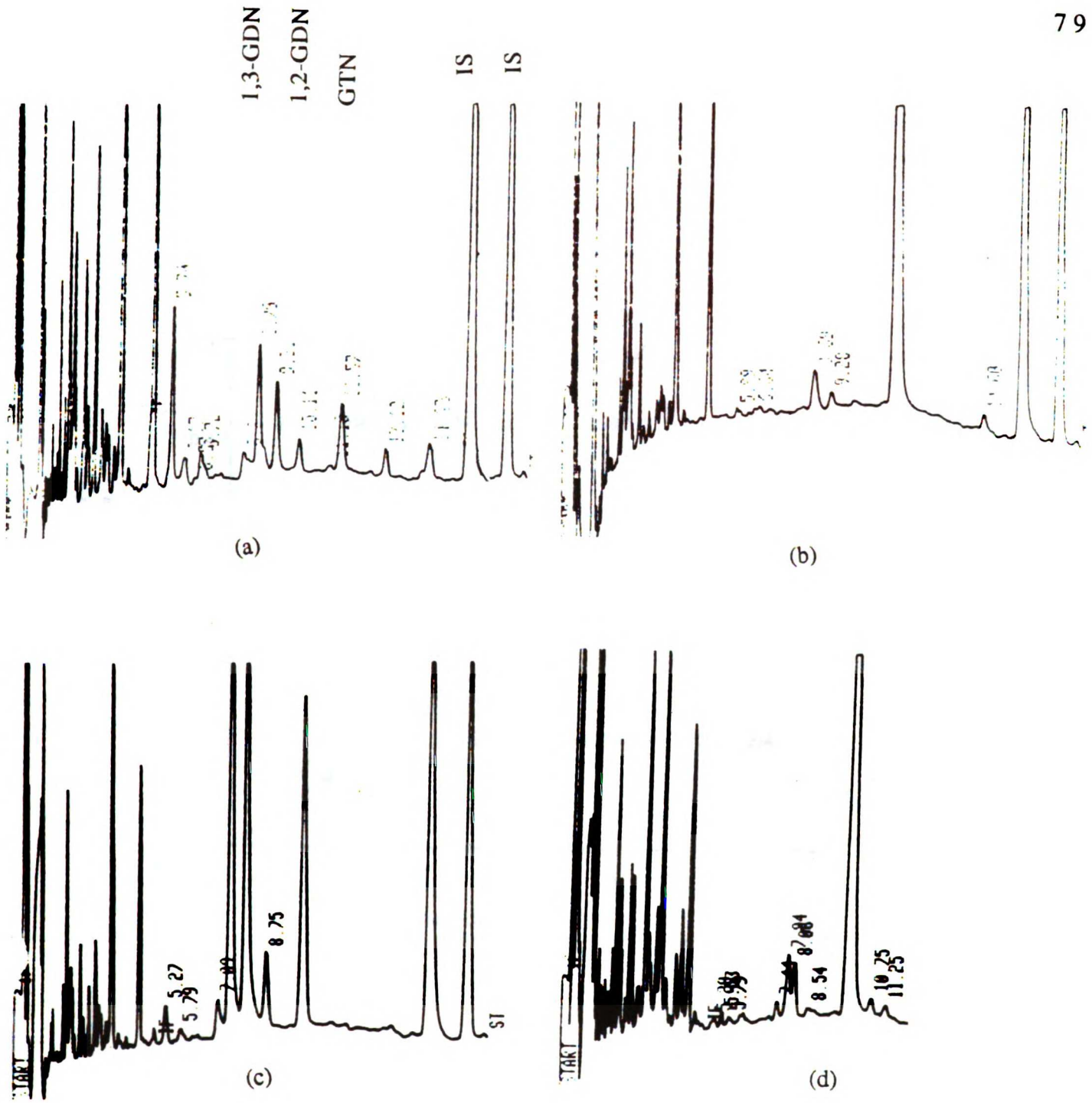


Fig. IV-5. The effect of heating on GTN metabolism and GDNs formation in subcellular fractions: (a) Control microsomes, (b) heated microsomes, (c) control cytosol, and (d) heated cytosol. Comparisons were made for the same incubation times.

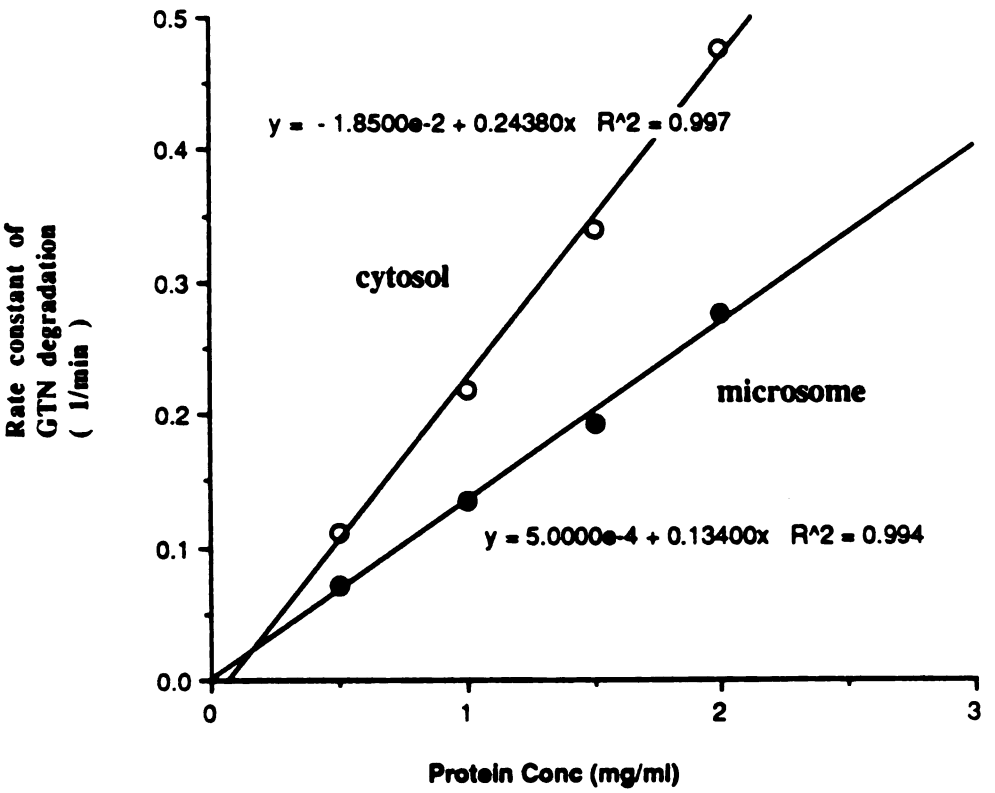


Fig. IV-6. Effect of diluting subcellular fractions on GTN metabolism.

from four rabbit livers were combined and the first-order degradation rate constants for GTN in both subcellular fractions were plotted against the protein concentration in the incubation media. Linearity in GTN degradation was observed from 0.5 to 2.0 mg/ml for both fractions.

4.2 Differential formation of GDN metabolites in cytosolic and microsomal fractions

The concentration profiles for GTN degradation and GDN formation in the cytosolic and microsomal fractions following incubations of 20 ng/ml GTN are shown in Fig. IV-7 a & b. GTN degradation was more rapid in the cytosol, with GTN concentrations diminishing below the detection limit at approximately 30 min. More interestingly, the preferential formation of GDNs from GTN in the two subcellular fractions differed. 1,2-GDN was the predominant metabolite in the cytosol, whereas in the microsomes, its structural isomer -- 1,3-GDN, was found to be preferentially formed. The resultant GDN ratios at various time points from 20 ng/ml (0.088 μM) GTN incubations with the cytosol and the microsomes are shown in Fig. IV-8. The enzymatic processes for GTN metabolism in the subcellular fractions were initially examined following co-incubations of the subcellular fractions with 17.6 μM SBP. A significant decrease in the rate of GTN degradation was observed in the cytosol (Fig. IV-9). Moreover, a change in the pattern of GDN formation was observed. The effect of SBP on the GDN ratios at various time points during the 2-hr incubation period were shown in Fig. IV-10. A significant decrease in the GDN ratios was observed from 5 min onwards, indicating a more significant inhibition on the 1,2-GDN formation by SBP. At 60 min, when GTN metabolism was complete in both the control and the inhibitor-treated incubations, there was a 77.4±9.6% decrease in 1,2-GDN concentrations, accompanied by a 155±26 increase in 1,3-GDN concentrations. Upon increasing SBP concentrations, the GDN ratios decreased further, indicating an increasingly greater contribution from the 1,3-GDN

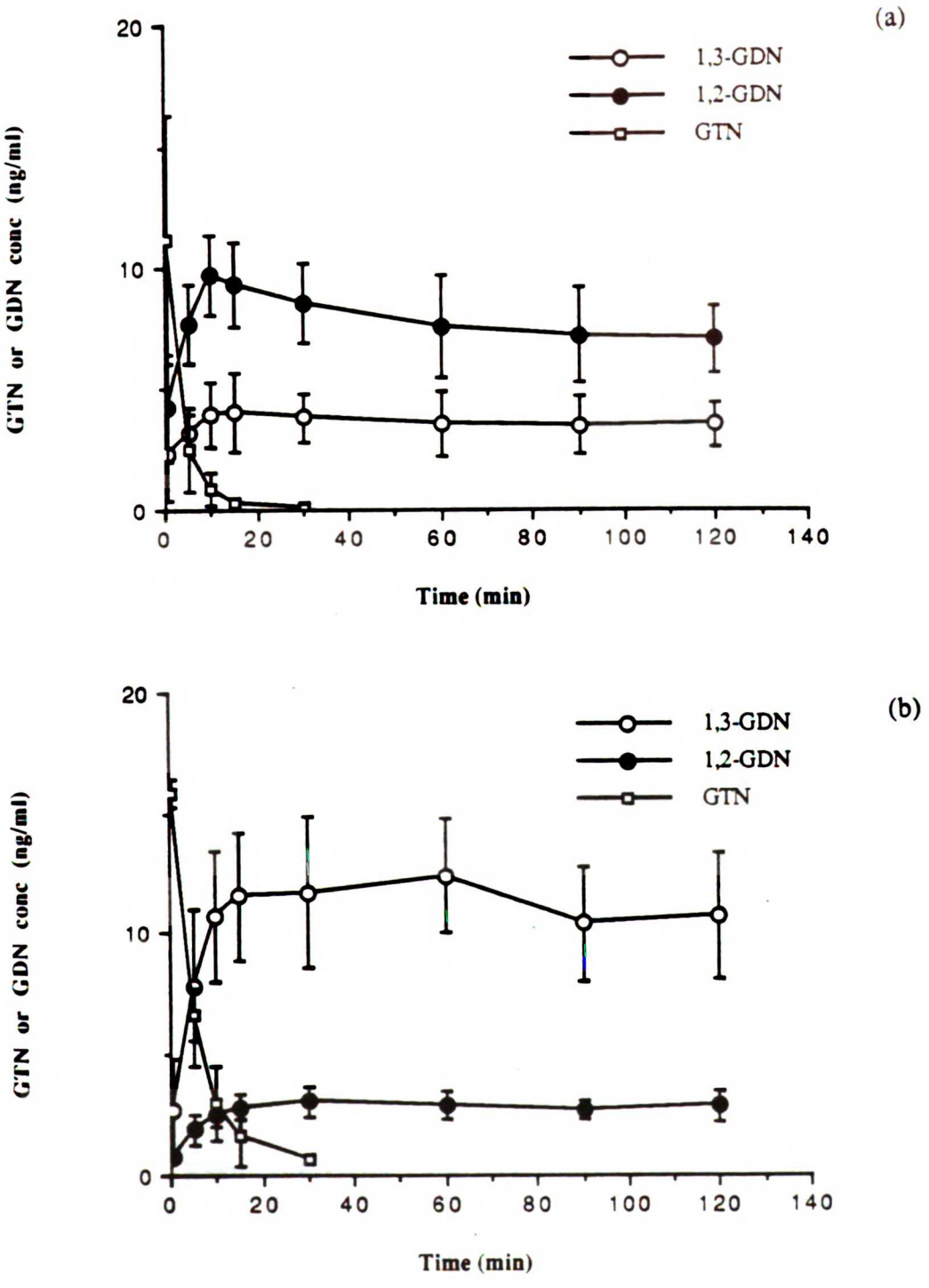


Fig. IV-7. Concentration-time profiles of GTN and GDNs in (a) the cytosolic (n=5) and (b) the microsomal fractions (n=4) following incubations of 20 ng/ml (0.88μM) GTN. (mean±SD)

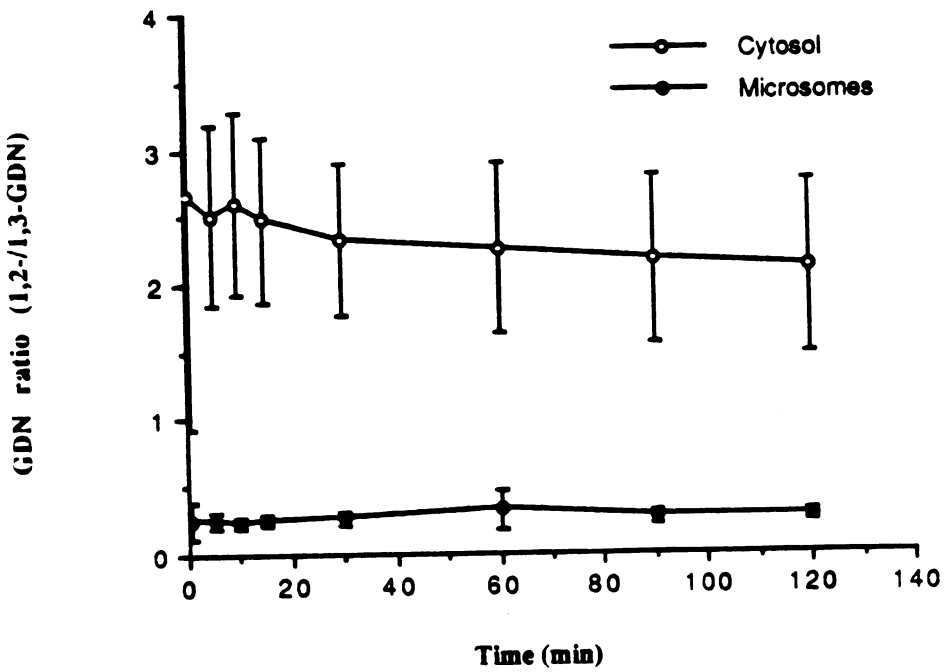


Fig. IV-8. Resultant GDN ratios (1,2-GDN/1,3-GDN) in the cytosolic (n=5) and microsomal fractions (n=4) following incubations of 20 ng/ml (0.88μM) GTN. (mean±SD)

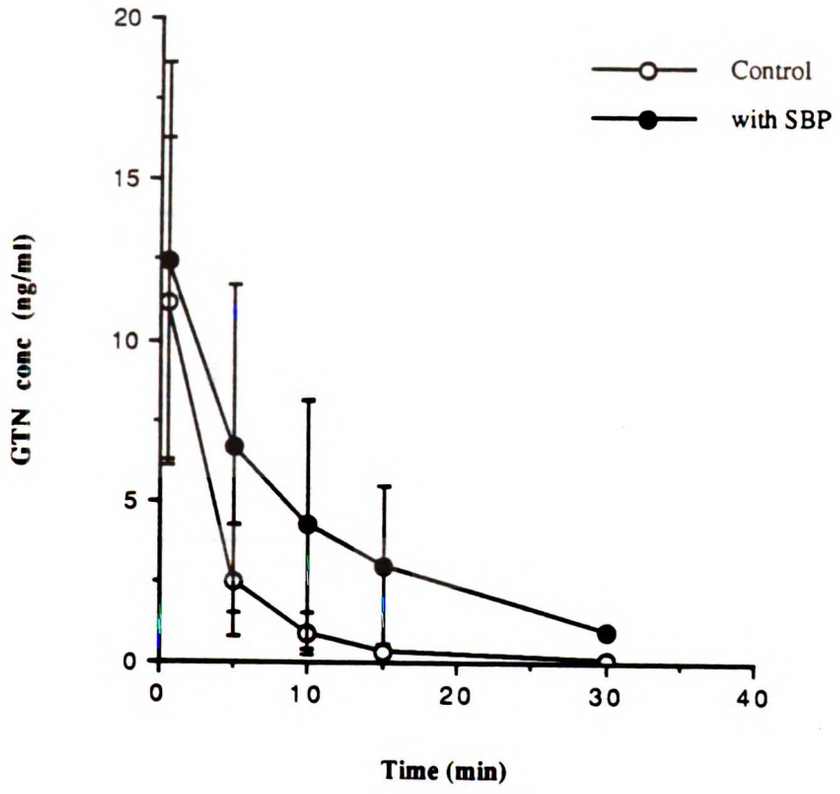


Fig. IV-9 The effect of sulfobromophthalein (SBP) on GTN degradation (20 ng/ml) in the cytosolic fractions (mean±SD, n=4).

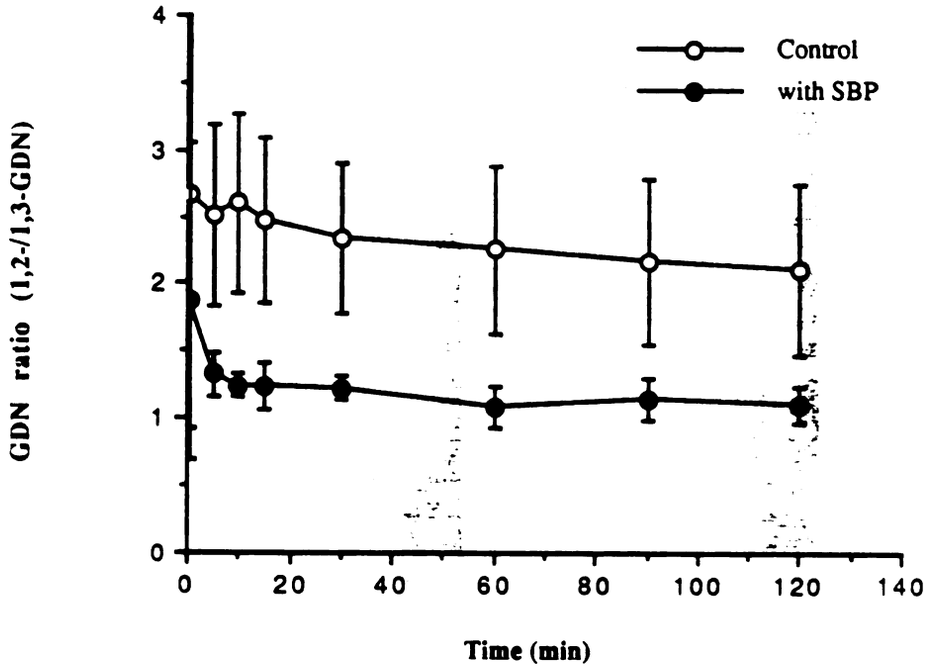


Fig. IV-10. Change of GDN ratio with time (1,2-GDN/1,3-GDN) upon addition of SBP at a concentration of 17.6 μM (200 times higher than GTN concentration) in the cytosol (mean±SD, n=4).

formation pathway (Fig. IV-11). In the microsomes, a slight change in the GTN degradation profile was observed (Fig. IV-12). However, the GDN ratios were not altered (Fig. IV-13), and remained approximately 4 to 1 in favor of 1,3-GDN production.

4.3 Concentration-dependence of GTN metabolism in subcellular fractions

In a series of shorter incubation studies with the subcellular fractions, the concentration dependence of GTN metabolism was investigated. Figure IV-14 demonstrates GTN metabolism following incubation of 0.08 μM (20 ng/1.1 ml) GTN in cytosol (1.5 mg/ml protein in 1.5 min) and microsomes (2.0 mg/ml protein in 2.0 min). Similar to the 2-hr incubation studies previously illustrated, a preferential formation of 1,2-GDN was observed in the cytosol. At a concentration of 0.08 μM, the GDN ratio (i.e., 1,2-GDN/1,3-GDN) was 2.91 ± 0.12. In microsomal fractions, the GDN ratio was 0.218 ± 0.045, indicating a predominant formation of 1,3-GDN. In the cytosolic fraction, despite the shorter time of incubation and lower protein concentration, a higher extent of GTN degradation was observed.

Different initial GTN concentrations incubated in the subcellular fractions also yielded differences in GTN metabolism. In Fig. IV-15, as the initial GTN concentrations increased, the percentage of GTN left unmetabolized in cytosolic fractions at 1.5 min increased from 45.5% ± 3.7% to 77.5% ± 0.3%, indicating that a saturable pathway for GTN metabolism exists in cytosolic fractions. In microsomal fractions, no significant difference in GTN degradation was observed over the range of GTN concentrations tested.

Due to the rapid rate of GTN metabolism in these subcellular fractions, true initial rates of GTN metabolism were almost impossible to obtain. However, when the rate of GTN degradation, normalized to the time of incubation as well as the protein concentration, were compared between the two subcellular fractions, the relative contribution of each fraction to the overall metabolism at different GTN concentrations can be estimated as

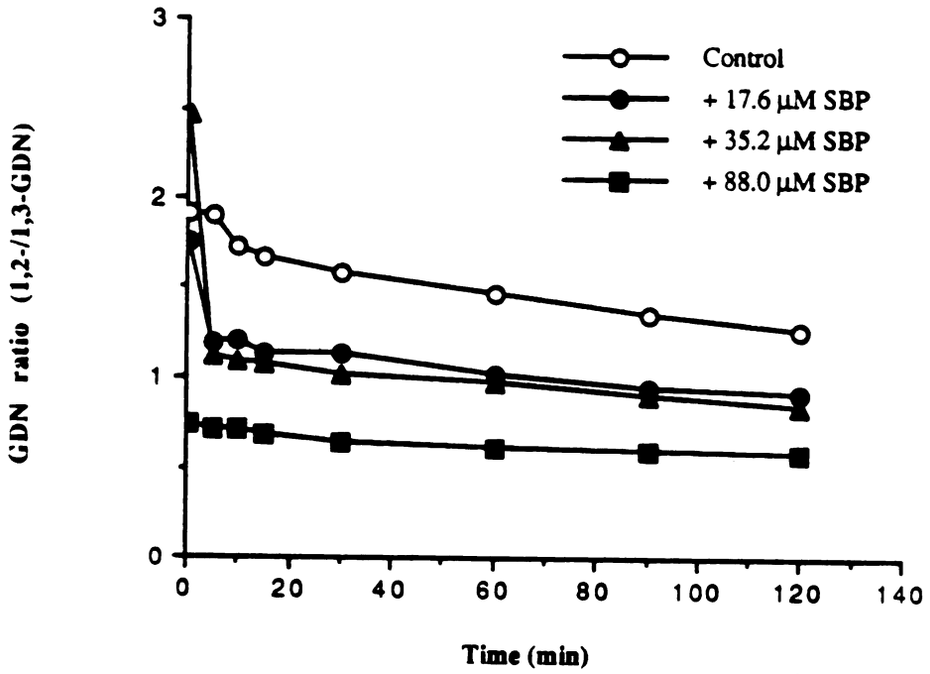


Fig. IV-11. Effect of increasing SBP concentration on the GDN ratio (1,2-GDN/1,3-GDN) in the cytosolic fraction of a rabbit liver.

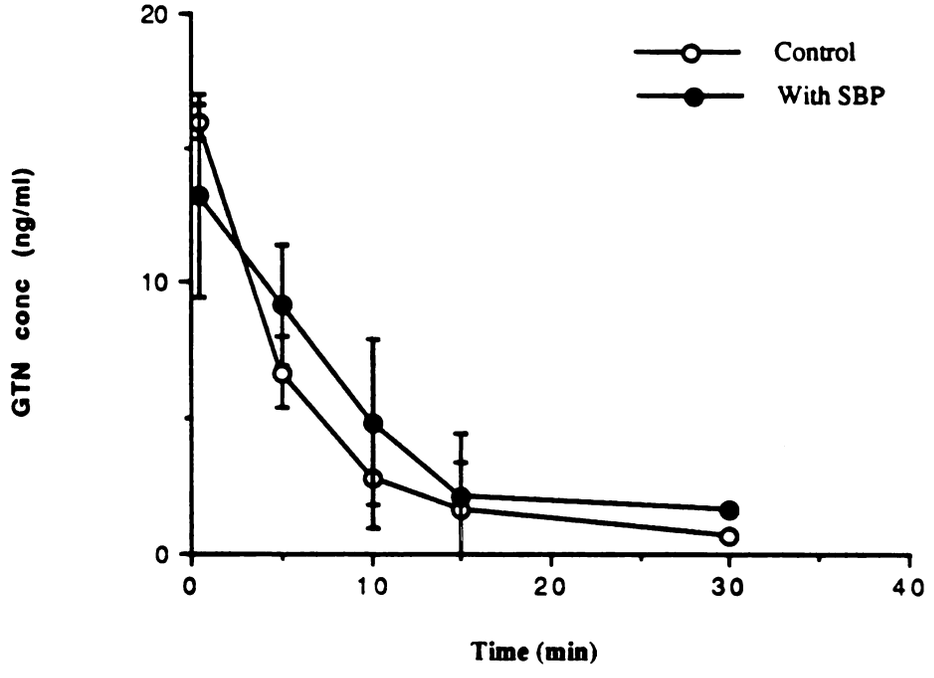


Fig. IV-12. The effect of SBP on GTN degradation (20 ng/ml) in the microsomal fractions (mean±SD, n=3).

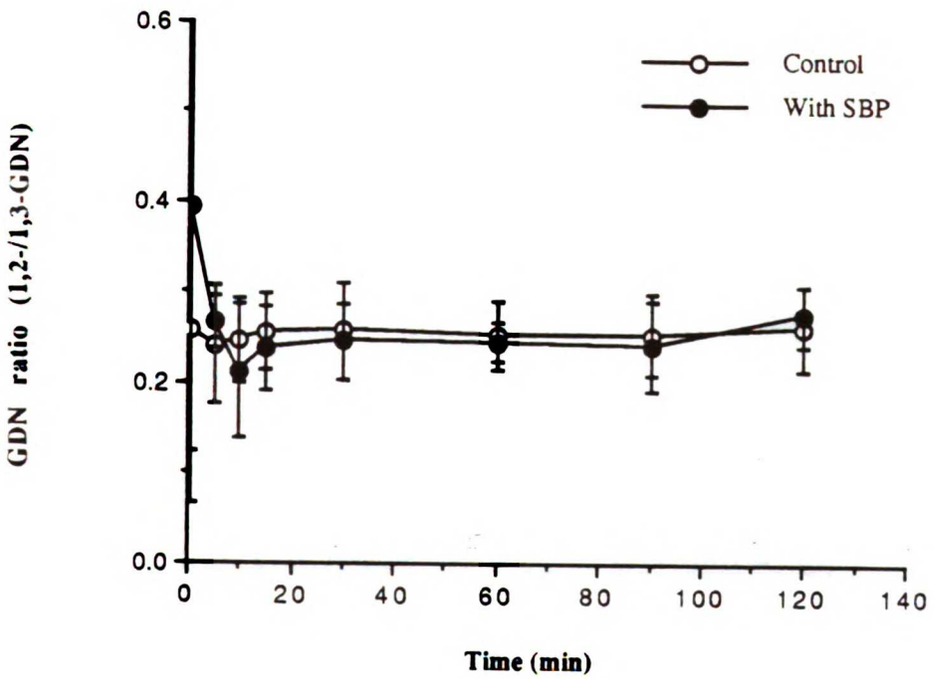


Fig. IV-13. Effect of SBP (17.6 μM) on the GDN ratio (1,2-GDN/1,3-GDN) in the microsomal fractions (mean±SD, n=3).

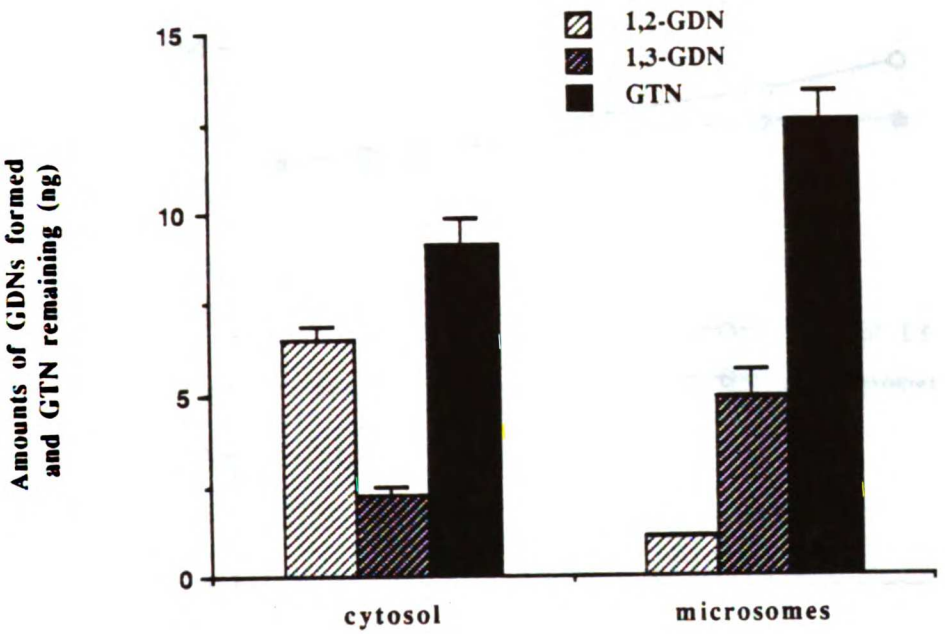


Fig. IV-14. GTN metabolism and GDNs formation in rabbit liver subcellular fractions after incubation of 0.08 μM GTN for 1.5 min with 1.5 mg/ml protein in cytosol and 2.0 min with 2.0 mg/ml protein in microsomes (mean±SD, experiments performed in triplicate)

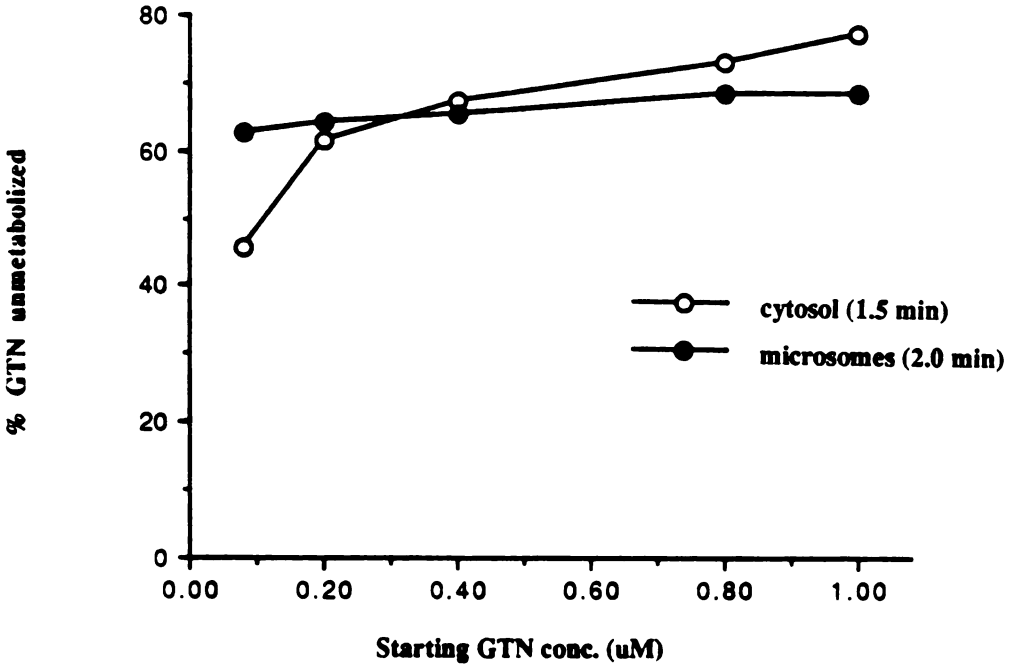


Fig. IV-15. Percentage of unmetabolized GTN in the cytosolic and the microsomal fractions vs. the initial concentrations of GTN added to the incubations (mean data from triplicates).

shown in Table IV-1. Over the 12.5 fold GTN concentration range, the ratio of the rates of GTN degradation in cytosolic to microsomal fractions decreased approximately 50%. These data suggest that the cytosolic fraction is more prone to saturability in GTN metabolism at lower substrate concentrations.

In addition to the dose-dependent effect on GTN metabolism, there was also a related change in the pattern of GDN formation in the cytosolic fraction. As shown in Fig. IV-16, the GDN ratio changed from about 2.91 ± 0.12 to 1.87 ± 0.06 when the initial GTN concentration was increased from 0.08 μM to 1.00 μM (250 ng/ 1.1 ml). Figure IV-17 shows that the decrease in the GDN ratio upon increasing GTN starting concentration was primarily due to a prominent saturation of the 1,2-GDN formation pathway. Increasing concentrations of GTN seemed to have a lesser effect on 1,3-GDN formation in the cytosolic fraction. In microsomal fractions, as depicted in Figs. IV-16 & 17, increasing initial GTN concentrations did not result in changes of either GDN formation pathway. The rates of individual GDN formation were estimated for the different incubations and were plotted following the Lineweaver-Burke relationships depicted in Fig. IV-18, so that the apparent V_{max}'s and K_m's of these processes could be estimated. The kinetic parameters obtained are shown in Table IV-2. The formations of individual GDN in each subcellular fraction were found to differ in their kinetic parameters.

4.4 Effects of various GST inhibitors on GTN metabolism in subcellular fractions

When various inhibitors of GST (16.0 μM) were added to GTN incubations, GTN degradation was inhibited to different extents in subcellular fractions. In cytosolic fractions, all inhibitors decreased GTN metabolism. The decrease in formation of each GDN at 0.8 μM (200 ng/1.1 ml) GTN upon addition of inhibitors is shown in Table IV-3. Statistically significant reductions in the formation of both GDNs were observed in cytosolic fractions for each inhibitor. In microsomes, only the ethacrynic acid-treated

Initial Conc. of GTN (μM)	Rate of GTN degradation (pmol/min/mg protein)		Ratio (cyt/mic)
	cytosol	microsome	
0.08	21.4	8.23	2.59
0.20	37.7	19.7	1.91
0.40	64.1	38.4	1.67
0.80	105	69.9	1.51
1.00	110	86.6	1.27

Table IV-1. Rates of GTN degradation in subcellular fractions of rabbit liver as a function of initial GTN concentrations (mean data from triplicate studies)

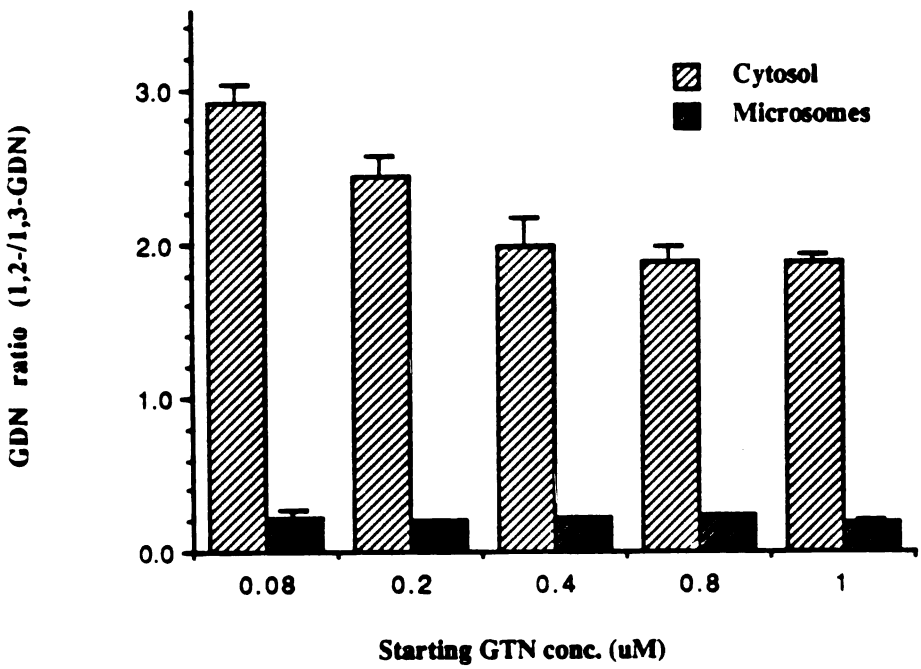


Fig. IV-16. GDN formation ratios (1,2-GDN/1,3-GDN) in cytosolic and microsomal subcellular fractions as a function of initial GTN concentrations (mean±SD, experiments performed in triplicate)

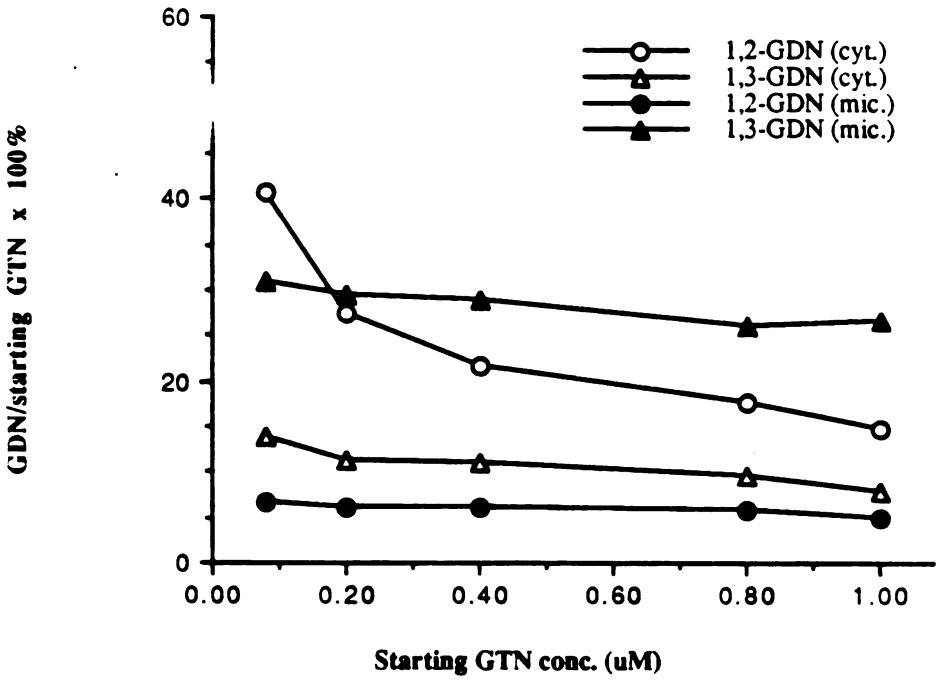


Fig. IV-17. Molar percentage of GTN dose accounted for by 1,2-GDN (circles) and 1,3-GDN (squares) in cytosolic (open symbols) and microsomal (solid symbols) fractions vs. increasing initial concentrations of GTN (mean data from triplicate studies).

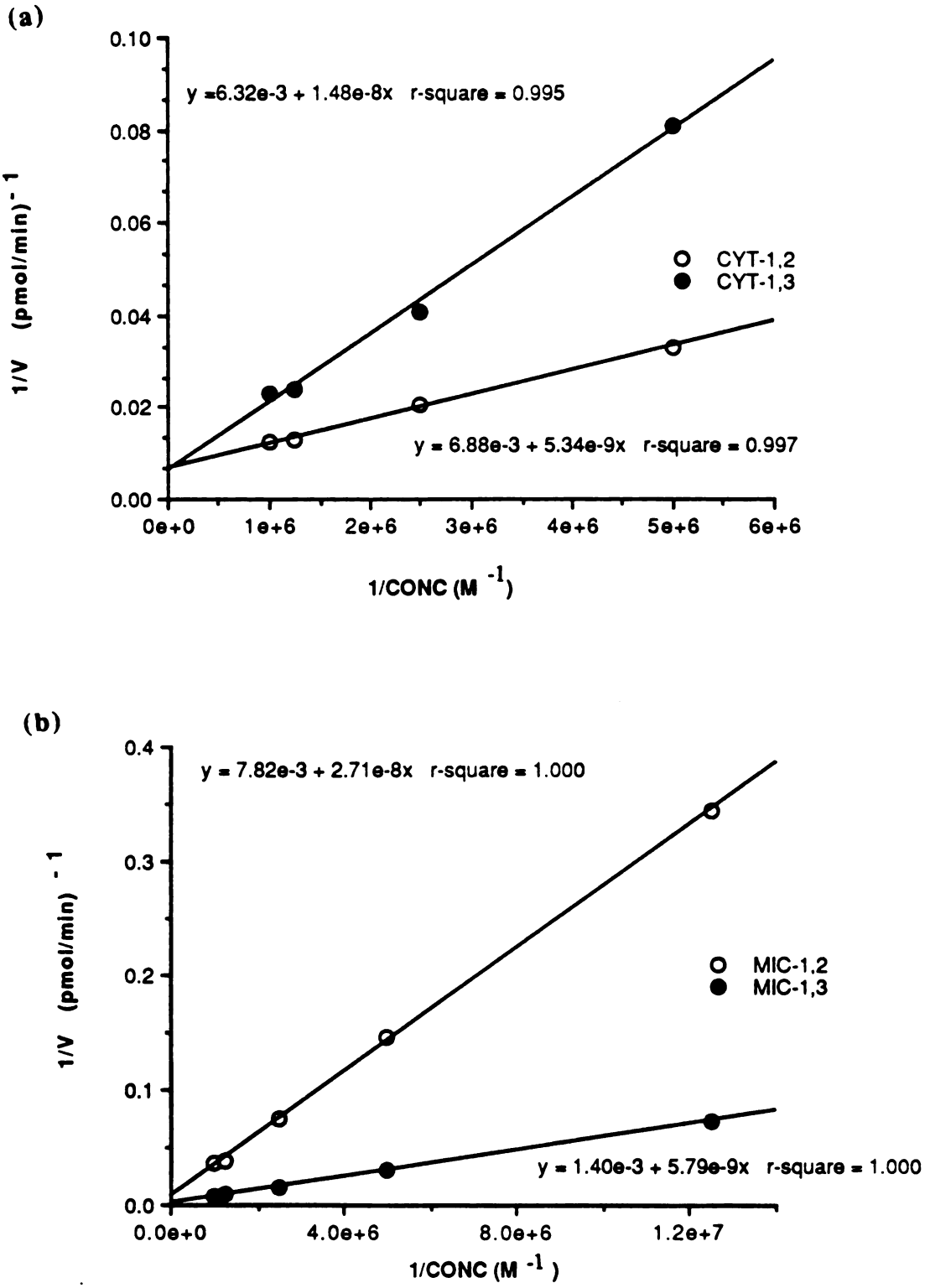


Fig. IV-18. Lineweaver-Burke plots of the rate of GTN degradation (1/v) vs. the concentration (1/conc) in the (a) cytosolic fractions and (b) the microsomal fractions. Data from the lowest concentration in the cytosolic fraction was not used (mean data from triplicate studies).

FRACTION	GDN	Vmax (pmol/min)	Km (μM)	Vmax/Km (10^{-6} min^{-1})
Cytosolic	1,2-GDN	194	0.972	200.0
	1,3-GDN	206	2.85	72.4
Microsomal	1,2-GDN	128	3.46	36.9
	1,3-GDN	715	4.15	172.6

Table IV-2. Kinetic parameters obtained from Lineweaver-Burke analysis (Fig. IV-18) of the formation rates of GDNs (mean data from triplicate studies).

	Cytosolic Fractions			Microsomal Fractions		
Treatment	1,2-GDN	1,3-GDN	GDN ratio	1,2-GDN	1,3-GDN	GDN ratio
Control	69.1±1.4	36.7±2.5	1.89±0.09	13.0±0.8	56.9±0.2	0.229±0.013
SBP	45.2±3.4[a]	32.9±1.5[a]	1.38±0.04[a]	12.5±0.3	48.9±5.4[a]	0.258±0.034
ECA	10.4±2.5[a]	21.2±1.1[a]	0.50±0.13[a]	8.92±0.2[a]	46.7±7.0[a]	0.193±0.024
p-NBC	26.4±0.9[a]	22.9±1.0[a]	1.15±0.05[a]	12.9±0.7	53.7±2.5	0.241±0.020
CDNB	52.4±3.5[a]	25.2±2.3[a]	2.08±0.08	12.3±0.9	52.1±3.1	0.235±0.010
IDM	58.8±2.0[a]	31.3±3.6[a]	1.89±0.15	12.6±1.6	52.0±3.0	0.244±0.040

[a] Significantly different from control values at $p < 0.05$.

Table IV-3. Changes in rate (pmol/min/mg protein) and ratio of formation of GDN metabolites (1,2-GDN/1,3-GDN) from 0.8 μM (200 ng) GTN upon addition of various GST inhibitors (16 μM) to the incubation medium (mean±SD, n=3)

samples show a statistically significant decrease in the formation of both GDNs. SBP caused a change only in 1,3-GDN formation. In cytosolic fractions, the resultant GDN ratios obtained suggest the inhibitors may act differently. A relatively higher inhibition of 1,2-GDN formation was observed in SBP, ECA, and p-NBC treated cytosolic fractions and the GDN ratio also decreased significantly. In the ECA-treated cytosolic incubations, 1,2-GDN formation was decreased to such an extent that the GDN ratio dropped below unity, i.e., more 1,3-GDN than 1,2-GDN was produced. Although CDNB-treated and IDM-treated incubations also showed reductions in the formation of both GDNs, the resultant GDN ratios were not significantly altered. Changes in GDN ratio after addition of inhibitors for different initial concentrations of GTN in cytosolic fractions are depicted in Fig. IV-19. It is clear that CDNB and IDM, though attenuating GTN degradation, did not alter the GDN ratios; whereas additions of SBP, p-NBC, and ECA resulted in significant reductions of the ratio, indicating a more specific inhibition of the 1,2-GDN formation pathway.

5. Discussion

Numerous animal studies have demonstrated that GTN metabolism occurs rapidly in various organs, and it is generally believed that the metabolism of the drug is crucially related to its pharmacologic effect (Needleman and Krantz, 1965; Needleman et al., 1969; Ignarro et al., 1981; Brien et al., 1986 & 1988). Tolerance to GTN upon repeated dosing might be explained by a decrease in the rate of GTN metabolism, possibly due to deprivation of thiol groups near the site of action (Ignarro et al., 1981; Axelsson and Ahlner, 1987). Although it has been shown that the liver may not be an important elimination organ after systemic GTN administration (Blei et al., 1984a), it could be of paramount significance in oral GTN administration. To compare the relative rates of GTN degradation in various organs, homogenates from various organs of one rabbit were

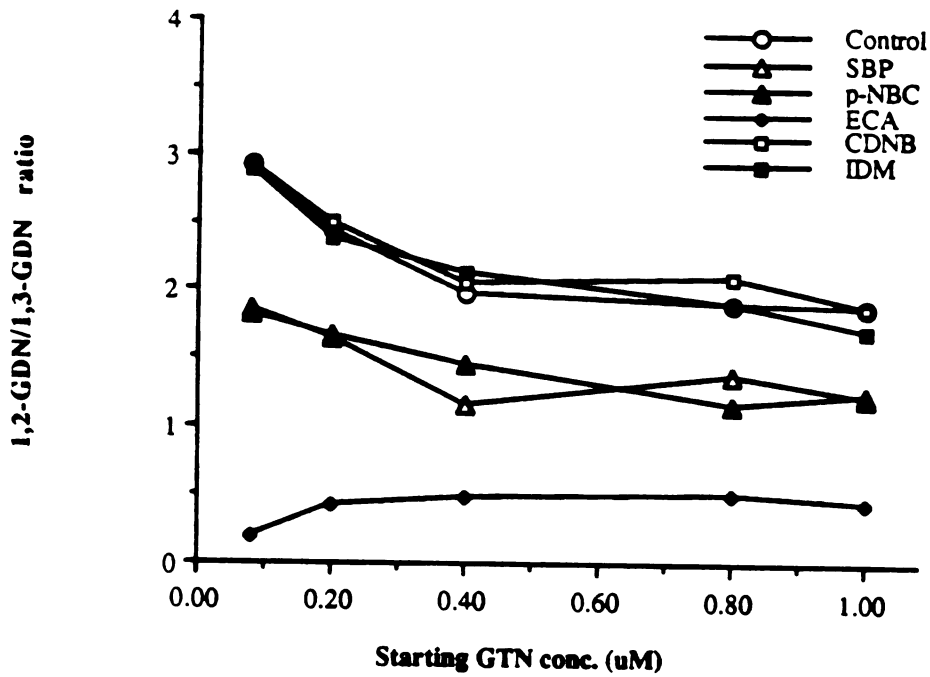


Fig. IV-19. Effect of GST inhibitors (16.0 μM) on GDN formation ratio (1,2-GDN/1,3-GDN) for different initial concentrations of GTN in cytosolic fractions (mean data from triplicate studies).

prepared and GTN was incubated in these homogenates at equimolar protein concentrations (2 mg/ml). These results are shown in Fig. IV-20, which demonstrates that the liver possesses the highest GTN eliminating activity, although GTN was found to degrade in all organ homogenates examined. Interestingly, the muscles from the hind limb exhibited the second highest rate of GTN degradation. It should be noted that in the in vivo GTN pharmacokinetic studies previously discussed in Chapter III, significant GTN extraction by the hind limb of the rat was observed. Homogenates from the kidney, the lung, and the intestine possessed intermediate metabolic activities, followed by only minor GTN degradation in the spleen and the heart. This confirms the results from various other reports, which demonstrate that in homogenate preparations, the liver exhibits the highest rate of degradation when compared to other organs (Fung et al., 1984a; Cossum and Roberts, 1985a). In in vivo studies, the extraction ratio of GTN in the liver approaches unity, so that after oral dosing, plasma concentrations of GTN are almost undetectable (Noonan and Benet, 1987). In spite of the low or no bioavailability, oral doses of GTN do yield antianginal effects in man (Winsor and Berger, 1975). In whole animal studies, GTN was found to be extracted by the liver extensively and in a dose-dependent manner (Cossum et al., 1986). The goal of the experiments described in this chapter was to explore the characteristics of hepatic GTN metabolism by means of dose-dependence studies and the use of multiple inhibitors of GTN metabolism in incubations of subcellular fractions of rabbit liver.

In the first part of the study, GTN metabolism in the cytosolic and microsomal fractions of livers from different animals were examined and compared on an equivalent protein concentration basis. In the second part of the study, subcellular fractions were obtained from livers of different animals, and then pooled together. In our experimental design the contribution of protein from each preparation was equivalent. Therefore, the data reported here should give a good estimate of the mean result from different preparations if the incubations were done separately with each preparation. From our

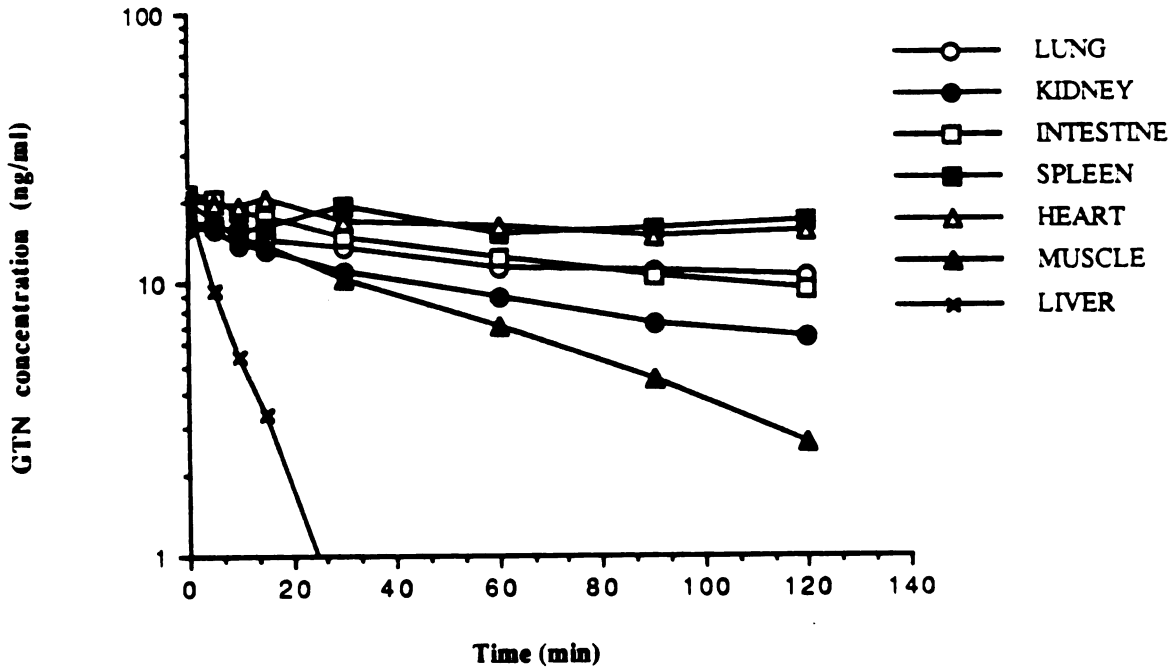


Fig. IV-20. Incubations of 20 ng/ml GTN with homogenates from various organs of a rabbit. Protein concentrations in each incubation was 2.0 mg/ml.

experience and as reported in the literature, GTN metabolism is highly variable from animal to animal, reflected by data possessing huge coefficients of variation. This variability makes detection of changes in metabolism, for example, with respect to substrate concentrations or addition of inhibitors, very difficult when separate incubations are performed utilizing preparations from different animals. Therefore, the pooled fractions experimental design was used here to minimize the influence of inter-individual variability. As shown in Fig. IV-6, GTN degradation was linear over the range of 0.5 to 2.0 mg/ml protein. Different protein concentrations and incubation times were selected for cytosolic and microsomal incubations in an attempt to produce similar extents of GTN metabolism in both fractions for comparison. This design was chosen in an attempt to negate the possibility that dose-dependence could be associated with differing capacities of each fraction to metabolize GTN. GSH (2mM) has been found to be optimal by other investigators (Tam et al., 1987 & 1988), when similar substrate concentrations were used. Since the cofactor concentration was 2000-fold higher than the highest concentration of GTN used in the study (1 μM), it is unlikely that the cofactor will be rate-limiting in the incubation.

In 1965, Needleman and Hunter (1965) reported the presence of a drug-class specific enzyme -- organic nitrate ester reductase, which is responsible for GTN metabolism in the liver. These investigators also reported that 1,3-GDN is formed in preference to its 1,2- isomer when purified organic nitrate reductase from hog liver is incubated with GTN. However, subsequent investigators (Cossum and Roberts, 1985; Tam et al., 1988) found contradictory results with liver homogenate incubations from other species. It is conceivable that these differences are due to inter-species differences in GTN metabolism. Lee (1973) compared GDN formation in the 9000g supernatant fractions from livers of several species. The investigator found that, with the exception of rats, all other animals tested (rabbits, dogs, and cats) produced 1,2-GDN preferably. Our preliminary experiments also showed that the rat liver cytosolic fraction, unlike that of the rabbit,

preferentially formed 1,3-GDN (Fig. IV-21); although the rat microsomal fraction exhibit similar GDN formation with that of rabbits. This species-dependent phenomenon may be explained by the presence of various isozymes of glutathione S-transferases, by which the process of GTN degradation might be carried out differently; or it may be due to the presence of other enzyme systems in rats which are capable of producing 1,3-GDN. It should be noted that in our pharmacokinetic studies discussed in Chapter III, portal vein infusion of GTN in rats also resulted in higher blood concentrations of 1,3-GDN than 1,2-GDN.

The data in this study clearly demonstrate that more than one pathway is present to metabolize GTN in liver subcellular fractions. Previously, the 105,000 x g supernatant of liver homogenates was used as the only site of measurement of 'organic nitrate ester reductase' activity (Needleman et al., 1969; Maier et al., 1980). If a microsomal metabolic pathway is present, as suggested by this study, previous assessments probably underestimate the capacity of the liver to metabolize GTN. As shown in Table IV-1, the ratio of rates of GTN degradation between the cytosol and microsomes decreases as the initial concentrations of GTN increase, as saturation was observed in the cytosolic fractions but not in microsomes. This suggests that the microsomal pathway for hepatic GTN metabolism should not be ignored.

The differential formation of GDN metabolites in the cytosolic and microsomal fractions is an interesting phenomenon, suggesting differences in regioselectivities of the two fractions with respect to the position of denitration. The heat-sensitive and GSH-dependent nature of microsomal GTN metabolism suggest that the microsomal pathway is enzymatic in nature and may result from microsomal GSTs. Although in the past, microsomal GSTs were believed to result from contamination from cytosolic GSTs during the microsome preparation procedures (Lee and McKinney, 1982), many reports have demonstrated the differences between these enzymes and their cytosolic counterparts. Microsomal GSTs have been isolated and purified (Friedberg et al., 1979; Morgenstern et

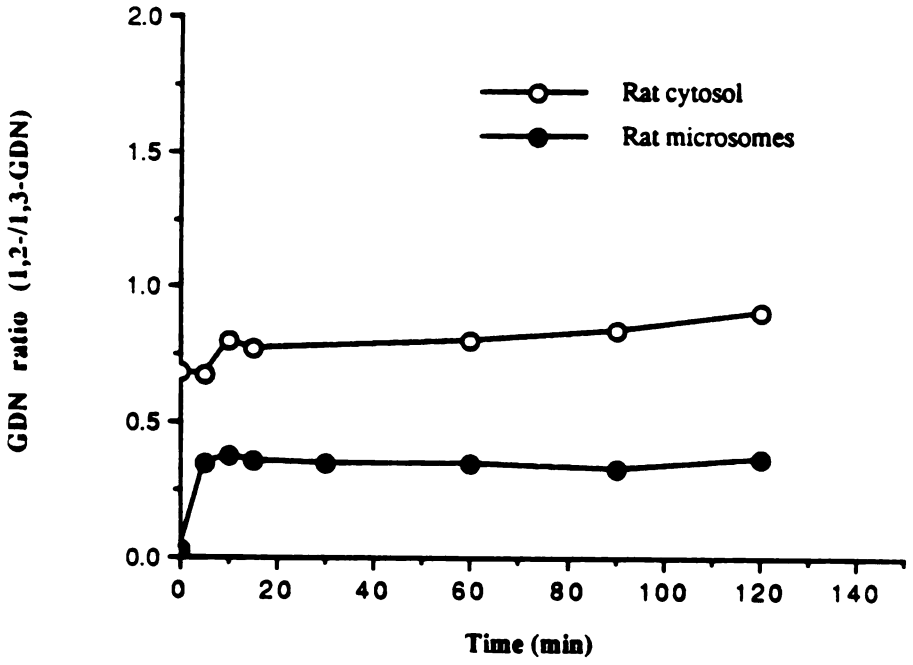


Fig. IV-21. Resultant GDN ratios from incubations of 20 ng/ml GTN in the cytosolic and the microsomal fractions of a pool of rat livers.

al., 1980). Moreover, various reports indicate that microsomal GSTs differ from cytosolic GSTs with respect to their inducibility (Morgenstern et al., 1980), immunoprecipitation with antibodies (Morgenstern et al., 1982), and the molecular weights of their subunits (Morgenstern et al., 1982). Up to now, microsomal GSTs have been found in all mammalian species investigated (Morgenstern et al., 1984), and it is believed that these enzymes may have various unique functions due to their location (Morgenstern and DePierre, 1987). In this study, microsomes were washed twice with buffer, so as to minimize the cytosolic contamination, as illustrated by Morgenstern et al. (1980). In our studies, there are differences in the metabolite formation patterns between the subcellular fractions (i.e. predominant 1,2-GDN formation in the cytosol versus predominant 1,3-GDN formation in the microsomes), indicate that the microsomal activity could not result from cytosolic contamination. It has been reported that the microsomal enzymes possess differences in substrate affinity and specificity when compared to the cytosolic isozymes (Morgenstern et al., 1982). This certainly appears to be true for the GTN substrate, where the active sites of these enzymes possess remarkable selectivity for the denitration process -- either at the C-1 or C-2 position of a GTN molecule for the production of 1,2-GDN or 1,3-GDN.

The nonlinearity of GTN metabolism and GDN formation exhibited in the cytosolic fractions might have significance in vivo. Recently, a human pharmacokinetic study of orally-administered GTN from our laboratory reported that when the GTN dose was increased from 0.4 mg to 1.6 mg, the GDN ratio decreased from a value greater than 3:1 to below 2:1, and then appeared to remain constant when the GTN dose was increased further (Nakashima et al., 1990). A similar phenomenon was also observed in these in vitro studies. The primary site for the observed dose dependent effect appears to be the 1,2-GDN formation pathway in the cytosolic fraction. The decrease in 1,3-GDN formation upon increasing GTN dose was relatively small when compared to that of 1,2-GDN. Several interesting observations were found when estimates of the V_{max} and K_m values

for the formation of the GDNs were calculated, as shown in Table IV-2. Although these values are not derived from true initial rate measurements, these results provide preliminary information about these two pathways. In the cytosolic fraction, the V_{max}'s for 1,2-GDN and 1,3-GDN formation are similar, indicating the total amount of protein available for the formation of either GDN may be very similar. However, the K_m for 1,3-GDN formation is three times higher than that of 1,2-GDN, suggesting that the predominant metabolite at low concentrations is 1,2-GDN. Moreover, when GTN concentrations approach or exceed the K_m, the 1,2-GDN formation pathway becomes non-linear. As a result, the GDN ratio decreases. According to these parameters, at much higher GTN concentrations, the K_m for 1,3-GDN formation will eventually be exceeded as well. The resultant GDN ratio will therefore remain at a constant value, as observed in the human study. In the microsomal fractions, non-linearity occurs at GTN concentrations which are much higher than the concentrations utilized in this study. It is interesting, however, that the V_{max} for 1,3-GDN formation is several-fold higher than that of 1,2-GDN. In humans, oral GTN administration usually results in a 2:1 GDN ratio (Noonan and Benet, 1987). However, when sustained-release preparations are used, the GDN ratio can be expected to be higher than for immediate release oral GTN preparations, as has been reported by different investigators (Laufen and Leitold, 1988; Yu et al., 1988). That is, the delivery of GTN from controlled release preparations is similar to a slow infusion of small doses of GTN though the gastro-intestinal tract to the liver, achieving hepatic portal vein concentrations that would minimize the saturability of the metabolic pathway to 1,2-GDN, thus resulting in higher GDN ratios. Therefore, under in vivo conditions, the rate of delivery of the drug to the metabolism site is also expected to affect the resultant GDN ratio.

From the glutathione-dependence and the multiple inhibitor studies, it is evident that GST enzymes play an important role in GTN metabolism. These enzymes have a wide spectrum of electrophilic and hydrophobic substrates (Habig et al., 1974). These compounds can be metabolized by GSTs to form glutathione conjugates, or they can just

bind to GST, as demonstrated for bilirubin (Kamisaka et al., 1975; Sugiyama et al., 1984) and bile acids (Sugiyama et al., 1984). Using various substrates as inhibitors, GTN metabolism in cytosolic fractions was inhibited. In microsomal incubations, however, only ECA produced a decrease in the formation of GDNs. In all treatments, however, there is no statistically significant alteration of GDN ratios in microsomal incubations. It should be noted that a diffusional barrier may exist for some of the substrates utilized, which would hinder their access to the active sites of the microsomal enzyme(s). In vitro experiments with ECA have shown an attenuation of the effect of GTN (Moffat et al., 1985) and other vasodilators (Rapaport and Murad, 1988), although the *in vivo* effect is not substantial and is hard to recognize (Moffat et al., 1985). Due to ECA's high lipophilicity, it may be relatively easier for this inhibitor to get to the active site and inhibit GTN metabolism. In any case, it is obvious that the microsomal pathway is GSH dependent and most probably involves microsomal GST in this subcellular fraction.

In the cytosolic fractions, differences in the selectivity of the inhibitors on the formation of either 1,2-GDN or 1,3-GDN were observed. Although the extents of inhibition were different for these inhibitors, they could be generally classified into two categories : CDNB and IDM decreased GDN formation but showed no selectivity; ECA, SBP and p-NBC preferentially inhibited the formation of 1,2-GDN. On the basis of the studies reported here, we hypothesize that at least two sites are present in the cytosolic fraction of rabbit livers, with one of them favoring the formation of 1,2-GDN (site I). The other site (site II) may be assumed to have relatively greater 1,3-GDN formation capacity than site I. CDNB and IDM have similar affinities for these two sites; therefore, the resultant GDN ratio is unaltered, although the extent of inhibition of GDN formation depends upon the K_I values for each inhibitor. In contrast, SBP, p-NBC, and ECA have greater selectivity in inhibiting site I. As a result, formation of GDNs from site II becomes more important and the resultant GDN ratio is decreased.

Due to the cross-reactivity of the substrates to different isozymes of GST, it is difficult to identify the enzyme(s) representing each site in the hypothesis. According to Habig et al. (Habig et al., 1974), p-NBC and SBP have greater affinities to GST A(3-3) (a class mu GST) than to GST-B(1-1) (a class alpha GST). Thus, we suggest that site I in our hypothesis may be GST A(3-3), or other closely related isozymes, e.g., GST C (3-4) (also a class mu GST). For site II, a possible candidate could be GST B(1-1, or 1-2, Ligandin, class alpha) because CDNB shows better affinity to the isozyme than SBP and p-NBC. It should also be noted that ECA, the inhibitor shown to be most effective in inhibiting GTN metabolism, also possesses high affinities to a GST which belongs to class pi (GST E, 7-7), suggesting that GST-pi may be responsible for a significant portion of overall GTN metabolism. However, a definite conclusion should not be made, until purified isozymes can be incubated directly with GTN as a substrate.

Recently, two groups of investigators reported that cytochrome P-450 in rat liver may be involved in GTN metabolism (Servent et al., 1989; MacDonald and Bennett, 1990). The reaction is believed to be an NADPH-dependent, reductive reaction, and predominantly occurring under anaerobic conditions. GTN metabolism mediated by this process was also shown to be affected by P-450 inhibitors such as carbon monoxide and SKF-525A. Interestingly, the predominant GDN metabolite from GTN degradation was 1,3-GDN, the same predominant metabolite found in microsomal incubations in our studies. Moreover, phenobarbital treatment results in a change in not just the rate of GTN metabolism, but the GDN ratio as well. Therefore, it is possible that various P-450 isozymes with different regioselectivities for GDN formation can participate in microsomal GTN metabolism. Using the incubation conditions of this study, cytochrome P-450-mediated GTN metabolism probably contributed minimally, because of the absence of an anaerobic environment. In addition, the mechanism of how cytochrome P-450 can be involved in this reductive reaction has not been deciphered. Nevertheless, these reports

present yet further evidence that GTN metabolism in a single organ can be mediated by more than one enzymatic pathway.

An interesting phenomenon relating the preferential formation of GDN metabolites in relation to the pharmacodynamic effects of GTN was reported by various investigators (Fung and Poliszczuk, 1986; Bennett et al., 1989; Slack et al., 1989). A decrease in production of 1,2-GDN was found in homogenates of tolerant aortic tissues when compared to control homogenates. However, the 1,3-GDN concentrations were unchanged. Although it is not clear at this point whether the metabolizing systems present in liver are the same as those in blood vessels, these studies also suggest that the GDN metabolites may be formed via different pathways, and that some of these pathways are more prone to 'tolerance' than others. Theoretically, formation of either GDN from GTN should be accompanied by the formation of an inorganic nitrite ion, which would lead to formation of the active intermediate -- S-nitrosothiol. Those studies suggest that the contribution of different pathways to the two dinitrate metabolites may lead to different pharmacologic effects, with the pharmacologically 'productive' pathway being formation of 1,2-GDN, the pathway mediated by cytosolic glutathione S-transferase in the present study. The importance of GSTs in mediating GTN metabolism and effects in blood vessels will be further discussed in subsequent chapters.

6. Summary

The studies described in this chapter represent the first report of the existence of a microsomal pathway for GTN metabolism, and that this pathway yields a pattern of GDN metabolite formation different from that found in cytosolic systems. Moreover, the possibility that multiple metabolism pathways for GTN exist in hepatic tissues was investigated and demonstrated, contrary to the previous belief, that GTN is mainly metabolized by a single cytosolic enzyme -- organic nitrate ester reductase. It is evident that

the microsomal metabolizing pathway cannot be ignored in hepatic tissues, especially at higher GTN concentrations, when the cytosolic pathway becomes saturated. Neglecting this alternate microsomal pathway leads to underestimation of GTN metabolism in an organ. Cytosolic GTN metabolism was found to be saturable at higher concentrations, and our data strongly suggests that the nonlinearity mainly results from saturation of a site which is more specific for 1,2-GDN production. Furthermore, this implies that more than one GTN metabolizing site exists in the cytosol, and our multiple inhibitor studies suggest that different GST isozymes are involved. Moreover, these GST isozymes may also possess different regioselectivities for denitration of the GTN molecule. Consequently, it is likely that multiple GTN metabolic sites are present in other organs, particularly at a site of action, such as vascular smooth muscle, which might then lead to different metabolically related pharmacologic effects of the drug.

CHAPTER FIVE

Metabolism of GTN in Homogenates and Subcellular Fractions of Bovine Coronary Arteries

1. Introduction

GTN metabolism by blood vessels is believed to be an important step in eliciting the vasodilatory effect of the drug (Chapter 2, Section 3.2). The denitration of GTN is proposed to lead to a release of nitrite ions, which subsequently undergo further biotransformation to produce the vasoactive species (Ignarro et al., 1981). However, the crucial enzyme(s) responsible for this metabolic process has not been identified. In Chapter 4, the role of GSTs in the hepatic metabolism of GTN was investigated. The results suggested that different GST isozymes could participate in GTN metabolism (Lau and Benet, 1989; 1990). In addition, different preferences in the formation of individual GDNs by these isozymes were observed. Both the cytosolic and the microsomal fractions were found to be capable of metabolizing GTN via glutathione-dependent enzymatic pathways. GST is known to be present in many tissues in the body (Corrigall and Kirsch, 1988), therefore, it can represent an important enzymatic pathway for organic nitrate metabolism and the mechanism of action for these agents in blood vessels. In this chapter, disrupted tissue preparations of bovine coronary artery (BCA) were used to investigate the vascular metabolism of GTN, and to examine the possible role of GST.

2. Objectives

The objectives of the studies described in this chapter are :

a. to examine the profiles for GTN degradation and GDN formation in BCA homogenates;

b. to compare the differences in GTN metabolism in the cytosolic and microsomal fractions of BCA;

c. with the use of two GST inhibitors, sulfobromophthalein (SBP) and ethacrynic acid (ECA), to investigate the role of GST isozymes in vascular GTN metabolism.

3. Materials and Methods

3.1 Chemicals

GTN solutions were diluted from Tridil solutions (American Critical Care, McGaw Park, IL), which are used for intravenous GTN administration (5mg/ml). 1,2-GDN and 1,3- GDN were generously supplied by Marion Laboratories (Kansas City, MO). Potassium phosphate (monobasic) and sodium phosphate (dibasic) were obtained from Fisher (Fairlawn, NJ). Reduced glutathione (GSH), sulfobromophthalein (SBP), and ethacrynic acid (ECA) were all purchased from Sigma (St. Louis, MO). All chemicals were purchased at the highest grade of purity obtainable.

3.2 Preparation of subcellular fractions

Fresh bovine hearts were obtained from Ferrara Meat Market (San Jose, CA). The interventricular and the circumflex coronary arteries were dissected and placed in ice cold phosphate buffer (0.13M KH_2PO_4-Na_2HPO_4). Blood vessels from six bovine hearts were pooled together to provide the tissues needed for one study. The blood vessels were then sliced, chopped, and homogenized in three volumes of phosphate buffer by a blade

homogenizer (Brinkman Instruments, Westbury, NY). The blood vessel homogenates were centrifuged at 9000g and the supernatant layer was decanted and saved for further ultracentrifugation at 105,000g at 4 °C. The 105,000g supernatant (cytosolic fraction) and pellet (microsomal fraction) were separated. The microsomal fractions were washed with the buffer and re-centrifuged two more times to minimize contamination of the content of each with the other fraction. The various BCA fractions were stored at -70 °C prior to the incubation experiments.

3.3 Incubation studies

In each incubation, 5 ml of BCA 9000g supernatant was used. In the studies intended for the comparison of GTN metabolism between the cytosolic and the microsomal fractions, the protein concentration in the incubates was normalized to 4.0 mg/ml, using the protein assay of Lowry et al. (1951), as discussed previously (Chapter 4, Section 3.4). The incubates were placed in a water bath with the temperature maintained at 37 °C. GSH (2 mM) was added as the co-factor for the metabolic reaction. The mixture was then pre-incubated for five minutes. For the inhibitor studies, 2×10^{-5} M SBP or ECA was added just before the 5 minute pre-incubation period. Following pre-incubation, 40 ng/ml (1.76×10^{-7} M) GTN was added to the incubates. 500-µl samples were taken at 10, 20, 40, 60, and 120 min, and were immediately frozen in a mixture of dry ice and methanol.

The concentrations of GTN and GDNs were determined as described previously (Chapter 3, Section 3.4). Briefly, samples were extracted three times with 10-ml mixtures of pentane and methyl-*t*-butyl ether (8:2). The organic phase was almost evaporated to dryness and 50 µl of *n*-butyl acetate was used for reconstitution of samples. A 0.3 µl aliquot was injected onto Varian 6000 & 6500 GCs equipped with electron capture detectors. A standard curve of 0.5-20 ng GTN or GDNs in BCA subcellular fractions was

constructed, with o-iodobenzyl alcohol as the internal standard. Linearity was observed over this range, and both GTN and GDNs were clearly separated.

Due to the presence of nonenzymatic GTN degradation in phosphate buffer, solution of the phosphate buffer and 2 mM GSH were incubated with GTN in each study as a control. The extent of nonenzymatic degradation at each sampling time was subtracted from the observed metabolism in the experimental samples to account for the metabolites resulting from nonenzymatic GTN degradation.

3.4 Statistical Analysis

One-way ANOVA was used to detect differences among various incubations. The Dunnett's test was used for comparison of a single group to the control. Data were expressed in mean ± SD.

4. Results

4.1 GTN metabolism in the 9000g supernatant of bovine coronary arteries

In phosphate buffer, nonenzymatic degradation of GTN was found to occur at an appreciable rate, and was observed to be both pH- and GSH- dependent. Figure V-1 depicts the total GDN metabolites formed at pH 6.8, 7.4, and 8.0, in the presence or absence of 2 mM GSH. In the absence of GSH, nonenzymatic degradation of GTN accounted for less than 4% of the total dose after 60 min at each of the pH's examined. No significant difference was observed among the 3 pH conditions. The rate of nonenzymatic GTN degradation was significantly higher at pH 8 in the presence of GSH, when compared to all other incubations. The extent of metabolism appeared to increase with increasing pH, suggesting that the nonenzymatic degradation of GTN was a base-catalyzed

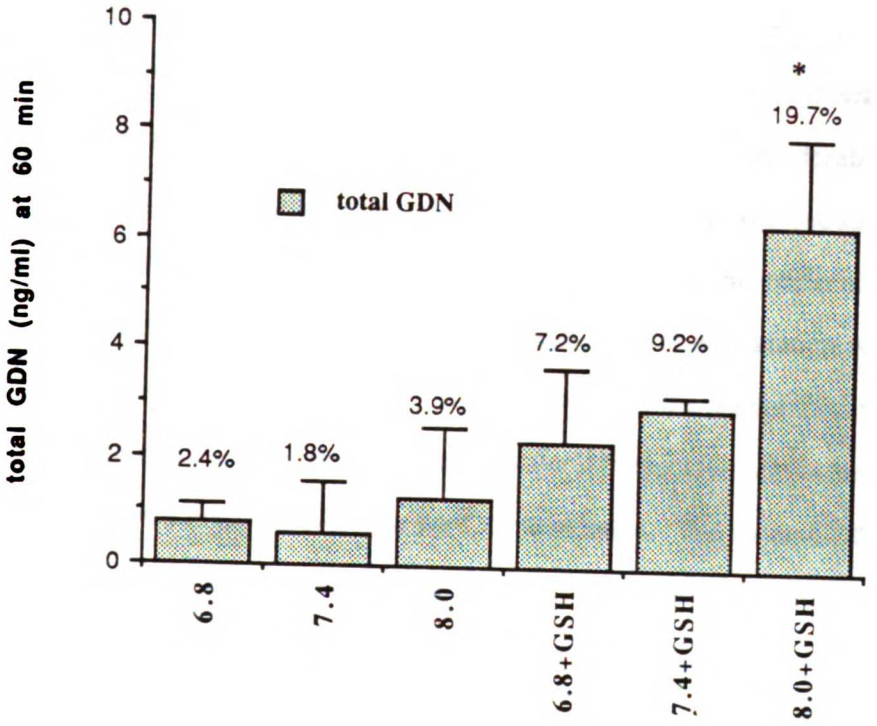


Fig. V-1. **Total concentration of GDN metabolites (1,2-GDN + 1,3-GDN) after 60-min incubation in phosphate buffer containing 40 ng/ml GTN at pH 6.8, 7.4, 8.0, and in the absence or presence of 2 mM GSH (mean±SD, n=3; * $p<0.05$, compared to other groups).**

reaction. At pH 7.4, the pH used for the subsequent metabolism studies described here, approximately 10% of the initial dose was degraded nonenzymatically after 60 min. Figure V-2 shows the formation of 1,2-GDN or 1,3-GDN from nonenzymatic GTN degradation. 1,3-GDN was found to be the predominant metabolite in incubations preformed at all three pH's.

Figure V-3 demonstrates GTN degradation and formation of GDNs in incubations with the 9000g supernatant fraction of BCA. GTN was metabolized via an apparent first-order degradation process (t1/2 = 25 min), which was considerably slower than the rate found in the liver studies described in Chapter 4. The concentrations of GDNs increased as GTN concentrations decreased, with 1,3-GDN being the predominant metabolite. When the amounts of GDNs resulting from nonenzymatic degradation were subtracted from these values, as shown in Fig. V-4, 1,3-GDN was still the predominant product. These results suggest that the enzyme(s) responsible for GTN metabolism in bovine coronary arteries may have a higher preference for C-2 denitration. The mean GDN ratio (1,2-/1,3-GDN) was found to be between 0.7 and 0.8. In the absence of 2.0 mM GSH, no significant GTN metabolism was observed, indicating that GTN was degraded via a glutathione-dependent enzymatic pathway.

4.2 GTN metabolism in the cytosolic and microsomal fractions

The cytosolic fraction and the microsomal fraction of BCA were compared for their ability to metabolize GTN at an equimolar protein concentration (4.0 mg/ml). Figure V-5 depicts the profiles of GTN degradation in buffer, microsomes, and cytosol. It is clear that the cytosolic fraction exhibits the highest rate of GTN degradation, where approximately 20% of the original GTN dose remained after 120 min. The microsomal fraction was also shown to possess some activity in metabolizing GTN, but only an additional 20% of the original dose was metabolized in the microsomes when compared to the buffer control at

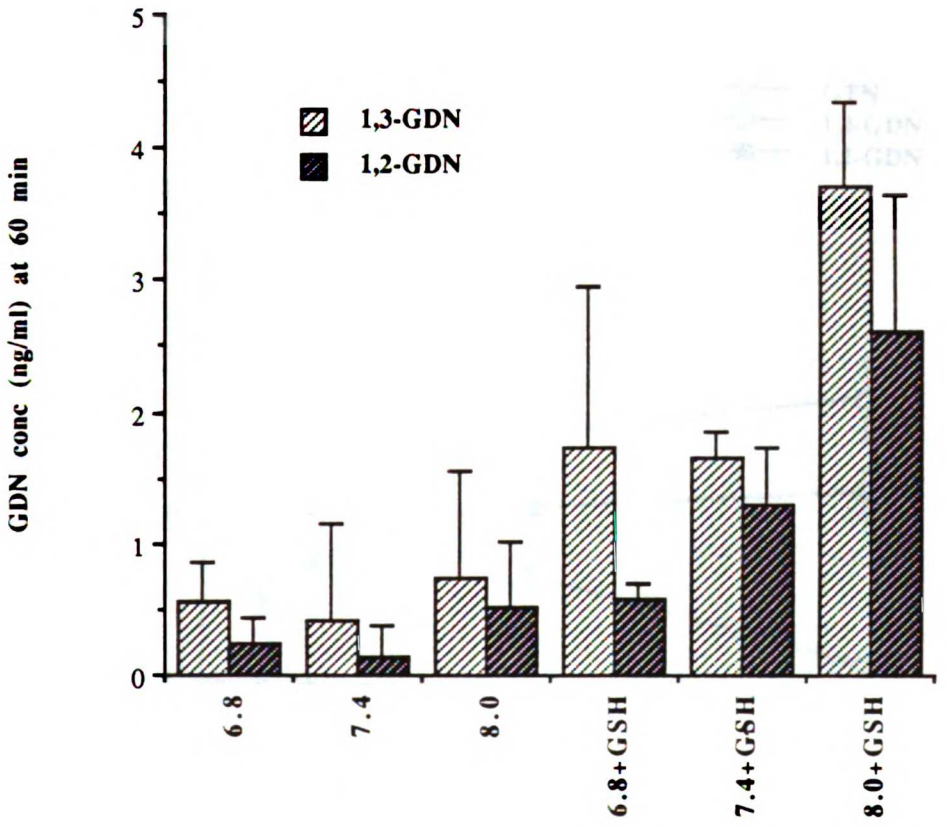


Fig. V-2. Concentration of 1,2-GDN and 1,3- GDN following 60-min incubation in phosphate buffer containing 40 ng/ml GTN at pH 6.8, 7.4, 8.0, and in the absence or presence of 2 mM GSH (mean±SD, n=3).

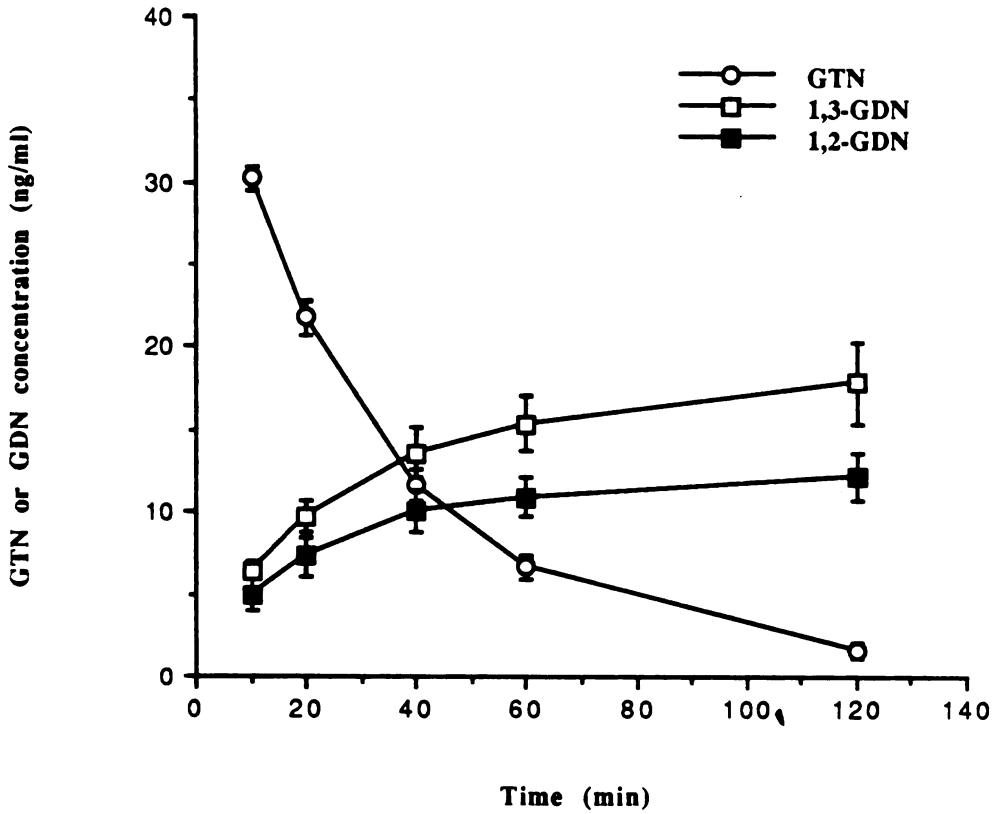


Fig. V-3. GTN degradation and GDN formation in 40 ng/ml GTN incubations with the 9000g supernatant fraction of BCA (mean±SD, n=3).

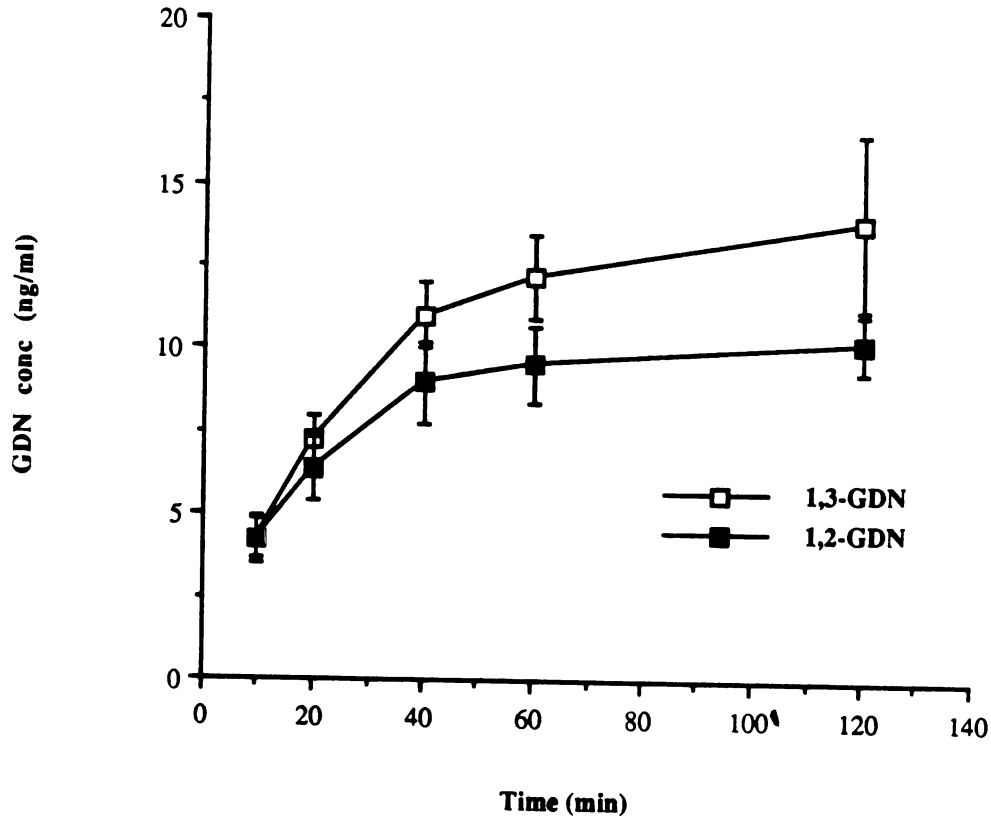


Fig. V-4. **GDN formation in 40 ng/ml GTN incubations with the 9000g supernatant fraction of BCA, corrected for the values of non-enzymatic GDN formation (mean±SD, n=3).**

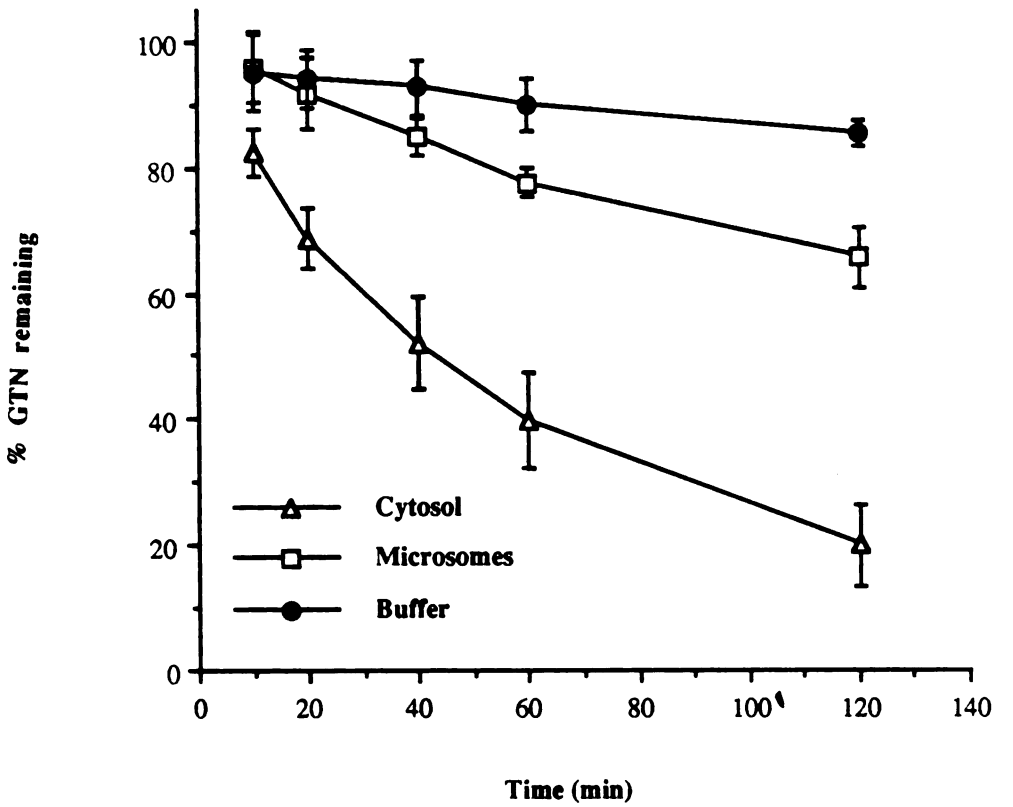


Fig. V-5. Precentage of GTN remaining unmetabolized in the cytosolic fraction and the microsomal fraction at different time points, compared with the buffer control. GTN concentration was 40 ng/ml and 2 mM GSH was added as the cofactor. Protein concentrations in the incubates were 4.0 mg/ml (mean±SD, n=3).

120 min. These data suggest that on an equivalent protein basis, the cytosol represents the more important site of GTN metabolism in vascular smooth muscles. Figure V-6 depicts the profile of GDNs generated from GTN metabolism in the two subcellular fractions, with the GDNs resulting from nonenzymatic GTN degradation in the control subtracted from the observed GDN concentrations. Differences in the amount of 1,3-GDN and 1,2-GDN seem to be better distinguished in the cytosol; whereas in the microsomes, no significant preference in the formation of the two GDNs was observed. In total, 65% of the GTN dose was enzymatically denitrated in the cytosol to form GDNs, compared to approximately 20% in the microsomes. Figure V-7 depicts the distribution of the amount of protein in the two subcellular fractions, as percentages of the total protein from the 9000g supernatant. The cytosol constituted the majority of the protein content in the 9000g supernatant, approximately 10-fold greater than the microsomal protein content. Thus, considering much higher rate of GTN metabolism observed in the cytosolic fraction and the predominance of cytosolic protein, these results suggest that the principal site of GTN metabolism in BCA is the cytosol. The cytosolic fraction of BCA was compared to the 9000g fraction for the rate of GTN metabolism without normalizing to protein content in 3 different studies (Fig. V-8). No difference was observed between the two subcellular fractions, further indicating that the cytosol is responsible for the majority of GTN metabolism in BCA.

4.3 Effect of GST inhibitors on GTN metabolism

The 9000g supernatant fraction was incubated with GTN in the presence ($2x10^{-5}$ M) of two GST substrates, SBP and ECA, as described in section 3.3. Figure V-9 demonstrates the effects of these compounds on the rate of GTN degradation. The rate of GTN elimination was attenuated in the presence of both SBP and ECA. The first-order half-life of GTN elimination increased from 25.7 min in the control to 66.0 min and 84.3

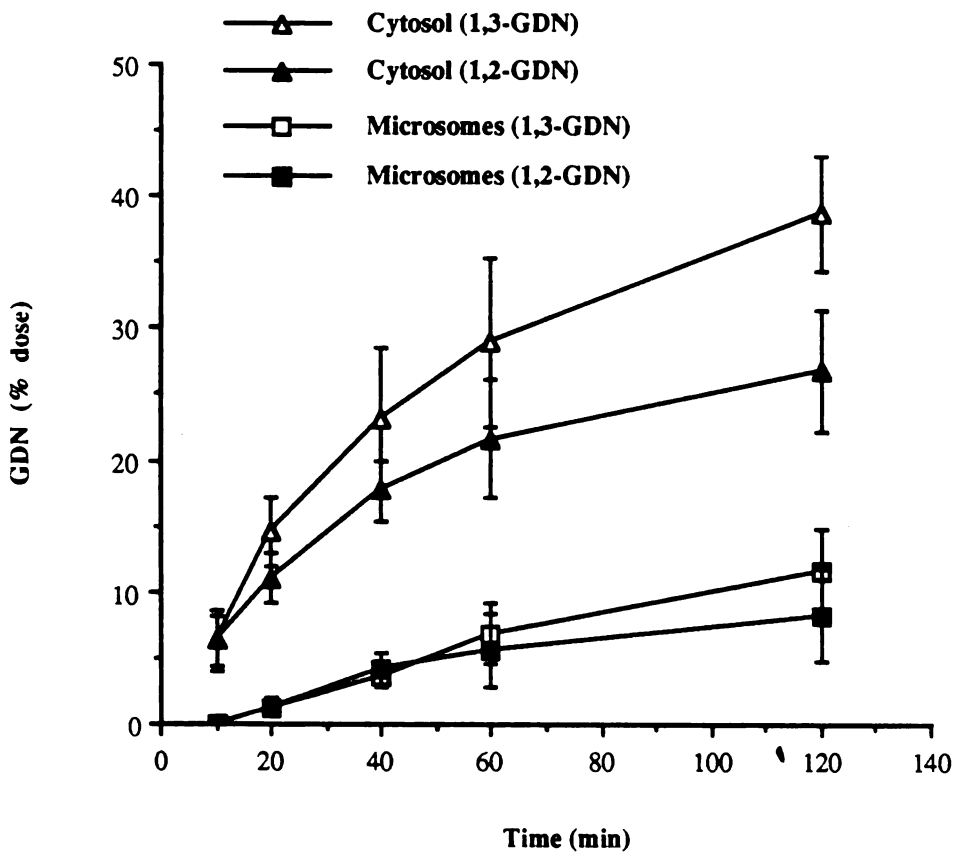


Fig. V-6. **GDN metabolites formed, as a percent of the dose, in the cytosolic fraction and the microsomal fraction, corrected for non-enzymatic GDN formation (mean±SD, n=3).**

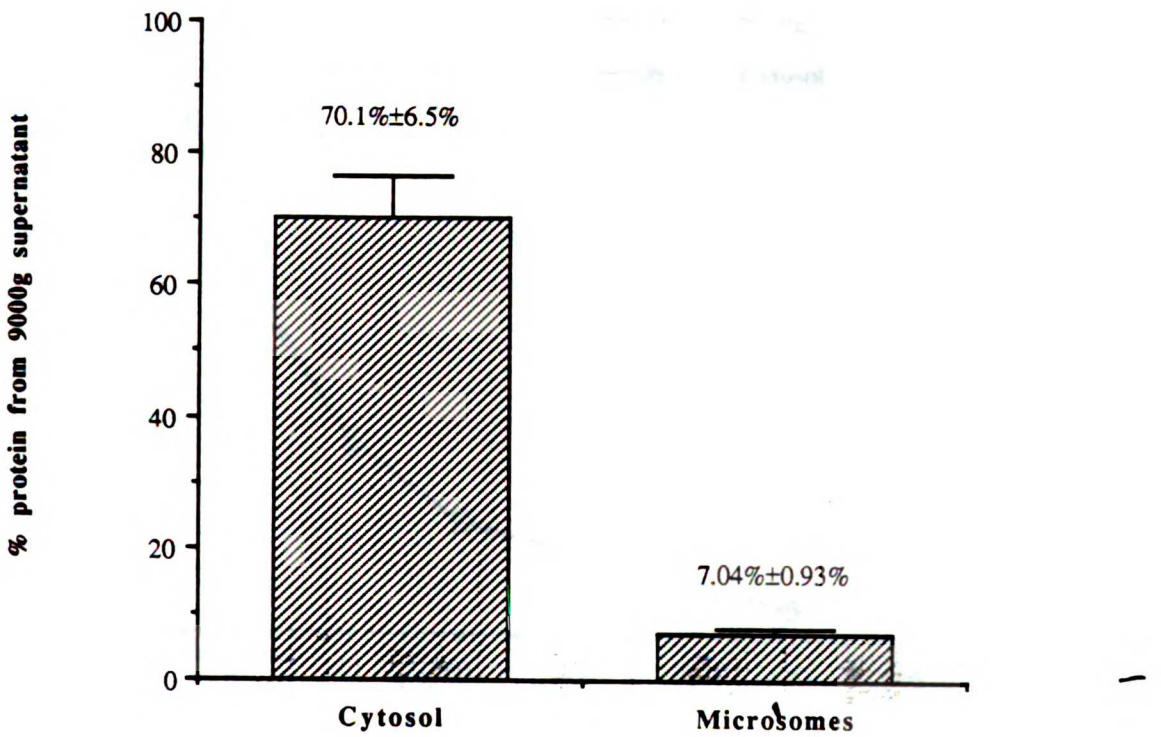


Fig. V-7. Percentage of protein in the BCA 9000g supernatant fraction recovered as the cytosolic and microsomal fractions (mean±SD, n=3).

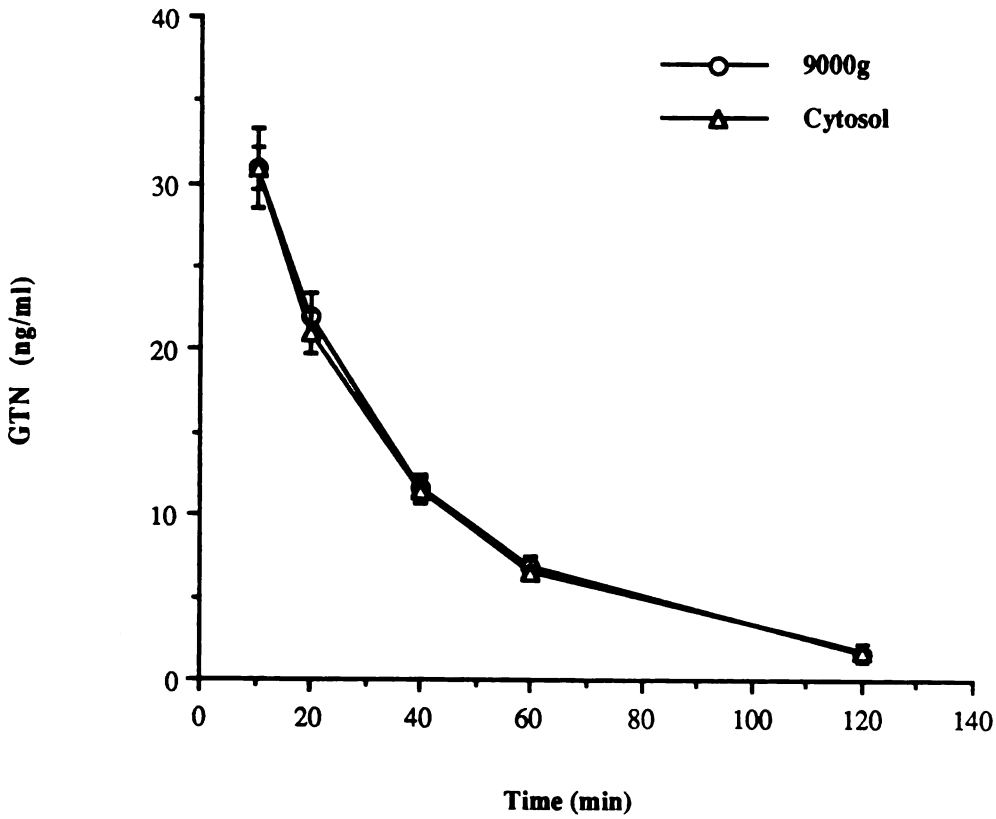


Fig. V-8. Comparison of the GTN degradation profiles in the 9000g supernatant and the cytosolic fraction, without normalization to protein concentration (mean±SD, n=3)

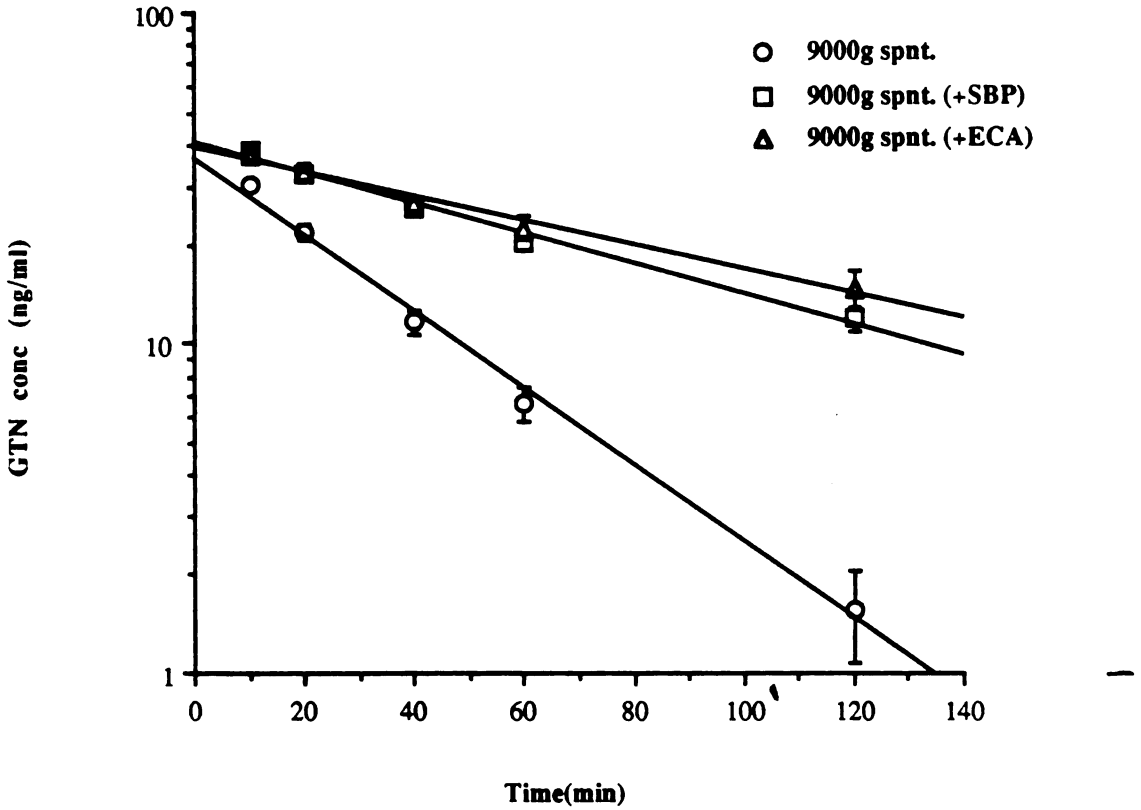


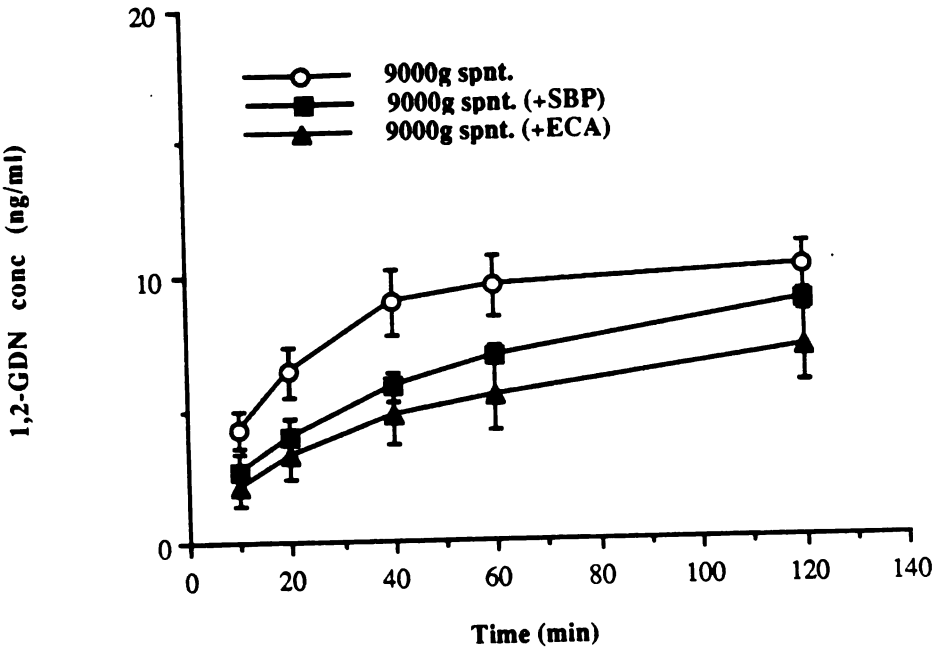
Fig. V-9 **The effects of 20 μM sulfobromophthalein (SBP) or ethacrynic acid (ECA) on GTN degradation (40 ng/ml) in the 9000g supernatant (9000g spnt.) fractions of BCA (mean±SD, n=3).**

and ECA on the formation of 1,2-GDN and 1,3-GDN, respectively. The concentrations of both GDNs were decreased when compared to the control; however, the decrease in 1,3-GDN formation was more extensive than that of 1,2-GDN formation. As a result, the GDN ratio increased in the presence of these GST inhibitors (Fig. V-11). The inhibition of GTN metabolism and the alteration in GDN formation by ECA were more prominent than those observed for SBP. The possible effects of SBP and ECA on nonenzymatic GTN degradation were examined in phosphate buffer at pH 7.4 (Figs. V-12a&b). No significant change in the extent of GTN degradation was observed at 60 min and 120 min; suggesting that the observed inhibitory effects of these agents were predominantly due to the inhibition of the enzymatic degradation of GTN.

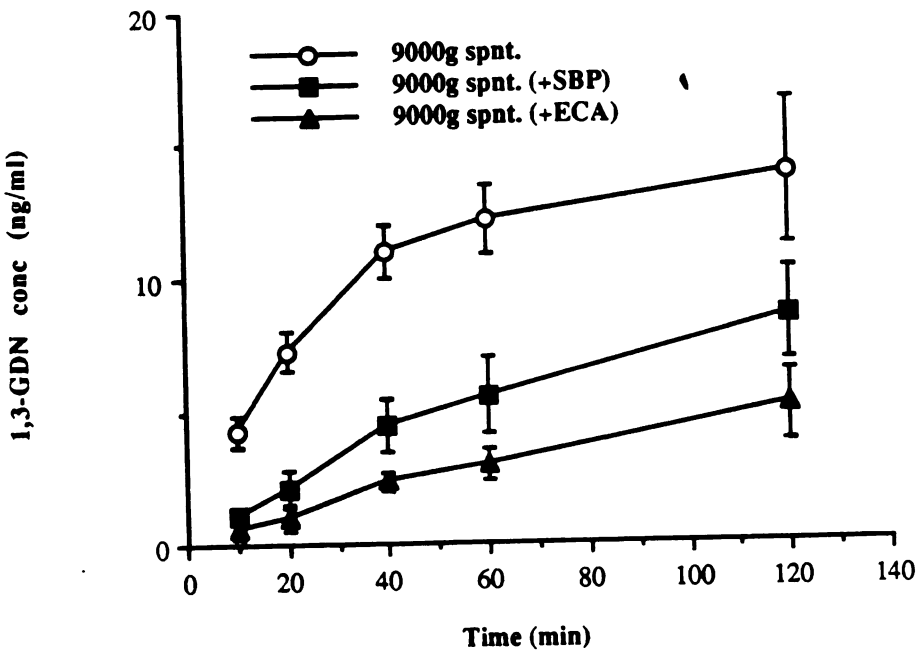
5. Discussion

According to the S-nitrosothiol hypothesis (Ignarro et al., 1981), GTN and other organic nitrates must undergo denitration for the subsequent generation of the pharmacologically active species (nitric oxide or S-nitrosothiol) which leads to vasodilatory effects. The relationship between organic nitrate metabolism and its induced-vasorelaxation has been reported by various investigators (Needleman et al., 1969; Brien et al., 1986, 1988; Slack et al., 1989). The enzyme(s) responsible for this denitration process has not been identified, although several possible candidates have been proposed (Marks, 1987; Bennett et al., 1990; Chung and Fung, 1990). In this study, GTN metabolism in BCA was examined, and the possibility of the involvement of GST in vascular GTN metabolism was explored.

GTN incubation studies with disrupted preparations of blood vessels from sheep (Cossum and Roberts, 1985a) and rats (Fung et al., 1984a) have been reported. A slight preferential formation of 1,3-GDN, similar to the results presented here, was observed in both of these studies. In addition, no difference in GTN metabolism was apparent when



(a)



(b)

Fig. V-10. Formation of 1,2-GDN and 1,3- GDN (a and b, respectively) in the 9000g supernatant fraction from incubations with 40 ng/ml GTN, corrected for nonenzymatic GDN formation

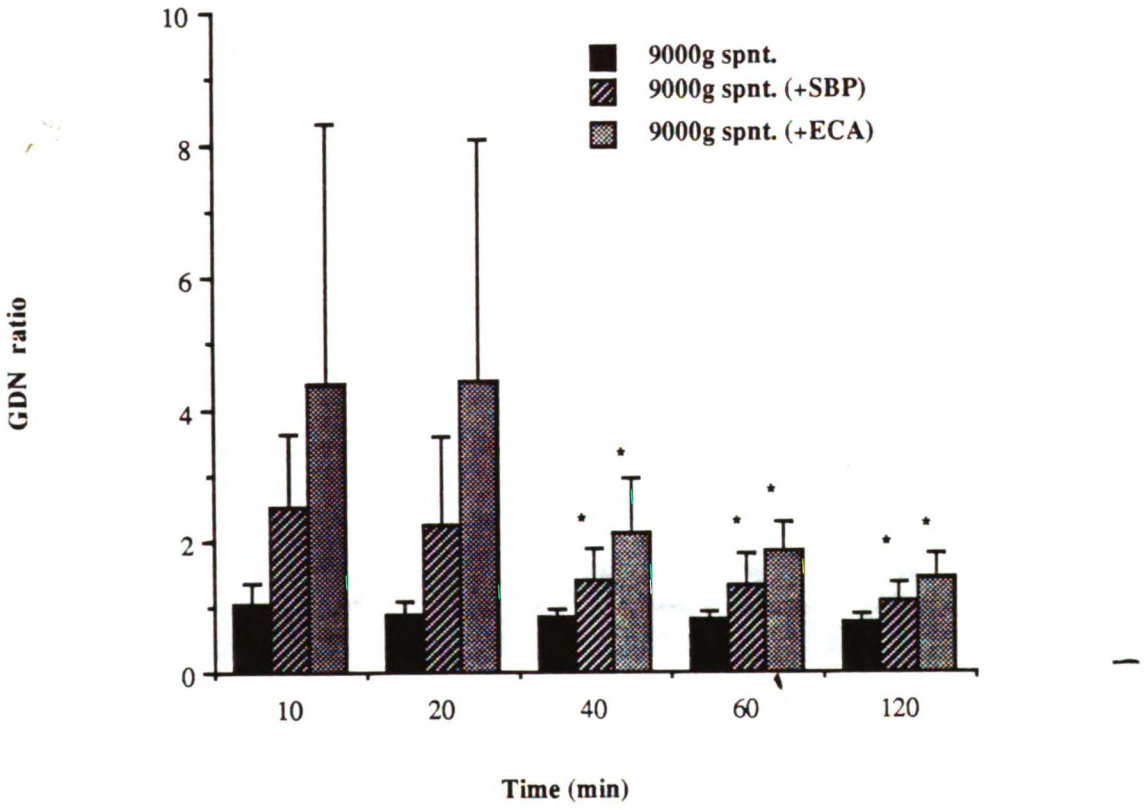
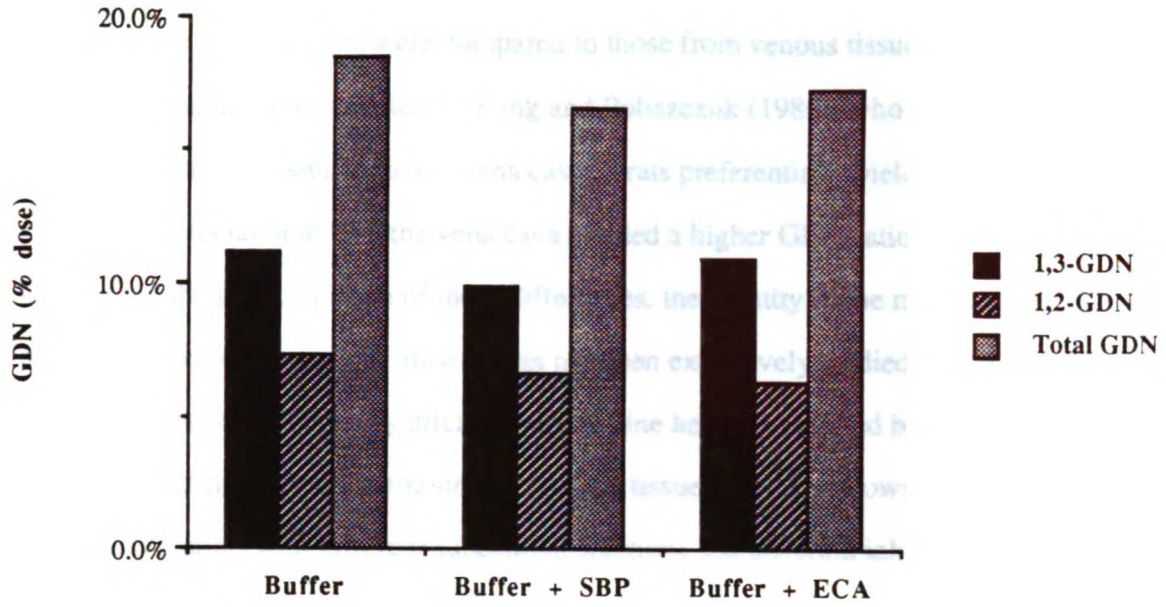
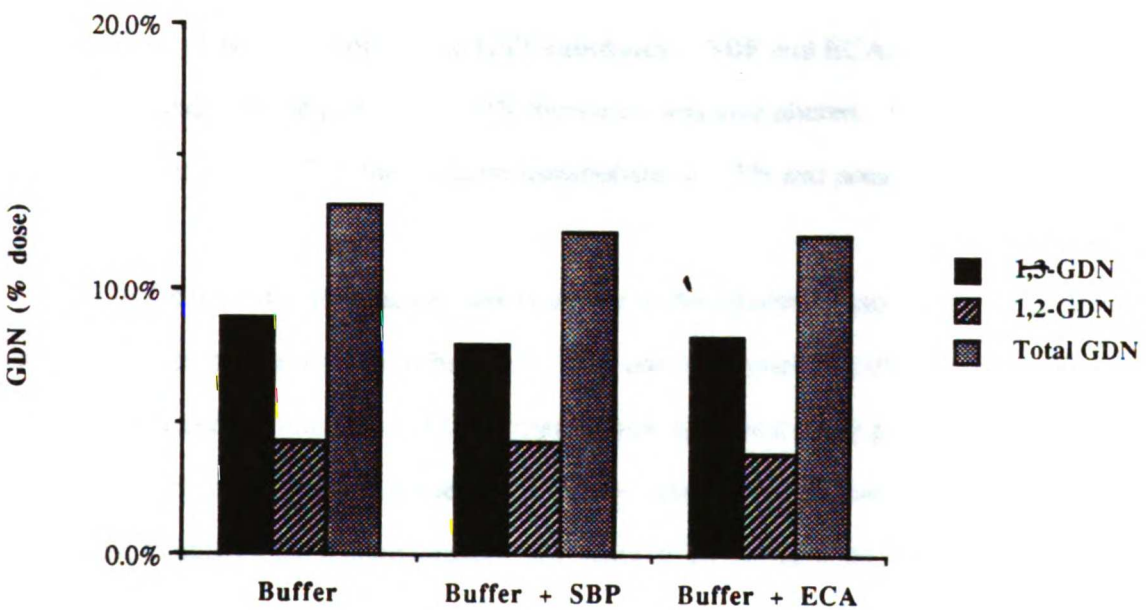


Fig. V-11. Resultant GDN formation ratios from SBP- and ECA-treated 9000g supernatant fraction, compared to the control (mean±SD, n=3; * $p<0.05$ versus control).



(a)



(b)

Fig. V-12. Percentages of GTN dose degraded non-enzymatically to GDNs in the presence of SBP and ECA at 60 min (a) and 120 min (b) (data represent the mean of duplicates).

arterial tissue homogenates were compared to those from venous tissues. An exception to the above findings was reported by Fung and Poliszczuk (1986), who observed that both the homogenates of both aorta and vena cava in rats preferentially yielded 1,2-GDN as the predominant metabolite, and the vena cava yielded a higher GDN ratio (1,2-GDN/1,3-GDN) than the aorta. In spite of these differences, the identity of the metabolizing pathways in vascular smooth muscles has not been extensively studied.

In this study, coronary arteries from bovine hearts were used because of the relatively higher yield in the amount of vascular tissues, thereby allowing various incubation studies with different subcellular fractions and different inhibitors of GTN metabolism. 1,3-GDN was found to be the slightly preferred metabolite from GTN denitration, indicating a regio-preference for C-2 denitration on the GTN molecule in these tissues. Moreover, the cytosolic fraction represents the major site where GTN is metabolized. Upon addition of two GST substrates -- SBP and ECA, GTN metabolism was attenuated, and the pattern of GDN formation was also altered. These results suggest the involvement of GST in the vascular metabolism of GTN and possibly other organic nitrates.

In the inhibitor studies, the concentration of the inhibitors used in the incubations was 100-times higher than the substrate (GTN), and 100-times less than the concentration of GSH. Therefore, inhibition of GTN metabolism observed in the presence of these agents should not be due to a depletion of the co-factors. In addition, the inhibitors were not found to change the nonenzymatic component of GTN degradation in buffer. Both SBP and ECA decreased the rate of GTN metabolism, and inhibited the formation of 1,3-GDN more extensively, indicating that these agents blocked an enzymatic pathway of GTN metabolism mediated by GST. The results in BCA are different from those of previous studies from our laboratory in rabbit liver cytosol (Chapter 4, section 4.4), where SBP and ECA inhibited 1,2-GDN formation more prominently (Lau and Benet, 1990); despite the fact that the results from both studies implicate the involvement of GST in GTN

metabolism. These contradictory results may be due to interspecies or intertissue differences in the GST enzyme, hence leading to different regioselectivities on GTN denitration. GST is known to consist of various isozymes in different organs (Corrigall and Kirsch, 1988) and different species (Boyer, 1989). The identity of the GST isozymes in blood vessels and their relationship to nitrate-induced vasorelaxation will be further examined in Chapter 6.

When compared to the liver, a relatively minor contribution of the microsomal fraction to the metabolism of the drug in BCA was noted. The rate and extent of GTN metabolism in the 9000g fraction of BCA were significantly lower in microsomes utilizing equivalent amounts of protein in the incubations (Fig. V-5). Moreover, the yield of microsomal protein from the 9000g supernatant fraction was approximately ten times lower than cytosolic protein. These data suggest that the glutathione-dependent pathway of GTN metabolism in vascular smooth muscles is in the cytosol. Although on the basis of the results presented here, it cannot be implied that the cytosolic GST-mediated metabolizing pathway also represents the pharmacologic activation process, results from this study warrant further examination of the role of GST isozymes in mediating vascular GTN metabolism, as well as their relationship to the production of pharmacologic effects.

Recently, GST isozymes were reported to be present in blood vessels (Mezzetti et al., 1990), and there are indications that a mu-isozyme of GST may be related to GTN metabolism and the generation of its effects (Tsuchida et al., 1990; Chern et al., 1991). SBP has historically been considered to be predominantly a GST-mu substrate (Habig et al., 1974; Mannervik et al., 1985). ECA, although previously identified as a GST-pi substrate (Mannervik et al., 1985), has recently been found to be more sensitive as an inhibitor of class mu isozymes of GST (Hansson et al., 1991; Ploemen et al., 1990). The inhibition of GTN metabolism by SBP and ECA, as observed here, may represent the inhibition of a GST enzyme responsible for both GTN metabolism and its pharmacologic effects.

6. Summary

In this study, GTN was found to be metabolized by disrupted tissue preparations of BCA, with 1,3-GDN as the predominant metabolite. The majority of GTN metabolism in BCA was carried out in the cytosolic fraction, which yields a similar ratio of GDN metabolites as found in the 9000g supernatant. Upon addition of SBP and ECA, the rate of GTN metabolism was drastically reduced, and the GDN ratio (1,2-GDN/1,3-GDN) was increased, suggesting the involvement of GST isozymes in the vascular metabolism of GTN.

CHAPTER SIX

Investigations on the Role of Glutathione S-Transferases in the Vascular Metabolism and Mechanism of Action of GTN

1. Introduction

The mechanism of action of organic nitrates has been discussed in detail in Chapter 2. It is generally believed that GTN and other nitro-vasodilators act as prodrugs (Ignarro et al., 1981), which are rapidly metabolized by vascular smooth muscles in the blood vessels to generate pharmacologically active intermediates that can elicit vasorelaxation, rather than exerting their actions directly as receptor-binding ligands (Kawamoto et al., 1988). The mechanism suggests that the presence of an enzyme, which exhibits the ability to denitrate organic nitrates, represents the first step in a cascade of reactions which in turn lead to the formation of pharmacologically active species, such as the proposed S-nitrosothiols (Ignarro and Gruetter, 1980; Ignarro et al., 1981) or nitric oxide (Feelisch and Noack, 1987; Henry et al., 1989; Marks et al., 1989b). The identity of the enzyme(s) which are responsible for metabolizing GTN and other organic nitrates within the blood vessels has not been universally agreed upon. The glutathione S-transferases (GSTs) is a possible class of enzymes that could be responsible for the denitration of organic nitrates. It has been reported (Kamisaka et al., 1975) that GSTs are involved in the hepatic metabolism of GTN. The reaction mechanism between GSTs and organic nitrates has also been proposed by Yeates et al. (1985). However, as yet there has not been any attempt to identify GSTs within blood vessels, nor to investigate their roles in the metabolism of organic nitrates and the generation of the vasorelaxative effects. Development of nitrate tolerance has also been linked to a decrease in the extent of nitrate metabolism in various in vitro studies (Fung and

Poliszczuk, 1986; Slack et al., 1989). Therefore, it is essential to clarify the role of GST isozymes in the vascular metabolism of organic nitrates.

2. Objectives

The objectives of the studies described in this chapter are:

a. to identify the presence of the different classes of GST isozymes in the rabbit aorta by immunoblots, i.e., with the use of antisera specific to different classes of GST isozymes;
b. to examine the effects of two GST substrate-inhibitors -- sulfobromophthalein (SBP) and ethacrynic acid (ECA), on the response of rabbit aorta strips to GTN;
c. to investigate and correlate the effects of SBP and ECA on GTN metabolism and GTN-induced vasorelaxation in rabbit aortic strips;
d. to investigate the effects of SBP and ECA on GTN metabolism and GDN formation in rabbit aorta homogenates.

3. Materials and Methods

3.1 Western Blots -- Identification of GST isozymes

3.1.1. Chemicals

The various chemicals used for the immunoblotting experiments, as well as the composition of the various buffers used, were listed in detail in Table VI-1. All chemicals were purchased at the highest available grade from Biorad (Richmond, CA).

3.1.2. Gel electrophoresis (SDS-PAGE)

Stock solutions for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

Acrylamide/bis-acrylamide	(30g / 0.8g/ 100ml)
Lower TRIS buffer	1.5 M Tris/HCl, 0.4 % SDS, pH at 8.8
Upper TRIS buffer	0.5 M Tris/HCl, 0.4 % SDS, pH at 6.8
Ammonium persulfate	2 %
Reservoir buffer	0.025 M Tris base, 0.2 M glycine, 0.1 % SDS
Bromophenol blue	0.04 % in 2.5 M Tris/HCl (pH at 6.8)
Pretreatment solution	200 µl 40% sucrose 400 µl 20% SDS 200 µl 0.04% bromophenol blue 200 µl β-mercaptoethanol

Running gel composition:

Distilled water	12.1 ml
Acrylamide-bis acrylamide	10.0 ml
Lower TRIS buffer	7.5 ml
Ammonium persulfate	0.45 ml
N, N, N', N'-tetramethylethylenediamine	7.5 ml

Stacking gel composition:

Distilled water	6.35 ml
Acrylamide-bis acrylamide	1.0 ml
Lower TRIS buffer	2.5 ml
Ammonium persulfate	0.15 ml
N, N, N', N'-tetramethylethylenediamine	10.0 ml

Composition of the electroblotting buffer:

TRIS base	0.025 M
Glycine	0.2 M
Methanol	20 %

Composition of the immunostaining solutions :

TRIS/saline buffer	
Sodium chloride	0.9 %
TRIS base	10 mM
(pH adjusted to 7.4 by conc. HCl)	
Diaminobenzidine	100 mg/ 267 ml of Tris/HCl (0.05M at pH 7.6)

Table VI-1. Chemicals, solutions, gels and buffers used in the immunoblotting experiments

The procedures for the separation of proteins by SDS-PAGE were adapted from Laemmli et al. (1970). The running gel was prepared the day before the electrophoresis experiment. The running gel solution was prepared (20-25 ml), and poured into the space between two glass plates, which were secured in a vertical gel-casting assembly. 2 ml of distilled water was applied on the top of the running gel to prevent dehydration, and the gel was allowed to form overnight. The stacking gel was prepared the next day, after the layer of water was removed, by pouring the stacking gel solution to fill up the space between the glass plates. A "comb" was inserted to create wells for the purpose of applying the samples. The stacking gel was allowed to set for 2 hours. Compositions for the running and the stacking gels are listed in Table VI-1.

The reservoir buffer was then prepared and poured into the electrophoresis cell (Protean II, Biorad). The cassette of glass plates containing the gel was assembled onto the cell, and a top reservoir was secured at the top of the cassette. The top and the bottom reservoirs were then filled with the reservoir buffer. The samples containing GSTs were added with the pretreatment solution. The samples were then boiled for 1-2 minutes to denature the proteins, and loaded into the wells with a Hamilton syringe (up to 50 µl for a 15-well gel). The unit was connected to the power supply, and was initially run at 20 mAmp for the stacking gel. After the front reached the running gel, the power was increased to 30 mA. When the dye front reached the bottom of the gel, the cassette was removed from the unit to prepare for electroblotting.

3.1.3. Nitrocellulose blotting

The procedures for the electrotransferring of proteins and their subsequent immunodetection of proteins by specific antisera were adapted from Towbin et al. (1979). Nitrocellulose (NC) papers were cut to the size of the gel (12x14 cm). The NC paper, as well as the sponge pads and blotting papers, were soaked in the Tris-saline buffer (Table VI-1). The NC paper was carefully aligned on the gel. The blotting papers and the sponge

pads were then placed on both sides of the gel and NC paper to create a "sandwich". The "sandwich" was then placed into the electroblotting unit (Trans-Blot Cell, Biorad), which was filled with the Tris-saline buffer. The unit was run at 150 mA for over 12 hours to allow adequate time for the transfer of proteins to the NC paper.

3.1.4. Immunodetection

The "peroxidase anti-peroxidase (PAP) bridge method" was used for the immunostaining and the detection of GST isozymes. The NC paper, which was electroblotted with proteins, was placed in a staining box. The paper was incubated with the blocking solution (3% fish gelatin oil) at 40 °C for 15 min to minimize nonspecific binding of the antisera to the NC paper. It was then incubated with the primary antisera (1:2000), i.e., antisera which can bind to the different classes of GST isozymes, for one hour at 37 °C. (These primary antisera were graciously supplied by our collaborators at Glaxo Inc.). The paper was then washed 4 times with the Tris/saline buffer (Table VI-1), with each wash consisting of 2 quick rinses and 1 slow rinse. The bridging antisera (1:200) was then incubated with the paper for 15 min. Following a second wash, the paper was incubated for 15 min with PAP (1:2000), which can recognize the bridging antibodies. Following another wash, hydrogen peroxide was added to the diaminobenzidine solution (Table VI-1), and was incubated with the NC paper until maximum contrast between the background and the stained spots was achieved. The blotted NC paper was washed and dried between paper towels. A brown band on the NC paper indicated the presence of a particular GST isozyme in the sample.

3.2. Rabbit aortic strip (RAS) experiments

3.2.1. Chemicals

GTN was purchased as 10-ml vials of Tridil® from DuPont Pharmaceuticals (Wilmington, DE). 1,2- and 1,3- GDNs (> 99% purity) standards were graciously supplied by Marion Laboratories (Kansas City, MO). *l*-Phenylephrine hydrochloride (PE), sulfobromophthalein (SBP), and ethacrynic acid (ECA) were obtained from Sigma Co. (St. Louis, MO). Ingredients of the Krebs-Henseleit buffer (NaCl, KCl, KH_2PO_4, $MgSO_4$, $NaHCO_3$, $CaCl_2$, and glucose) were also obtained from Sigma. The organic solvents used in the extraction procedure, i.e., pentane, methyl-*t*-butyl ether and butyl acetate, were purchased at the highest purity available from EM Sciences (Cherry Hill, NJ).

3.2.2. Preparation of the aortic strips

New Zealand White rabbits (Nitabell Rabbitry, Hayward, CA; male, 2-3 kg) were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and decapitated 15 minutes later. The thoracic cavity of the animal was immediately opened, and the descending aorta was quickly removed from the body. Adipose and connective tissues were removed, and the aortic tissue was immersed in a beaker of Krebs-Henseleit buffer (NaCl 119mM, KCl 4.8mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM, $CaCl_2$ 2.5mM, glucose 11.1mM), bubbled continuously with carbogen (95% oxygen, 5% carbon dioxide). The tissue was then cut into three helical strips of dimensions 3 cm x 4 mm. The strips were individually suspended in a buffer medium contained in a jacketed circulating water bath maintained at 37 °C. The contraction and relaxation of the tissues were recorded via transducers that were coupled to a Grass model 7 polygraph (Quincy, MA). The tension on the strips was maintained at 1 g. The tissues were allowed to equilibrate for at least 30 min before addition of any chemicals.

Upon stabilization, 100 μM PE was added to contract the strips maximally. The strips were then washed four times, 3 min apart, with the buffer. This was then followed by four longer washes for 15 min, and the strips were then allowed to stabilize. 100 μM PE was added for a second time, and the maximal tension achieved by each strip was

recorded. Interestingly, the second PE addition always resulted in a greater contraction than the first. The strips were then washed as described following the first PE addition. Upon stabilization, the strips were treated with consecutive additions of 0.1 μM PE, until the tension reached 60%-80% of the maximal contraction previously recorded (in most cases, 0.3-0.5 μM PE was necessary). At this point, the strips were ready for either the time-course experiment (Section 3.2.3.), or the dose-response experiment (Section 3.2.4.)

3.2.3. Time-course study

After the tension of the strips reached 60% to 80% of the maximal PE-induced contraction, one of the three strips was randomly selected as the control, while the other two were incubated for at least 10 min with inhibitors SBP or ECA at final concentrations of 0.1 mM. Upon stabilization, GTN was then added to each of the strips at a concentration of 0.5 μM, and the relaxation was recorded at 0.5, 1, 2, and 5 min. At 5 min, the strips were taken out of the tissue baths, wrapped in a piece of aluminum foil, dipped into a jar of liquid nitrogen, weighed, and stored at -70 °C until analysis.

GDN metabolites were assayed as reported previously (Chapter 3, Section 3.4), with slight modifications. Briefly, the strips were extracted 3 times with 10-ml mixtures of pentane and methyl-t-butyl ether (8:2). The organic extract was evaporated under a nitrogen stream, and reconstituted in n-butyl-acetate. o-Iodobenzyl alcohol was used as the internal standard. 0.2 μl of the reconstituted sample was injected into a Varian 6000/6500 gas chromatograph equipped with a Ni-63 ECD detector (Varian, Sugar Land, TX). The detection limit of the assay for the GDNs is 0.1 ng, and the amounts of GDNs measured were normalized to strip weights for comparison.

The three groups were compared using the repeated measure ANOVA test. The Dunnett's test was performed in comparing the two treatment groups to the control. For the correlation studies, linear regression was performed using geometric mean analysis,

which assumes errors are possible for both the x- and y- axes measurements. The correlation coefficient was tested for significance using the Student's t-test.

3.2.4. Dose-response studies

In these experiments, one of the three strips was randomly assigned as the control, and the other two strips were incubated with SBP or ECA at two different inhibitor concentrations (low : 0.01 mM, or high : 0.1 mM). The strips were allowed to stabilize for 10 min. Different concentrations of GTN were then added to the strips, from approximately 0.1 nM to 4 μM (10 different concentrations), in an increasing order. The relaxation of the strips caused by each of these GTN additions was recorded. A dose-response curve was constructed, using the cumulative percentage of relaxation from PE-induced contraction of these strips as the response measurement. A sigmoidal E_{max} model, was used to fit the concentration-response relationship for GTN:

$$E = \frac{E_{max} * C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}$$

where E = Effect generated by GTN, as measured by reversal of PE-induced vasoconstriction;
C = Concentration of GTN;
E_{max} = The maximum effect that can be generated by GTN;
EC_{50} = The concentration of GTN which can generate 50% of E_{max}; and
γ = The Hill coefficient.

The fitted parameters, E_{max}, EC_{50} and γ, were then compared among the control and the inhibitor-treated groups.

3.3 Incubation of rabbit aorta homogenate

3.3.1 Chemicals

GTN, GDNs, SBP, and ECA were obtained as previously described in Section 3.1.1. Reduced glutathione (GSH) was obtained from Sigma. Potassium phosphate (monobasic) and sodium phosphate (dibasic) were obtained from Fisher (Fairlawn, NJ). All chemicals were purchased at the highest grade of purity obtainable.

3.3.2 Preparation of 9000g fraction of rabbit aorta

The thoracic aorta of rabbits were obtained as described in Section 3.2.2., but were placed immediately in ice cold phosphate buffer (0.13M KH_2PO_4-Na_2HPO_4). Blood vessels from 15 rabbits were pooled together to provide enough tissues for the incubation study. The blood vessels were then sliced, chopped, and homogenized in three volumes of phosphate buffer by a blade homogenizer (Brinkman Instruments, Westbury, NY). The blood vessel homogenates were centrifuged at 9000g and the supernatant layer was decanted and saved. The 9000g fraction was stored at -70 °C prior to the incubation experiment.

3.3.3 Incubation studies

In each incubation, 3 ml of the 9000g supernatant was used. The incubates were placed in a water bath with the temperature maintained at 37 °C. GSH (2 mM) was added as the co-factor for the metabolic reaction. The mixture was then pre-incubated for five minutes. For the inhibitor studies, 2×10^{-5} M SBP or ECA was added just before the 5 minute pre-incubation period. Following pre-incubation, 40 ng/ml (1.76×10^{-7} M) GTN was added to the incubates. 500 µl samples were taken at 30 and 60 min, and were frozen immediately in a mixture of dry ice and methanol. The concentrations of GTN and GDNs were determined as described previously (Chapter 3, Section 3.4). Due to the presence of nonenzymatic GTN degradation in phosphate buffer, a vial containing just phosphate

buffer and 2 mM GSH was incubated with GTN as the control. The extent of non-enzymatic degradation at each sampling time was subtracted from the observed metabolism in the experimental samples in order to account for the metabolites resulting from non-enzymatic GTN degradation.

4. Results

4.1 Western Blots

Figure VI-1 shows the results of the Western blots of rabbit aorta 9000g and 105,000g supernatants with antisera recognizing the alpha, mu, and pi classes of GSTs. The most intense bands were observed with the anti-pi blot. However, both the anti-alpha and anti-mu blots showed the presence of GST isozymes belonging to these two classes. The antisera for alpha and pi GSTs were directed against rabbit lung GSTs, while the antiserum for mu GSTs were directed against rat liver GST-mu. Therefore, the intensity of the band cannot be interpreted quantitatively as to reflect the abundance of the isozymes. However, it is clear that all three classes of GSTs exist in the 9000g and 105,000g supernatants of rabbit aorta.

4.2. Aortic strips -- time-course study

No difference in the extents of PE-induced contraction in the 3 groups of aortic strips was observed, as shown in Table VI-2. However, there were differences in the magnitudes and the extents of GTN-induced relaxation among the 3 groups. The ECA-treated strips exhibited a significantly attenuated response to GTN when compared to the control and the SBP-treated groups. SBP did not seem to exert any effect on GTN relaxation. The time courses of relaxation during the first 5 min after GTN addition for the three treatments are shown in Fig. VI-2. For the ECA-treated strips, significant attenuation in the relaxation response was initially observed at 30 sec, and the effects of GTN reached

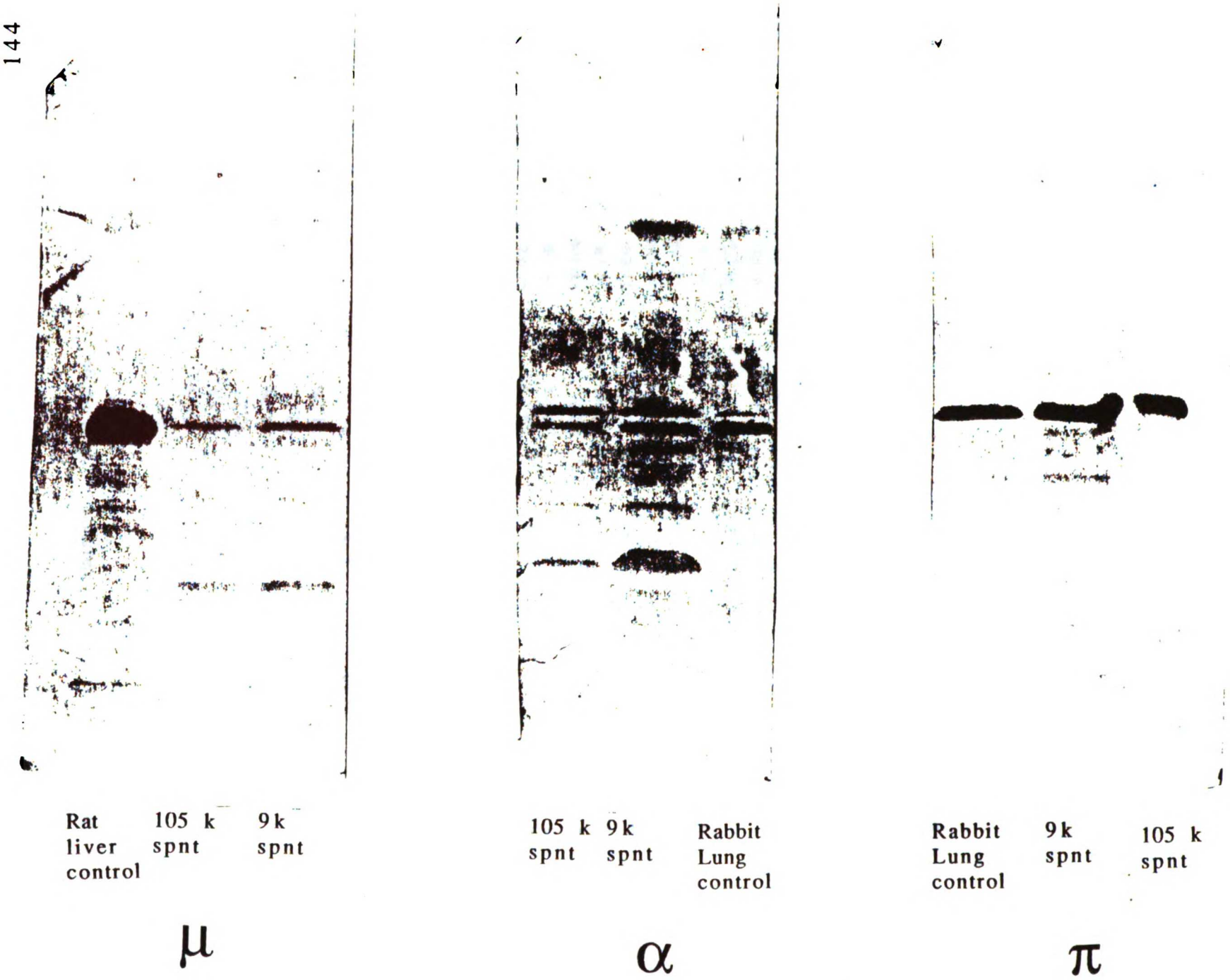


Fig. VI-1. **Western blot analysis of the various GST isozymes present in the 9000g and 105,000g supernatants of rabbit aorta. Each lane was loaded with 50 μg protein.**

	EXPERIMENT	PE-Contraction (g)	Relaxation (g)	% Relaxation (%)	% Relaxation (% control)
CONTROL	1	1.89	1.51	79.8%	
(C)	2	3.13	2.40	76.6%	
	3	2.40	2.27	94.4%	
	4	2.66	2.45	92.2%	
	5	2.43	2.05	84.2%	
	6	2.58	2.38	92.1%	
	7	1.06	0.76	71.7%	
	8	1.85	1.48	80.0%	
	9	3.91	3.16	80.8%	
	Mean	2.43	2.05	83.5%	
	±SD	0.81	0.70	7.8%	
	%CV	33.3%	34.3%	9.4%	
SBP-TREATED	1	1.10	0.90	81.9%	102.6%
(S)	2	2.23	1.66	74.2%	96.9%
	3	3.14	2.92	92.8%	98.3%
	4	2.95	2.74	92.8%	100.6%
	5	2.41	1.89	78.3%	93.1%
	6	3.30	2.79	84.6%	91.8%
	7	2.24	1.67	74.6%	104.0%
	8	1.91	1.53	80.1%	100.1%
	9	3.84	3.00	78.1%	96.7%
	Mean	2.57	2.12	81.9%	98.2%
	±SD	0.83	0.75	7.0%	4.1%
	%CV	32.1%	35.5%	8.5%	4.2%
ECA-TREATED	1	1.37	0.56	41.0%	51.4%
(E)	2	2.35	0.99	42.1%	54.9%
	3	3.29	2.54	77.2%	81.8%
	4	2.88	1.73	60.2%	65.3%
	5	2.56	1.13	43.9%	52.1%
	6	2.88	1.78	61.8%	67.1%
	7	3.13	2.13	68.1%	94.9%
	8	1.94	1.19	61.3%	76.7%
	9	2.93	1.57	53.6%	66.3%
	Mean	2.59	1.51	56.6%	67.8%
	±SD	0.62	0.61	12.5%	14.6%
	%CV	23.7%	40.4%	22.0%	21.5%
STATISTICS :					
	ANOVA	NS	p<0.05	p<0.001	p<0.001
	Multiple Comparisons		S=C>E	C=S>E	S>E

Table VI-2. The contraction (PE-induced) and the relaxation (5 min after 0.5 μM GTN addition) of rabbit aortic strips in the presence of 0.1 mM SBP or ECA, compared to the control.

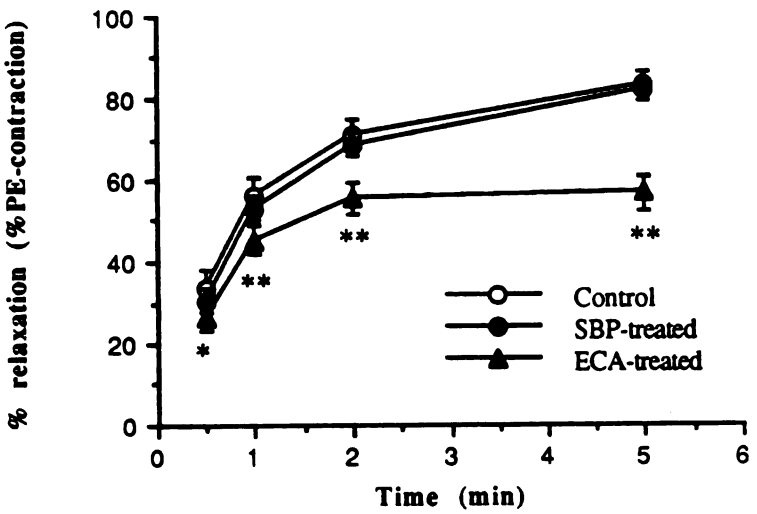


Fig. VI-2. Time-courses of relaxation, as percent of PE-induced contraction, in control, SBP-treated, and ECA-treated groups (mean±SE, n=9; * : $p<0.05$, ** : $p<0.01$ vs. control).

maximum values at 2 min, whereas in the control and the SBP-treated strips, relaxation continued to increase up to 5 min. There were no detectable differences between the SBP-treated strips and the control at any time point. For ECA-treated strips, the relaxation observed 5 min after GTN addition was 67.8%±4.8% of the control, whereas in the SBP-treated strips, there was no statistical difference from the control (98.3%±1.4%).

The amounts of the GDN metabolites of GTN were also measured in the aortic strips, and are listed in Table VI-3. The weights of the aortic strips were not significantly different. However, considerable variability existed in the amount of GDNs and GTN recovered from these tissues. Using repeated measures ANOVA, it was found that there was no statistically significant difference between the SBP-treated and the control strips. However, there were statistically significant reductions in the amount of total GDNs, as well as that of 1,2-GDN, in the ECA-treated strips when compared to the control group, indicating a decreased extent of GTN metabolism. When the amount of GDNs in the inhibitor-treated groups were normalized to the amount in the control for each animal, significant reductions in the formation of 1,2-GDN and 1,3-GDN were observed in the ECA-treated group, as shown in Fig. VI-3. For the SBP-treated group, no statistically significant difference in the formation of either GDN was observed. For both inhibitor-treated groups, however, there seemed to be no change in the pattern of GDN formation.

According to the proposed scheme of action of organic nitrates, GTN metabolism is tightly coupled to the generation of effects. Therefore, in order to explore if any correlation exists between the attenuation of GTN effects and reduction in GTN metabolism for the ECA-treated group, the relaxation caused by GTN addition was plotted against the formation of GDNs, each as percentages of the control values. Strong correlations were found between the GTN effects with the formation of 1,2-GDN (r^2=0.848, $p<0.01$), 1,3-GDN (r^2=0.805, $p<0.01$), and the sum of the two GDNs (r^2=0.809, $p<0.01$), as illustrated in Figs. VI-4 a, b and c, respectively.

	CONTROL (C)	SBP-treated (S)	ECA-treated (E)	ANOVA (p=)	Multiple Comparisons
Weight (g)	0.0631 ± 0.0095	0.0642 ± 0.014	0.0704 ± 0.012	0.242	
1,3-GDN/wt (ng/g tissue)	10.9 ± 12	8.18 ± 6.6	6.91 ± 6.6	0.066	
1,2-GDN/wt (ng/g tissue)	24.9 ± 12	26.7 ± 10	16.5 ± 10	0.006	S=C>E
Total GDN/wt (ng/g tissue)	35.8 ± 22	34.9 ± 14	23.5 ± 16	0.016	C=S>E
GTN/ wt (ng/g tissue)	303 ± 163	223 ± 110	325 ± 135	0.22	
Total nitrate/wt (ng/g tissue)	1.53 ± 0.70	1.17 ± 0.50	1.56 ± 0.57	0.287	

Table VI-3. Recovery of GTN and GDN metabolites from rabbit aortic strips, in tissues treated in the presence or absence of GST inhibitors (SBPor ECA). (mean±SD, n=9).

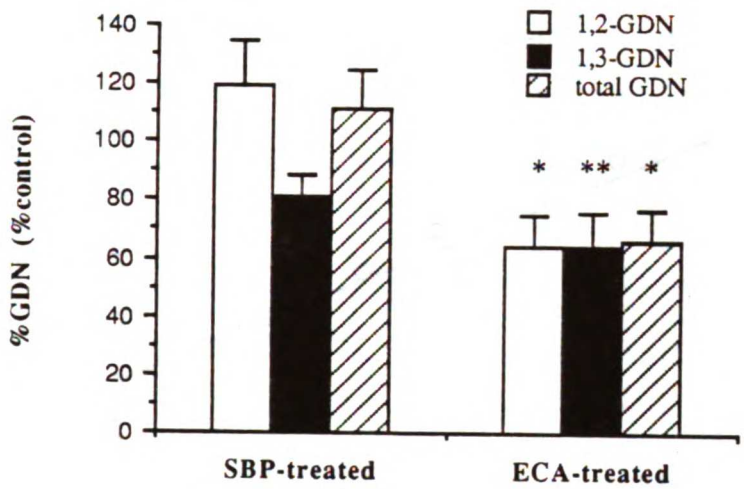


Fig. VI-3. Weight-normalized GDN measurements in the strips treated with SBP and ECA, as percentages of the control. (mean±SE, n=9; * : $p<0.05$, ** : $p<0.01$ vs. control).

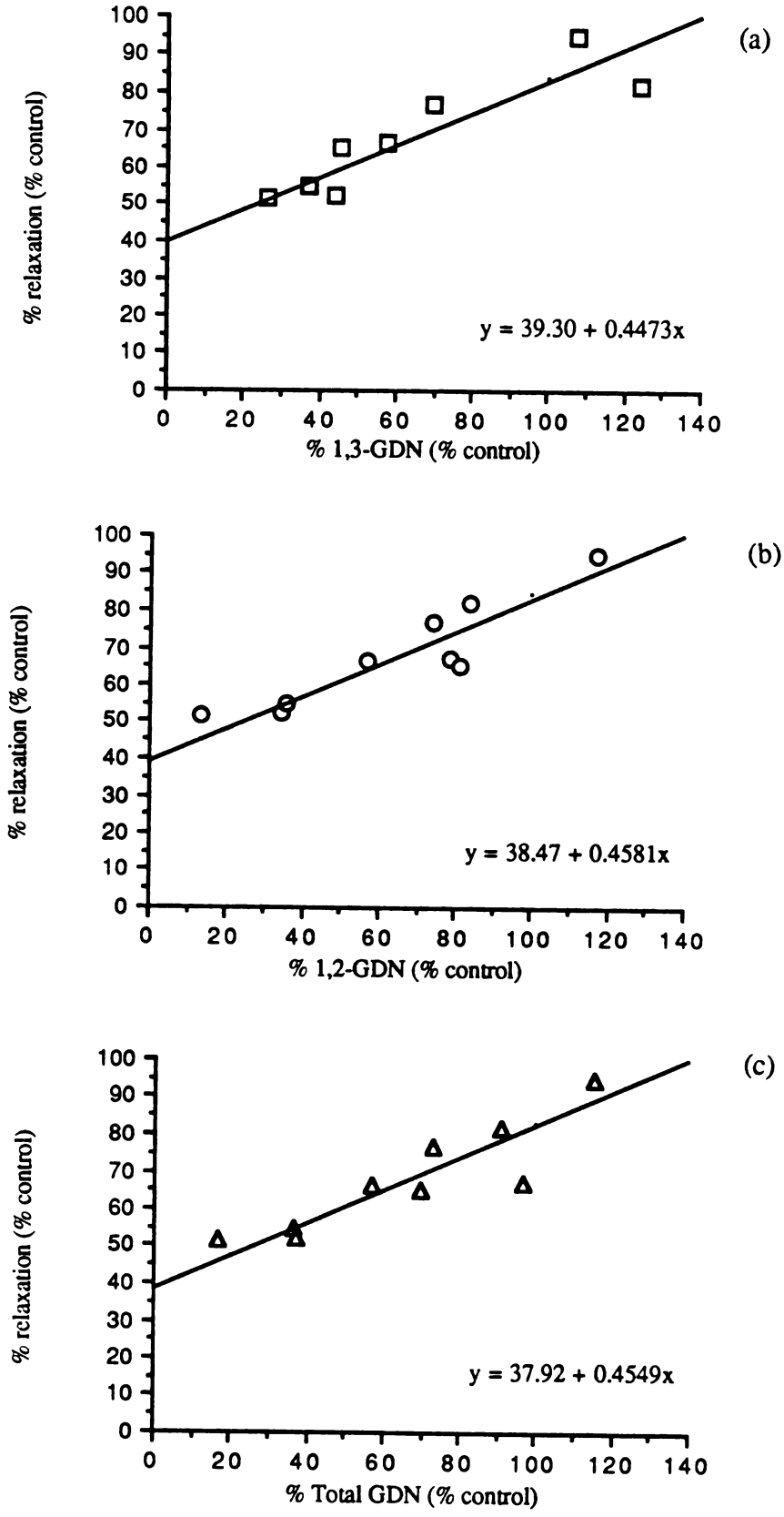


Fig. VI-4. Relationship in the presence of ECA between the aortic strip relaxation with the weight-normalized formation of : (a) 1,3-GDN (r^2=0.805), (b) 1,2-GDN (r^2=0.848), and (c) total GDN (1,2-GDN + 1,3-GDN) (r^2=0.809). All values are presented as the percentages of the corresponding control values. (n=9, except in (a), where one set of data was ignored because the 1,3-GDN measurement was below detection limits.)

4.3. Dose-response studies

To demonstrate that the effects of the inhibitors did not exist for one particular concentration of GTN, the dose-response relationships for GTN-induced relaxation were obtained in the presence and absence of SBP and ECA, as shown in Figs. VI-5 a & b, respectively. It is obvious that ECA resulted in a more apparent change in the dose-response relationship, especially at the higher inhibitor concentration, while SBP caused only minor alterations. As shown in Table VI-4, a mild, significant decrease in E_{max} (~10%), but no other alterations in the EC_{50} or Hill coefficient, were observed with the SBP-treated groups. With the ECA-treated strips, a similar magnitude of change in E_{max} was observed (Table VI-5). However, in addition to the change in E_{max}, there was a significant reduction in the slope factor (γ) of the Hill equation. The parameters obtained from the inhibitor-treated groups were normalized to those from the control group, and the ratios are depicted in Fig. VI-6. The attenuations in the Emax of the ECA- and SBP-treated strips were statistically significant from the control when normalized as ratio. Moreover, in strips treated with ECA at low (0.01 mM) or high (0.1 mM) concentrations, significant decreases in the slope factor was observed, which was not observed in the SBP-treated groups. In addition, the magnitude of alteration seems to increase as the concentration increases, indicating a concentration-dependent inhibition of the effects of GTN on the aortic strips by ECA. Although considerable variability existed in the EC_{50} values of both inhibitor-treated groups, the EC_{50}'s of the ECA-treated group almost achieved statistically significant differences with the control.

4.4. Incubation of rabbit aorta 9000g fraction with GTN

In order to compare the effects of these inhibitors on homogenized tissues versus the aortic strips, the 9000g fraction of a pool of 15 rabbit aorta was incubated with 40 ng/ml GTN in the presence or absence of these inhibitors. The reported concentrations of GDNs were corrected for nonenzymatic GTN degradation (in a vial containing GTN and

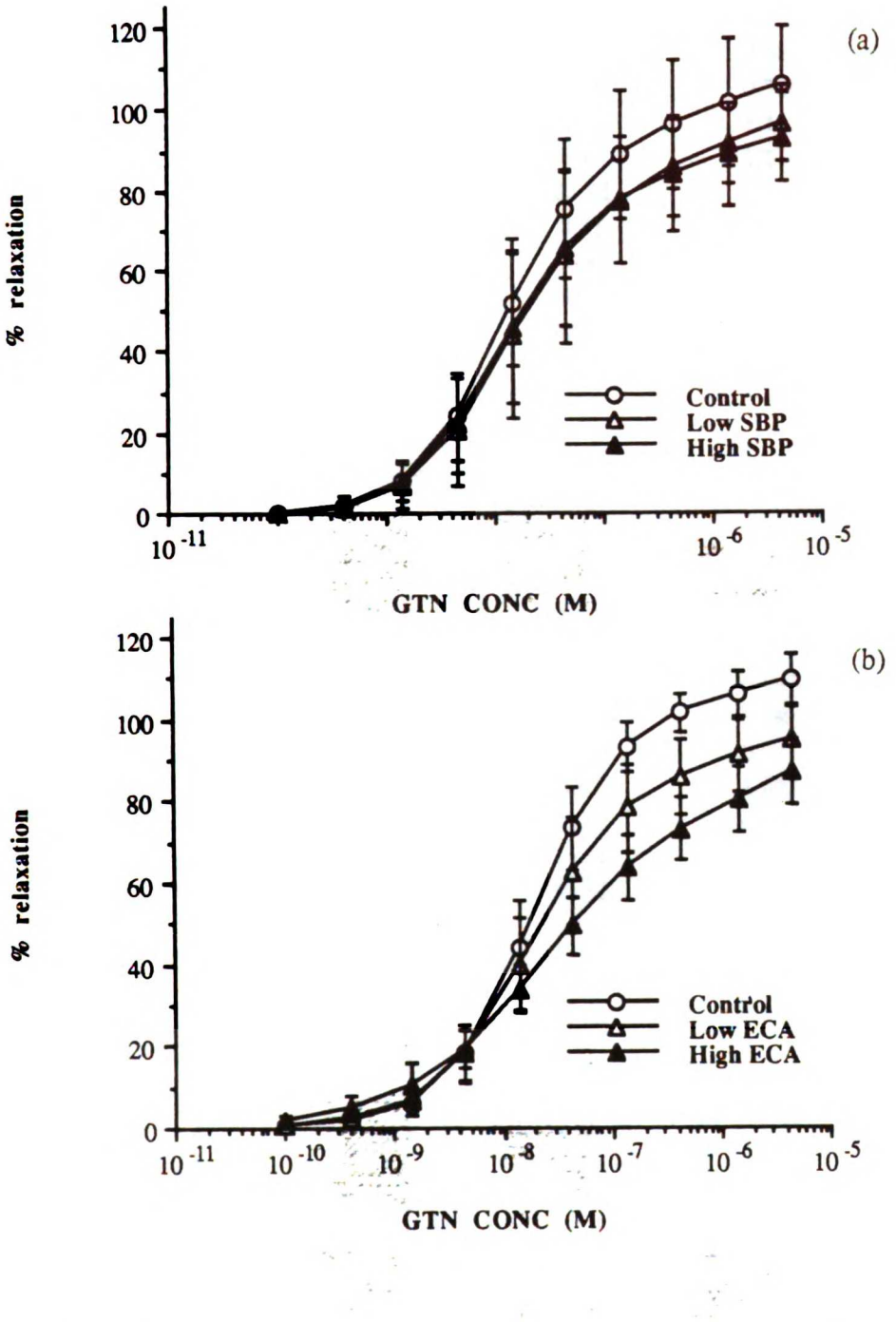


Fig. VI-5. Dose-response curves of rabbit aortic strips to GTN-induced relaxation. Tissues were treated with either low (0.01 mM) or high (0.1 mM) concentrations of (a) SBP (n=10) or (b) ECA (n=8), and were compared to the control in each study.

	EXPERIMENT	Emax (% relaxation)	EC50 (nM)	γ
CONTROL	1	112.5	41.72	0.887
(C)	2	114.8	23.84	0.780
	3	98.3	8.01	1.112
	4	95.9	6.19	1.118
	5	78.5	25.92	0.863
	6	107.9	8.85	1.155
	7	134.9	16.87	1.035
	8	104.8	9.92	1.072
	9	92.8	34.15	0.942
	10	94.9	17.11	0.908
	Mean	103.5	19.26	0.987
	±SD	15.3	12.0	0.128
	%CV	14.8%	62.3%	12.9%
LOW SBP	1	106.5	136.90	1.031
(0.01 mM)	2	94.9	132.80	0.750
(L)	3	96.6	8.88	1.034
	4	97.4	7.50	1.068
	5	89.6	22.56	0.887
	6	97.6	10.20	1.182
	7	109.1	13.06	0.952
	8	95.4	6.62	1.027
	9	76.9	31.90	0.979
	10	80.4	26.31	1.036
	Mean	94.5	39.67	0.995
	±SD	10.1	50.9	0.115
	%CV	10.7%	128.3%	11.6%
HIGH SBP	1	90.2	41.90	0.835
(0.1 mM)	2	97.8	44.43	0.731
(H)	3	95.7	9.46	1.091
	4	98.0	5.81	1.116
	5	85.4	19.85	0.913
	6	98.8	7.21	1.119
	7	99.9	9.40	0.913
	8	96.7	12.91	0.947
	9	63.3	102.20	0.850
	10	86.6	24.15	1.111
	Mean	91.2	27.73	0.963
	±SD	11.1	29.6	0.139
	%CV	12.1%	106.8%	14.5%
STATISTICS :				
	ANOVA	0.006	0.282	0.500
	Multiple Comparisons	C>L=H		

Table VI-4. The parameters obtained from fitting the Hill equation to the GTN concentration-response curves, in rabbit aortic strips treated with low (0.01 mM) or high (0.1 mM) concentrations of SBP, compared to the control.

	EXPERIMENT	Emax (% relaxation)	EC50 (nM)	γ
CONTROL	1	113.3	19.87	0.998
(C)	2	113.0	43.72	1.027
	3	105.8	31.85	1.043
	4	106.8	11.66	1.068
	5	106.4	13.53	1.107
	6	112.6	24.43	0.860
	7	104.1	23.75	1.104
	8	103.7	19.97	1.060
	Mean	108.2	23.60	1.033
	±SD	4.09	10.3	0.079
	%CV	3.8%	43.7%	7.7%
LOW ECA	1	99.4	17.12	0.879
(0.01 mM)	2	97.5	107.44	0.898
(L)	3	85.3	27.73	0.955
	4	100.2	13.74	0.918
	5	99.8	15.98	0.943
	6	84.4	25.97	0.727
	7	101.5	13.88	0.999
	8	82.9	18.94	0.906
	Mean	93.9	30.10	0.903
	±SD	8.11	31.7	0.080
	%CV	8.6%	105.3%	8.9%
HIGH ECA	1	91.3	36.33	0.712
(0.1 mM)	2	105.7	177.20	0.612
(H)	3	92.0	120.10	0.527
	4	75.4	24.96	0.643
	5	83.6	13.95	0.646
	6	101.6	27.18	0.523
	7	83.9	25.02	0.718
	8	86.7	29.34	0.718
	Mean	90.0	56.76	0.637
	±SD	9.91	59.1	0.080
	%CV	11.0%	104.0%	12.5%
STATISTICS :				
	ANOVA	p<0.001	p=0.059	p<0.001
	Multiple Comparisons	C>L=H		C>L>H

Table VI-5. The parameters obtained from fitting the Hill equation to the GTN concentration-response curves, in rabbit aortic strips treated with low (0.01 mM) or high (0.1 mM) concentrations of ECA, compared to the control.

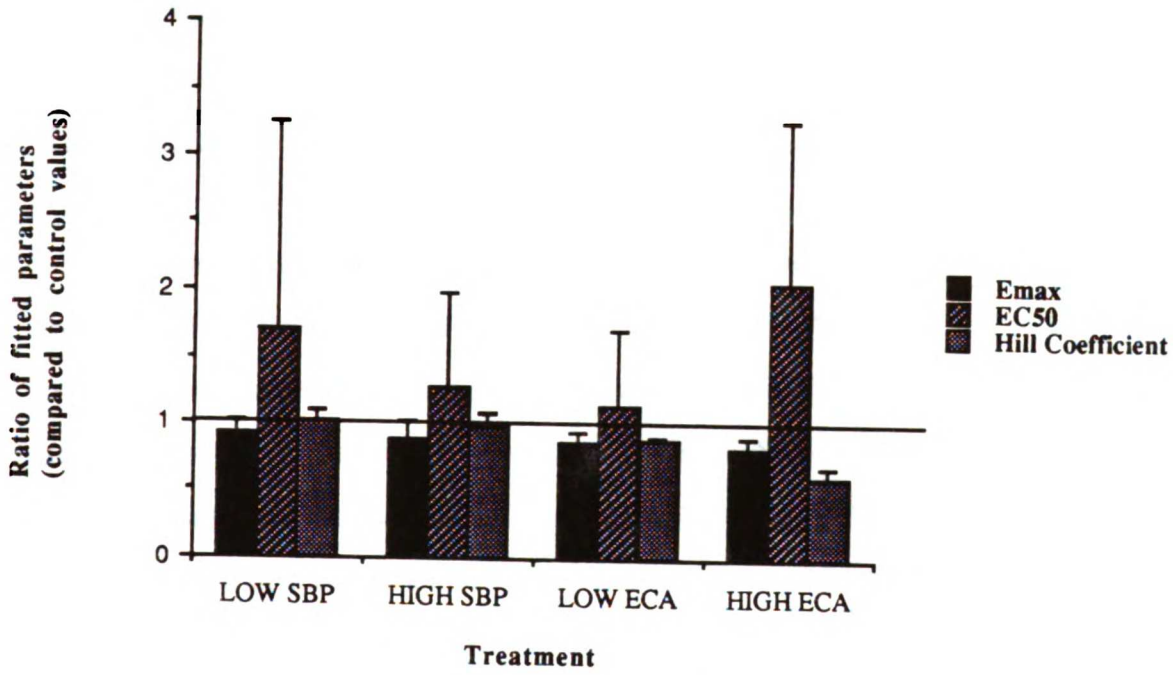


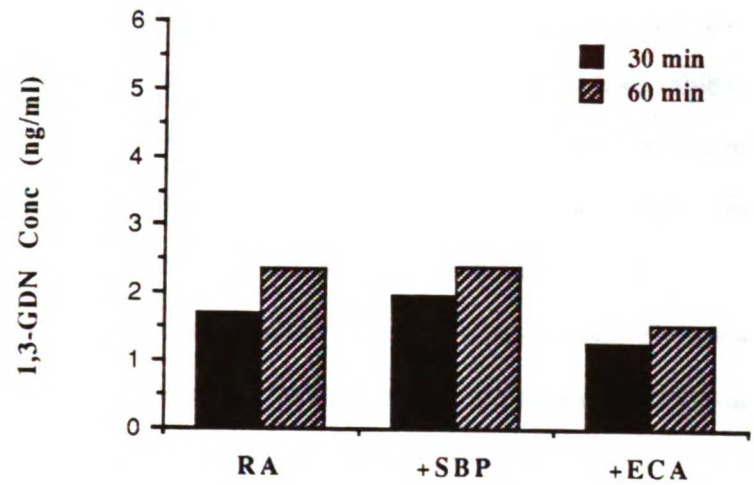
Fig. VI-6. **Ratios of the parameters obtained from the Hill equation fit, compared to the control values, for each inhibitor-treated group.**

GSH only), as previously described for the studies in Chapter 5, are depicted in Fig. VI-7. The changes in total GDN concentrations were more prominent in the ECA-treated than the SBP- treated group at both the 30- and 60 min time points, consistent with the more prominent inhibition of GTN metabolism and effects observed with the ECA-treated group in the aortic strip studies. Moreover, both inhibitors seemed to decrease the formation of 1,2-GDN slightly more extensively than that of 1,3-GDN.

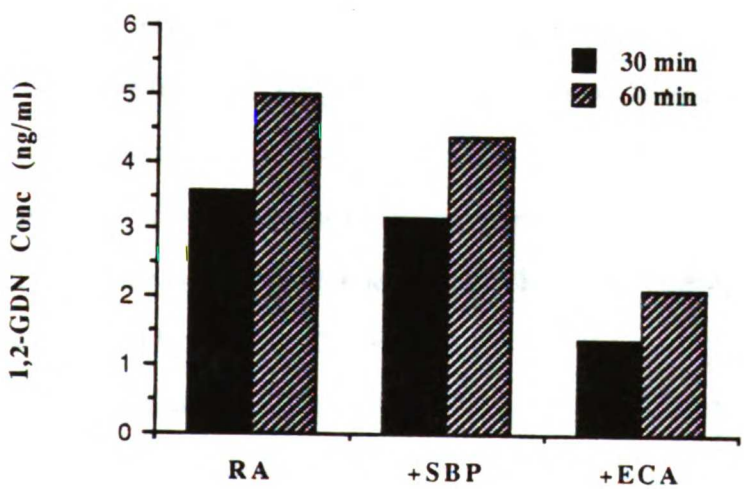
5. Discussion

According to their proposed scheme of action, organic nitrates must first be metabolized (Ignarro et al., 1981) in order to elicit their pharmacologic effects. Numerousreports have demonstrated that the metabolism of organic nitrates in vascular smooth muscle cells precedes their vasodilating effects (Kawamoto et al., 1986; Brien et al., 1988; Kawamoto et al., 1990). Tolerance to organic nitrates was also found to be associated with an attenuation of nitrate metabolism in tolerant tissues (Fung and Poliszczuk, 1986; Bennett et al., 1989; Slack et al., 1989). It is generally believed that the metabolism of nitrates in vascular smooth muscle cells is carried out enzymatically, although the enzyme responsible for the activation of these agents has not been identified.

In this study, the possible involvement of GSTs in the scheme of nitrate action was investigated. Previously, the role of GST in GTN metabolism in rabbit liver was investigated (Chapter 4). It was observed that hepatic GTN metabolism is a glutathione-dependent process (Lau and Benet, 1989), which can be inhibited by various GST substrates (Lau and Benet, 1990). There is also evidence that more than one GST isozyme may be involved, and that these isozymes may exhibit different regioselectivities for the denitration reaction of GTN. In Chapter 5, it was found that a cytosolic, GSH-dependent, enzymatic process responsible for GTN metabolism was present in bovine coronary artery homogenates. In addition, the rate of GTN metabolism was significantly reduced by two



(a)



(b)

Fig. VI-7. **1,3-GDN (a) and 1,2-GDN (b) recovered from 40 ng/ml GTN incubations in the 9000g supernatant of rabbit aorta, corrected for nonenzymatic degradation. (RA : control; +SBP : RA with 0.02 mM SBP; +ECA : RA with 0.02 mM ECA). Results represent mean of duplicate values, measured at 30 min or 60 min after GTN addition.**

GST substrate-inhibitors -- ECA and SBP. If GSTs are indeed involved in vascular GTN metabolism, these enzymes may also have a role in mediating nitrate-induced relaxation. In this study, using the rabbit aortic strip model, a well-established in vitro model for studying the pharmacologic actions of vasoactive compounds, the possible involvement of GST in the action of GTN was investigated by using two inhibitors (ECA and SBP) which have been shown to be able to reduce GTN metabolism in a homogenate system (Chapter 5).

At the time of the study, there was no report in the literature which could confirm the presence of GSTs in blood vessels of various animals, although it is observed that GSTs exist in almost every organ investigated. The presence of the three classes of GST isozymes -- alpha, mu, and pi, was examined by using antisera which were selective to the isozymes. Both anti-alpha and anti-pi sera were directed against rabbit GST isozymes. Unfortunately, an anti-mu serum for rabbit mu isozymes was not available, and the antiserum used for GST-mu detection was an anti-mu serum against rat isozymes. The immunoreactivity of this antiserum to the GST-mu isozymes in the rabbit aorta is not known. Therefore, the presence of mu-isozyme in rabbit aorta may only be inferred qualitatively. A faint band was observed for the mu-isozyme in the rabbit aorta 9000g and 105,000g fractions, suggesting the presence of GST-mu in those tissues. As for the alpha and pi classes of GST isozymes, results of the Western blots clearly demonstrate the presence of both isozymes. The more intense band of the anti-pi blot may suggest that the pi-isozyme is more predominant than GST-alpha. The results of this study were later confirmed by a report (Chern et al., 1990) showing that GST-pi was the predominant isozyme in rabbit aorta (>80%). GST-mu and GST-alpha made up the remaining GST activities in the tissue (approximately 10% and 3 %, respectively). In addition, glutathione-dependent enzymatic activities, including those of peroxidase, reductase, and transferase, have recently been reported in human blood vessels (Mezzetti et al., 1990), although the distribution of the three classes of isozymes was not determined in that study.

To examine the role of these GST isozymes in the mechanism of generation of the vasodilating effects by GTN, the effects of two GST inhibitors, ECA and SBP, on GTN-induced relaxation of rabbit aortic strip were investigated. The results indicated that ECA addition led to attenuation in GTN activity, suggesting that ECA inhibits the activation of GTN in blood vessels, although considerable variability in the extent of inhibition was observed. GTN-induced relaxation upon ECA treatment, as a percentage of the control value, ranged from 51.4% to 94.7%. However, this variability also makes it possible to examine the correlation between the reduction in GTN metabolism and its attenuated effect over a range of values. The strong correlations found suggest that ECA inhibits GTN metabolism, which leads to a reduction in the formation of GDNs and the pharmacologically active species, which in turn leads to attenuation in GTN effects. The significant change in the shape of the dose-response relationship of GTN following ECA addition also suggest an alteration in the kinetics of the formation of vasoactive intermediates from GTN in the presence of ECA.

The results here agree with previous *in vitro* experiments (Moffat et al., 1985; Rapaport and Murad, 1988) where ECA was shown to attenuate the effects of GTN and other vasodilators. However, it cannot be concluded that ECA inhibits GTN-induced relaxation solely via inhibition of GST activity towards GTN. In fact, if there were no reduction in GTN metabolism by ECA (i.e., GDN formation is 100% of control values), one would expect a 16.6% decrease in the capacity of the aortic strips to be relaxed by GTN (i.e. y-axis intercept for 100% on the x-axis is 83.4%). This suggests that ECA may also affect some other steps in the process of GTN activation. Rapaport and Murad (1988) have shown that 0.1 mM ECA did not affect either the cGMP level or guanylate cyclase activities in such tissues. However, ECA was shown to be able to inhibit sodium nitroprusside-induced relaxation of aortic strips. It was suggested that the inhibitor could affect certain unspecified sulfhydryl groups that are not contained within guanylate cyclase. It is possible that some of the effects of ECA may be related to this non-specific

phenomenon. However, most of the preparations (7 out of 9) demonstrated 20% or more reduction in GTN-induced relaxation, suggesting that this explanation cannot account for all the GTN activity that was lost upon ECA treatment. Moreover, the strong correlation between the decrease in GTN metabolism and its effect suggests that inhibition of GTN metabolism is an essential mechanism by which ECA inhibits the pharmacologic effects of GTN. It can also be argued that the glutathione pool in the aortic strip may be depleted. It has been shown that depleting glutathione in blood vessels by pretreatment with buthionine sulfoximine did not cause any change in the sensitivity of rabbit aortic strips towards GTN (Yeates et al., 1989), suggesting that glutathione is normally present at an excess concentration. In addition, if these inhibitors serve only as sulfhydryl depletors, one would expect GTN effects to be antagonized to a certain extent in the SBP-treated strips, unless the two inhibitors differ vastly in the rates of their metabolism. Rather, we believe that the lack of inhibition of GTN effects by SBP indicates that the attenuation in GTN effects for ECA-treated strips probably results from a specific inhibition of a certain enzymatic pathway, instead of nonspecific depletion of co-factors.

It should be noted that the results here contradict those of an earlier publication (Yeates et al., 1989), where it was reported that GTN-induced relaxation could be antagonized by SBP. It is not clear why different results were found in this work. However, there is a noticeable difference in the sensitivity of the aortic strips to GTN relaxation. In this study, more than 80% relaxation was observed at 5 min after GTN addition, whereas the earlier report showed only about 35% relaxation with the same GTN concentration, although the time of measurement was not specified. The difference in the dose-response relationship is apparently caused by the difference in the method of eliciting vasoconstriction before GTN addition. In the earlier study, KCl was used to elicit vasoconstriction, whereas in this study, phenylephrine was used instead. It could be possible that SBP was not significantly taken up from the buffer medium in this experiment, whereas in the earlier study, its uptake was enhanced by the KCl-depolarized

tissues. Moreover, the mechanism of KCl- and PE- induced contraction may be different so that a higher concentration of GTN may be needed to antagonize the contraction state of the tissues. In any case, it is not clear whether the resultant differences in the baseline GTN dose-response curves affect the results of the inhibitor studies. However, it can be reasoned that since the EC_{50} for GTN relaxation reported in this study is much lower, the tissues should be more sensitive to any inhibition by the inhibitors. In addition, GTN dose-response studies were also performed in the presence of SBP. There was no change in the EC_{50}, and only a slight change in the E_{max} -- approximately 10% . Moreover, the extent of SBP inhibition of GTN metabolism in the rabbit aorta homogenate was found to be much less than that for ECA. Therefore, we believe that the effect of SBP on GTN relaxation, if there is any, is much less pronounced than the effect of ECA observed here. This implies that the crucial GST isozyme involved in vascular GTN metabolism may possess a higher activity towards ECA than SBP.

One limitation of this study is the cross-reactivities of the inhibitors to individual isozymes of GSTs. For example, SBP was known to be a relatively specific substrate and inhibitor for class mu-isozymes; however, at higher micromolar concentrations, it has also been shown to inhibit the alpha-form (Mannervik et al., 1985). Similarly for ECA, which has been regarded as a GST-pi substrate, it was demonstrated recently that ECA is a better inhibitor to mu-isozymes (Ploemen et al., 1990; Hansson et al., 1991). Therefore, it is difficult to determine the identity of the GST isozyme which is involved in GTN metabolism. However, it can be concluded that the GST present in rabbit aorta exhibit a higher activity to ECA than SBP. Recently, our collaborators have found that in tissues made GTN tolerant both in vitro or in vivo, 1-chloro-2,4-dinitrobenzene (CDNB) activity, an indicator of total GST activity, was not different from that in nontolerant control tissues. However, the activities to trans-stilbene oxide (TSO) and GTN were significantly reduced to the same extent -- 30% for tolerant tissues prepared in vitro and 50% in tolerant tissues prepared in vivo (Chern et al., 1991). Since TSO is a GST-mu specific substrate, it can be

implied that the mu-isozyme may be responsible for the action of nitrates. Tsuchida et al. (1990) recently reported the isolation and purification of GST isozymes that exhibit GTN metabolic activities. Some of these isozymes was found to exhibit immunoreactivity to anti-mu serum. However, the GTN-metabolizing activity of this purified isozyme can also be inhibited by SBP. It is possible that although GST isozymes may belong to the same class, the activities to individual substrates may be different. It is also possible that species-differences in GST-substrate reactivities can exist. For example, in bovine coronary artery homogenates (Chapter 5), GTN metabolism was inhibited to similar extents by ECA and SBP, whereas in rabbit aorta homogenates, the effect of SBP on GTN metabolism was minimal, and was substantially less than that of ECA. Nevertheless, the role of GSTs in tolerance development of organic nitrates warrants further investigation of the identity of the GST isozymes which are responsible for GTN metabolism and effect.

6. Summary

In summary, the alpha, mu and pi classes of GST isozymes were found in rabbit aorta. Addition of ECA inhibits the generation of effects to GTN, and the extents of the decrease in effects were well-correlated to the extent of its inhibition of GTN metabolism. In contrast, SBP does not seem to affect GTN metabolism and pharmacologic effects in rabbit aorta. It can be concluded that the crucial enzyme, which is important for nitrate metabolism as well as the scheme of action of these agents, possesses enzymatic activities towards ECA. The results from this study, coupled to recent reports on the role of GSTs in vascular GTN metabolism, suggest that the mu class of GST may be the crucial enzyme involved.

CHAPTER SEVEN

In Vitro Studies on the Pharmacological Effects of GTN and Its Denitrated Metabolites

1. Introduction

Substantial evidence exists to suggest that GTN does not exert its pharmacological action by the drug alone, but rather, in combination with contributions from its dinitrate (GDNs) and mononitrate (GMNs) metabolites. According to the scheme of action of organic nitrates, these agents are denitrated to release an inorganic nitrite ion, which is then further processed to generate vasoactive intermediates (Ignarro et al., 1981). It is conceivable that the metabolites of GTN, since nitrate moieties remain intact in their structures, can be metabolized by the same enzyme as GTN, so as to exert the same vasorelaxing effects. In fact, in studies where human or animals were dosed orally with GTN, GTN blood concentrations are usually very low or undetectable, due to the extensive first-pass metabolism carried out mainly by the liver (Noonan and Benet, 1986; Nakashima et al., 1990). However, oral GTN dosing has proven to yield effective antianginal therapy (Krantz and Leake, 1975; Winsor and Berger, 1975; Heinzow and Zeigler, 1981). Moreover, following acute dosing of GTN, as with sublingual GTN administration, the pharmacological effects are usually more sustained than the rapid fall-off of the concentration profile of GTN (Armstrong et al., 1979; Noonan and Benet, 1987). Since the metabolites are eliminated at much slower rates than GTN, it is rational to speculate that these metabolites may in fact contribute to the longer-term effects of GTN dosing.

Tolerance is a common clinical problem with the dosing of organic nitrates (Flaherty, 1989; Fung et al., 1989; Katz, 1990). One theory suggests that nitrate tolerance is a result of an attenuation of GTN biotransformation in tolerant tissues. It has been

suggested that in GTN-tolerant tissues, the C-1 denitration of GTN was decreased more extensively than that of C-2 denitration (Fung and Poliszczuk, 1986). The authors proposed that the C-1 denitration pathway may in fact represent the "pharmacologically effective" pathway for the production of vasoactive intermediates. Therefore, the attenuation of GTN metabolism via this pathway may also be responsible as the biochemical cause of nitrate tolerance. Since GMNs can only be denitrated at one position, it would be interesting to examine if there are any significant differences in the dose-response relationship to these agents in GTN-tolerant tissues. The cross-tolerance properties of GDNs, which are potential therapeutic agents, with GTN will also be examined.

2. Objectives

The objectives of the studies described in this chapter are as follows:

a. to compare the concentration-response relationships of rabbit aortic strips to GTN with its denitrated metabolites -- 1,2-GDN, 1,3-GDN, 1-GMN, and 2-GMN;
b. in rabbit aortic strips, to examine the cross-tolerance properties of GTN and its denitrated metabolites.

3. Materials and Methods

3.1 Chemicals

GTN was purchased as 10-ml vials of Tridil® from DuPont Pharmaceuticals (Wilmington, DE). Authentic samples of 1,2-GDN, 1,3- GDN, 1-GMN, and 2-GMN (> 99% purity) standards were graciously supplied by Marion Laboratories (Kansas City, MO). *l*-Phenylephrine hydrochloride (PE), as well as the ingredients of the Krebs-

Henseleit buffer (NaCl, KCl, KH_2PO_4, $MgSO_4$, $NaHCO_3$, $CaCl_2$, and glucose), were obtained from Sigma (St. Louis, MO).

3.2. Preparation of the aortic strips

Aortic strips were obtained and prepared from New Zealand White rabbits as described in Section 3.2.2, Chapter 6. Upon stabilization of the strips, 100 μM PE was added to contract the strips maximally. The strips were then washed four times, 3 min apart, with the buffer. This was then followed by four longer washes for 15 min, and the strips were then allowed to stabilize. 100 μM PE was added for a second time, and the maximal tension achieved by each strip was recorded. The strips were then washed as described following the first PE addition. Upon stabilization, the strips were treated with consecutive additions of 0.1 μM PE, until the tension reached 60%-80% of the maximal contraction previously recorded (in most cases, 0.3-0.5 μM PE was necessary). GTN and its GDN and GMN metabolites, were then added from the lowest to the highest concentrations. The relaxation in the tension of the strips following the addition of each concentration of organic nitrate was recorded. A concentration-response curve was then constructed, using the cumulative percentage of relaxation from PE-induced contraction of these strips as the response measurement. A sigmoidal E_{max} model was used to fit the dose-response relationship, using the software The fitted parameters, E_{max}, EC_{50}, and the Hill coefficient (γ), were then compared among the various organic nitrates.

In the cross-tolerance studies, the strips were induced to GTN tolerance in vitro as described previously by other investigators (Rösen et al., 1987; Marks et al., 1989b; Slack et al., 1989; Kowaluk and Fung, 1990). Briefly, the strips were incubated with 0.22 mM GTN for an hour, after the second PE (100 μM) addition. These strips were then washed with the buffer for at least 30 min (8-10 washes), before the consecutive additions of PE (0.1 μM). No observable alteration in PE-induced contraction was observed with the tolerant strips. When the contraction of each strip reached 60%-80% of the maximal

contraction, the organic nitrates were added from the lowest to the highest concentrations, and the concentration-response relationships for these GTN-tolerant strips to the various metabolites of GTN were obtained as discussed above.

4. Results

4.1 Comparative potencies of GTN and its denitrated metabolites

The dose-response relationship of GTN and its dinitrate and mononitrate metabolites in rabbit aortic strips are depicted in Fig, VII-1. GTN displayed the highest potency of all nitrates. The two GDNs exhibited almost equivalent dose-response relationships. The mononitrates, however, differed significantly in their pharmacodynamic properties. 1-GMN was observed to be more potent than its structural isomer, 2-GMN. The dose-response curves of individual strips were fitted to the Hill equation, and the fitted parameters -- E_{max}, EC_{50}, and the Hill Coefficient (γ), are listed in Table VII-1. There were significant differences in the potencies of the various glyceryl nitrates, as reflected by their corresponding EC_{50}'s. The ratios of the EC_{50}'s of the various nitrates were 1:44:40:7400:59000 (GTN:1,2-GDN:1,3-GDN:1-GMN:2-GMN). The fitted E_{max}'s and γs values were not statistically different among the different agents.

4.2. Cross-tolerance of GTN and its denitrated metabolites

Figure VII-2 depicts the change in the dose-response relationships of rabbit aortic strips to GTN following induction of tolerance in vitro, i.e. following one hour incubation with GTN at a high concentration. There was an apparent 2 to 3 orders of magnitude right-shift of the dose-response curves. A markedly increased variability in the response to GTN was noted in the tolerant tissues. Using this method of inducing GTN tolerance to rabbit aortic strips, the changes in response to GDNs and GMNs in GTN-tolerant strips were investigated as depicted in Figs. VII-3 & 4, respectively. Due to the lack of higher

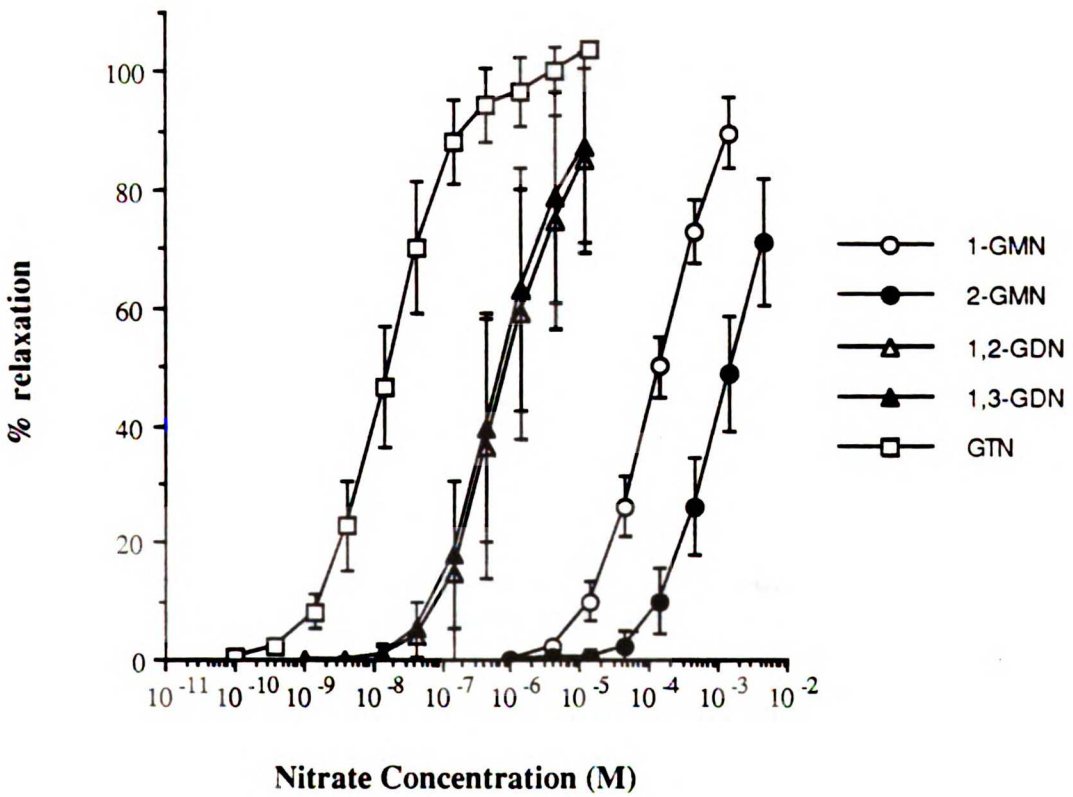


Fig. VII-1. Concentration-response relationships, as measured by the percentage of relaxation of PE-induced contraction for GTN and its dinitrate and mononitrate metabolites (mean±SD, n=6, except for GTN (n=5))

	E_{max} (%relaxation)	EC_{50} (μM)	γ
GTN	101±3	0.0194±0.0083	0.925±0.085
1,2-GDN	88.4±14	0.857±0.410	1.08±0.09
1,3-GDN	90.7±15	0.772±0.420	1.05±0.06
1-GMN	99.4±7	144±34	0.937±0.046
2-GMN	88.5±14	1150±320	1.03±0.14

Table VII-1. Parameters obtained from the fitting of concentration response curves in Fig. VII-1 (mean±SD, n=6, except for GTN (n=5)).

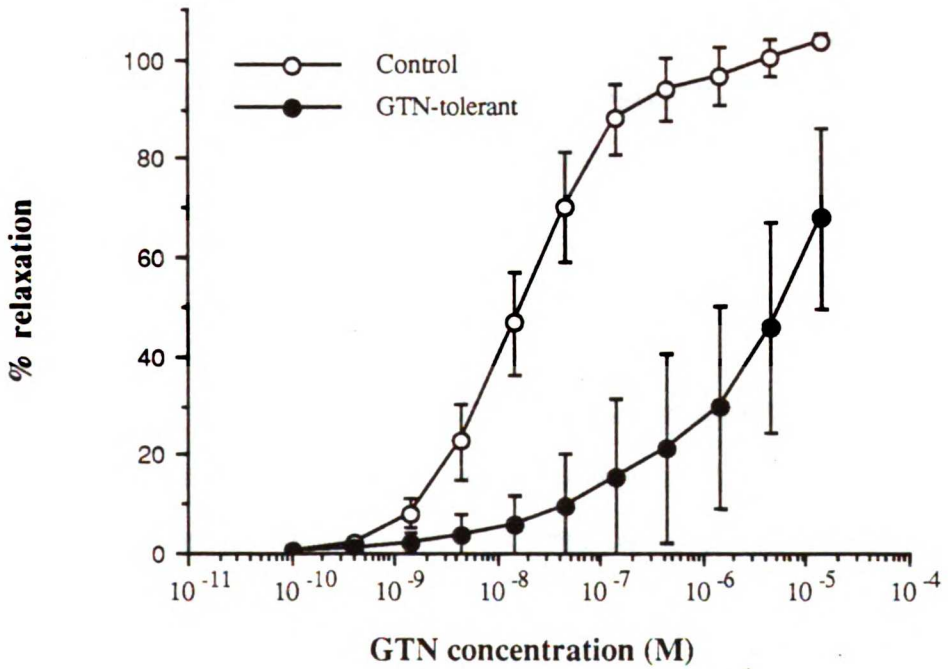


Fig. VII-2. Concentration-response relationships of rabbit aortic strips to GTN in tissues which have been made tolerant in vitro with GTN (n=6) versus the control (n=5).

concentrations of these metabolites, complete dose-response relationships for all the metabolites could not be obtained. However, it is apparent that for the range of concentrations tested, the two GDNs exhibited similar changes in dose-response relationships in tissues which had acquired GTN tolerance. The magnitude of change in the dose-response curves was approximately 100-fold for both GDNs. For 1-GMN, there was an apparent 10-fold right shift in the dose-response curves in GTN-tolerant strips. The change in the curves for 2-GMN seemed to be less. Interestingly, the GTN-tolerant strips displayed almost equivalent responses to 1-GMN when compared to the response of the control strips to 2-GMN (Fig. VII-4).

5. Discussion

Although it is generally agreed that the metabolites of GTN possess pharmacological activities, most of the reports in the literature on the comparative potencies of these metabolites are based on single time-point measurements. Needleman et al. (1969) observed that 1,2- and 1,3- GDNs were 10 and 14 times less potent than GTN, respectively. The GMNs did not yield any measurable pharmacological effects (blood pressure depression) in that study. In another study, Bogaert et al.(1968) concluded that the GDNs were 40-50 times less potent than GTN. Recently, Leitold et al. (1986b), using T-wave suppression of EKG in rats as the measurement of pharmacological activity, compared the potencies of GTN and its dinitrate and mononitrate metabolites over a four- to five- fold change in dose for each agent. On a molar basis, the ratios of the ED_{50}'s for GTN, versus that of 1,2-GDN, 1,3-GDN, 1-GMN, and 2-GMN were 1:1250:770:2000:9800.

In this study, using the relaxation of PE-precontracted rabbit aortic strips as the measurement of pharmacologic activity, the concentration-response relationships for GTN and its metabolites were compared utilizing concentrations over several orders of

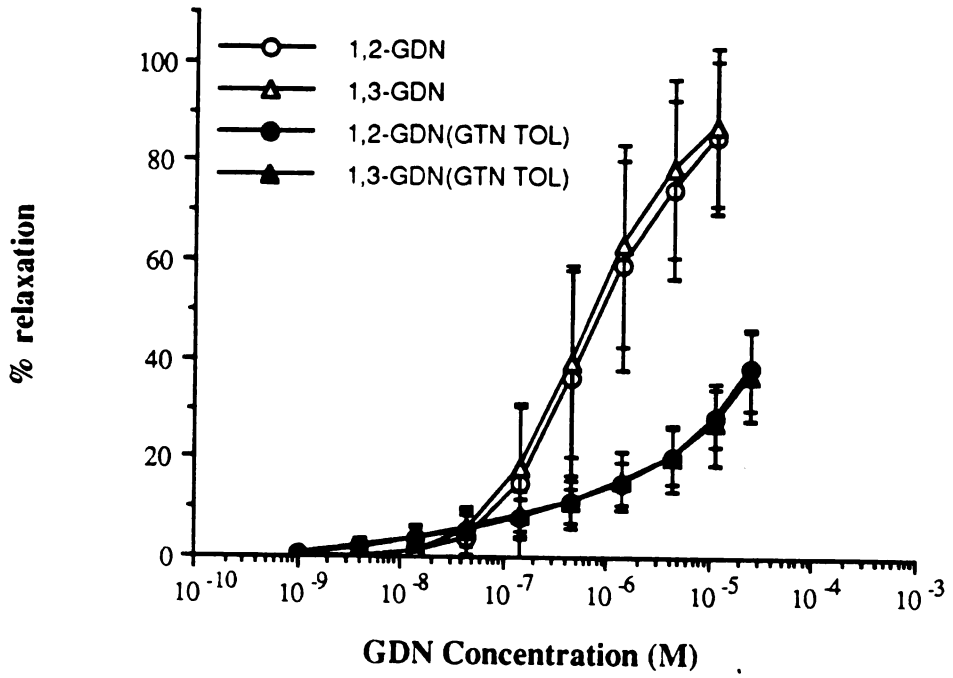


Fig. VII-3. Concentration-response relationships of rabbit aortic strips to 1,2- and 1,3-GDNs in tissues have been made tolerant in vitro with GTN, versus the control (mean±SD, n=6).

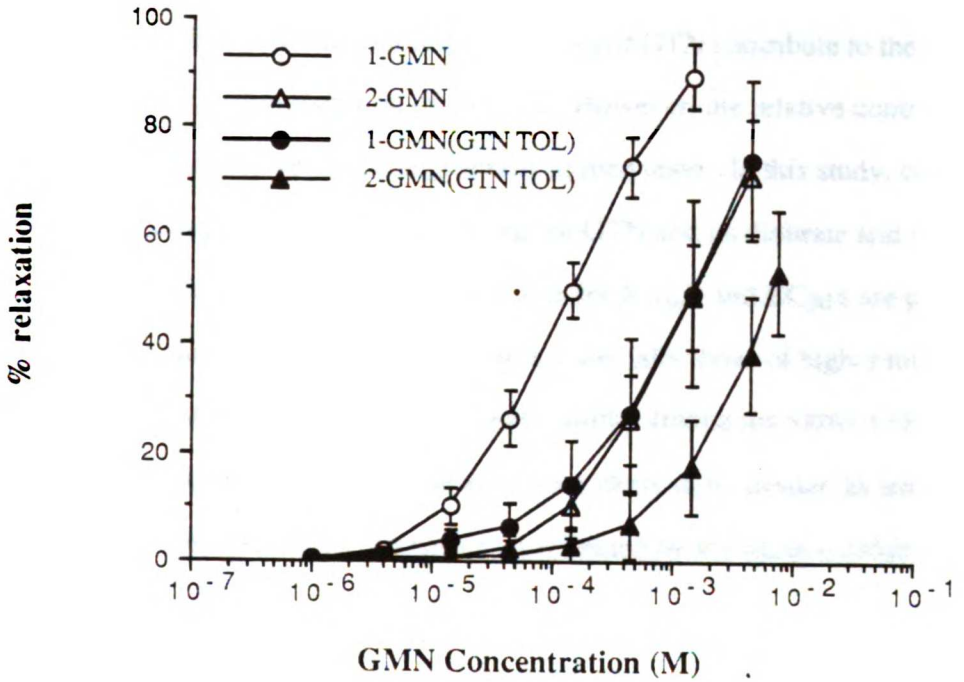


Fig. VII-4. Concentration-response relationships of rabbit aortic strips to 1- and 2-GMNs in tissues which have been made tolerant in vitro with GTN, versus the control (mean±SD, n=6).

magnitude. The results in this study reveal that the GDNs are approximately 40 times less potent than GTN, and that there is no observable difference in the concentration-response relationships between the two GDNs. However, there were obvious differences among the two GMNs, with 1-GMN exhibiting approximately an order of magnitude higher potency than 2-GMN. Leitold et al. (1986a, 1986b) has previously reported that 1-GMN possesses higher activities than 2-GMN.

It is believed that the metabolites of GTN contribute to the effects observed following administration of the drug. However, the relative contributions of the various metabolites have not been generally agreed upon. In this study, concentration-response relationships have been established for GTN and its dinitrate and mononitrate metabolites, although for the GMNs, the estimates for E_{max}'s and EC_{50}'s are probably subject to larger errors due to insufficient data points, especially those of higher nitrate concentrations. The E_{max}'s and Hill coefficients (γ) were similar among the various nitrates, indicating that the mechanism of action of these agents are likely to be similar, as least qualitatively. However, the relative potencies, as reflected by the EC_{50}'s, exhibit differences of several magnitudes. Laufen and Leitold (1988) examined the pharmacokinetics of glyceryl nitrates following oral GTN administration in man. No unchanged GTN was found in the plasma. All GDNs and GMNs were found, however, with the areas under the curve ratios of 1,3-GDN:1,2-GDN:1-GMN:2-GMN being 1:4:19:64. Accounting for the differences in molecular weights, the ratios become 1:4:25:85. Assuming the relative potencies of GTN and its metabolites are the same in rabbit and human tissues, and incorporating the ratios of the EC_{50}'s found in this study (44:40:7400:59000), the relative contributions to the pharmacological effects of these metabolites can be estimated to be 17:63:2.3:1, i.e. the GDNs contributed over 96% of the total effects accounted for by the metabolites. In the same study (Laufen and Leitold, 1988), the peak molar plasma concentration ratios were 1:3.4:10.7:28.4. Using these ratios of the C_{max}'s, the relative contributions of the metabolites to the pharmacologic effects are 52:161:30:1, again suggesting a more

significant contribution from the dinitrate metabolites. According to the results from this concentration-response study, for GMNs to contribute significantly to the observed effects of GTN, the concentration of GMNs has to be at least in the 10^{-5} M range (approximately 1.37 μg/ml), which is much higher than that obtained from in vivo GTN studies reported in animals (Carlin et al., 1989), as well as in clinical situations, where GTN and its metabolites were found to be in the nanogram per milliliter range (unpublished observations). Therefore, it can be concluded that the contribution of GMNs to the observed GTN effects is minimal. GDNs, however, may play a more significant role, especially in instances of oral or sublingual GTN administration, where GTN cannot be found in the circulation. In addition, previous results from our laboratory suggest that following intravenous GTN administration to dogs, when the AUC's of the effect (measured as systolic blood pressure decrease) is normalized to the dose of nitrates, 1,2-GDN was found to be twice as potent as GTN, whereas 1,3-GDN was at least equipotent with GTN (Lee et al., 1990). Therefore, the advantages related to the pharmacokinetic properties of GDNs may compensate for their lower potencies when compared to GTN. The in vivo pharmacodynamics of GDNs and GMNs have been examined in the past few years. Recently in our laboratory, GDNs were administered to human healthy volunteers at doses resulting in concentrations similar to that following GTN dosing, and significant blood pressure attenuation was observed (Gumbleton and Benet, 1991), suggesting the potential use of GDNs as therapeutic agents in their own right. Efficacy trials for GMNs, administered at doses at least 1000-fold higher than normal GTN doses, have been conducted as well, and qualitatively similar pharmacologic effects were also observed. GDNs and GMNs may prove to be advantageous in therapy because of their longer duration (residence time) in the body, where compared to GTN. However, the potential for tolerance development of these agents have not been examined thoroughly.

In this study, rabbit aortic strips were induced to GTN tolerance in vitro by incubating the tissues with high concentrations of GTN. It is apparent that all the

metabolites exhibited some cross-tolerance with GTN, although the magnitude of the shift in EC_{50}'s were different, i.e. 500-fold for GTN, 100-fold for GDNs, 10-fold for 1-GMN, and approximately 5-fold for 2-GMN. The extents for the shift in the concentration-response curves seemed to correlate with the relative potencies of the nitrates. The comparison of the concentration-response relationships for 1- and 2- GMNs are especially interesting. The rationale behind the higher potency of 1-GMN over 2-GMN is not known. One may suggest that 2-GMN is not taken up by blood vessels as readily as 1-GMN. It is believed that organic nitrates are taken up by the vasculature by simple diffusion as a result of their low molecular weight and high lipophilicity. According to published results where relative lipophilicities were compared, 2-GMN is approximately 20% more lipophilic than 1-GMN -- 0.32 vs 0.26 for partitioning between n-octanol and an aqueous buffer (Laufen et al., 1986). Using methyl-t-butyl ether as the organic solvent to extract various organic nitrates, 2-GMN was also more readily extracted into the organic layer (Carlin et al., 1988), suggesting again that the lipophilicity of 2-GMN is probably higher than that of 1-GMN. Therefore, the differences in the observed EC_{50}'s should not be due to any differences in lipophilicity; but rather, a result of the different rates of denitration of these mononitrates.

Although the identity of the denitrating enzyme for organic nitrates may not be clear, its affinity for C-1 denitration is apparently higher than that for C-2 denitration. Fung and Poliszczuk (1986) observed that in tolerant rat aorta homogenates, formation of 1,2-GDN from GTN was specifically decreased. Other studies have also indicated that C-1 denitration is attenuated to a greater extent in tolerant tissues than C-2 denitration (Bennett et al., 1989; Slack et al., 1989). Two hypotheses are available to explain these results. Firstly, one may suggest that only C-1 denitration leads to the formation of pharmacologically active species. This is probably not the case, since 2-GMN, which possesses only a C-2 nitrate moiety, has also been shown to be active both in vitro, as shown in this study, and in other studies. The second hypothesis postulates that the crucial

enzyme which leads to the denitration of glyceryl nitrates and subsequent generation of effects possesses a higher affinity for the C-1 nitrate than the C-2 nitrate. The results with GMNs from this study are consistent with this hypothesis. According to this hypothesis, this enzymatic pathway of GTN degradation may be more extensively inhibited in tolerance. Moreover, in tolerance, the regioselectivity for denitration is also lost; therefore, the inhibition on C-1 denitration may be more extensive than that on C-2 denitration. Since the mononitrates can only be denitrated at one position, the relative decrease in potencies (or increases in EC_{50}'s) for 1-GMN and 2-GMN should be different, if the loss of regioselectivity is related to the loss in effect. In this study, 2-GMN was shown to exhibit cross-tolerance to GTN; therefore, the "crucial enzyme" should be a C-1 selective, but not specific denitrating enzyme. Although responses to higher concentrations for GMNs are not available in the study to allow fits to obtain EC_{50}'s, especially in the tolerant tissues, it is apparent that the shifts in the concentration-response curve for 2-GMN is less than that for 1-GMN (Fig. VII-4); suggesting in agreement with other studies that the regioselectivity may have diminished in tolerant tissues. Coincidentally, the tolerant tissues yield the same response to 1-GMN as the control tissues to 2-GMN. The mechanistic relationship between the regioselectivity of denitration and the pharmacological effects of glyceryl nitrates is not clear at this point. Biochemical characterization of the enzymes involved in the biotransformation of GTN in blood vessels will be helpful for the understanding of this phenomenon.

6. Summary

In this study, the concentration-response relationships of GTN and its dinitrate and mononitrate metabolites were compared, and the ratios of the EC_{50}'s of these agents were found to be 1:40:44:7400:59000 (GTN:1,2-GDN:1,3-GDN:1-GMN:2-GMN). According to the results in this study, it can be rationalized that the GDNs can account for a significant

portion of the activities observed following GTN administration, particularly via routes of administration which results in rapid disappearance of the parent drug. The GMNs, however, are less likely to contribute to the effects of GTN; although when given at significantly higher doses, it is still possible for these agents to act as potential therapeutic agents. All the metabolites of GTN exhibit cross-tolerance with the parent drug. The observation that 1-GMN possesses higher potencies and extents of cross-tolerance to GTN than 2-GMN may suggest that the enzymatic pathway crucial for denitrating GTN may possess a higher preference in C-1 over C-2 denitration.

CHAPTER EIGHT

Summary of Findings

1. Pharmacokinetics of GTN and its dinitrate metabolites

The pharmacokinetics of GTN and GDNs following intravascular GTN infusions via various vascular beds (left femoral vein (LFV), left jugular vein (LJV), left femoral artery (LFA), and hepatic portal vein (HPV)) were investigated in rats. Different steady-state concentrations for GTN were obtained from these various infusions. The arterial clearance for GTN was calculated using the resulting steady-state concentrations of GTN. LFV and LJV infusions yielded clearances values which approximate the cardiac output of the rat. However, LFA and HPV infusions resulted in GTN clearances much higher than the cardiac output, suggesting that significant GTN extraction had occurred in the leg(hind-limb) and the liver, respectively. The availability of GTN through the liver, leg, and veins were estimated to be 18.3%, 37.5% and 79.1%. In addition, the concentrations of GDNs, and the ratios between the two GDNs (1,2-GDN/1,3-GDN) varied among the different infusions. The different GDN ratios indicate that various tissue beds may exhibit differences in their preferential formation of GDNs, in addition to their different capabilities in extracting the parent drug. In particular, HPV infusions yielded higher 1,3-GDN steady-state concentrations relative to 1,2-GDN, which was different from the GDN ratios resulting from other routes of infusion, confirming results from previous in vitro studies which suggested that rat liver predominantly denitrates GTN at the C-2 position. LFA infusions yielded the lowest total nitrate concentrations and recovery of dose (as calculated by the percentage of GTN clearance which can be accounted for by the sum of the formation clearances of the GDN metabolites), suggesting that the leg may be an important

organ for sequential metabolism of GTN. The extraction by the venous compartment may be important for the mechanism of action of organic nitrates, since it is believed that biotransformation of these drugs follow vascular uptake, and leading subsequently to the formation of vasoactive intermediates.

The pharmacokinetics of 1,2- and 1,3-GDNs in rats were also examined in rats. The GDNs were administered intravenously via the right femoral vein, and blood samples were obtained from the left femoral artery. The GDNs exhibited similar pharmacokinetic properties, although 1,2-GDN was found to possess slightly higher, though not statistically significant, clearance and volume of distribution. The half-lives and the mean residence times of the two GDNs did not differ, and were much prolonged over the reported values for GTN. No dose-dependent change in the pharmacokinetics of the GDNs was observed over a 8-fold change in infusion rate. In addition, the possible interaction in pharmacokinetics between the GDNs was examined, and no detectable change in the pharmacokinetics of either GDN was observed upon co-administration of an excess of the other isomer.

2. Metabolism of GTN in subcellular fractions of the liver

Cytosolic and microsomal fractions were prepared from rabbit livers, and the metabolism of GTN in these subcellular fractions was examined. Both the cytosolic and the microsomal fractions were found to be able to metabolize GTN, with the cytosol exhibiting a more rapid rate of GTN degradation. Interestingly, the preferential formation of GDN metabolites from GTN varied between the two fractions. The cytosol preferred 1,2-GDN formation, whereas in the microsomes, 1,3-GDN was the predominant metabolite from GTN degradation (the resultant GDN ratios were approximately 2.5 in the cytosol and 0.25 in the microsomes). Both of these degradation pathways for GTN were glutathione-dependent and enzymatic in nature. Upon a 12.5 fold increase in GTN

concentration, the cytosol exhibited a dose-dependent decrease in the rate of GTN degradation. The rate of GTN degradation in the microsomes, however, was relatively unaffected by increases in GTN concentration. Therefore, the contribution of the microsomal pathway to total hepatic metabolism is important, especially at higher GTN doses or concentrations. Moreover, the preferential 1,2-GDN formation in the cytosol was reduced at higher GTN concentrations, leading to a decrease in the resultant GDN ratio. Enzyme kinetic analyses revealed that in the cytosol, the affinity constant (K_m) for 1,2-GDN formation was approximately three times lower than that for 1,3-GDN formation, and hence, 1,2-GDN was more prone to concentration-dependent changes in the rate of its formation. The microsomes demonstrated a much higher capacity for 1,3-GDN than 1,2-GDN formation; however, the K_m's for both processes were much higher than the GTN concentrations tested. As a result, no apparent nonlinearities were observed in the microsomal fractions.

Various substrates for glutathione S-transferases (GSTs) were found to be able to decrease the rate of GTN degradation in the cytosolic fraction. However, some inhibitors, besides causing the attenuation in the rate of GTN degradation, also altered the ratios of GDN formation. For instance, sulfobromophthalein (SBP) and p-nitrobenzyl chloride reduced the formation of 1,2-GDN more extensively than that of 1,3-GDN. Co-incubation of ethacrynic acid (ECA) decreased the GDN ratio so drastically that 1,3-GDN became the preferential product. 1-chloro-2,4-dinitrobenzene and iodomethane did not alter the GDN ratios, although significant reductions in the rate of GTN degradation were observed. These results suggest that multiple pathways for GTN metabolism exist in the liver, contrary to the previous belief that a drug class-specific enzyme -- organic nitrate ester reductase, is the sole enzyme responsible for GTN metabolism. These results also indicate that various isozymes of GSTs, which may exhibit different regioselectivities for denitration on the molecule, are responsible for the metabolism of GTN.

3. Metabolism of GTN in blood vessel homogenates and subcellular fractions

The metabolism of GTN and the subsequent formation of GDNs were investigated in bovine coronary artery homogenates (BCA). GTN was found to be metabolized by the 9000g supernatant of the BCA homogenates, with 1,3-GDN as the predominant metabolite. Unlike the results from the liver studies, the majority of GTN metabolism was shown to be carried out by the cytosolic fractions. The ratio of GDNs (1,2-GDN/1,3-GDN), generated from GTN degradation was approximately 0.7. Upon additions of GST inhibitors -- SBP and ECA, GTN degradation was significantly inhibited to a similar extent by both inhibitors as compared to controls. In addition, there was a more prominent inhibition on 1,3-GDN than 1,2-GDN formation. These results suggested that GSTs may also be involved in the vascular metabolism of GTN. Since GTN metabolism in vascular tissue may be tightly coupled to the mechanism of vasorelaxation, the role of GSTs in eliciting the pharmacological actions of organic nitrates needs to be elucidated.

When rabbit aorta 9000g supernatant was incubated with GTN, the predominant metabolite obtained was 1,2-GDN. Addition of ECA resulted in a more prominent decrease in the extent of GTN metabolism than SBP. Moreover, ECA also caused a change in the pattern of GDN formation -- the reduction in 1,2-GDN formation was more extensive than that of 1,3-GDN. These results suggest that although GSTs may be involved in the vascular metabolism of GTN in different animals, possible species-difference in GST-mediated GTN metabolism may exist, leading to differences observed in the pattern of GDN formation.

4. Role of GSTs in the mechanism of nitrate action

The three major classes of GST isozymes -- alpha, mu, and pi classes of GSTs were found in homogenates of rabbit aorta, using immunoblotting techniques. To

investigate the role of GSTs in the scheme of nitrate action, two classical GST substrate-inhibitors, which have been previously shown to inhibit GTN metabolism in blood vessel homogenates -- SBP and ECA, were used in the study. The effects of SBP and ECA on GTN metabolism and GTN-induced pharmacological effects were examined. ECA-treated strips were found to exhibit a decrease in the response to 0.5 μM GTN, whereas SBP did not seem to affect GTN-induced vasorelaxation of the strips. The amounts of GDNs recovered in the ECA-treated strips were also significantly less than the control and the SBP-treated groups. In addition, the reductions in the extent of GTN metabolism was well-correlated to the inhibitions of GTN effect in individual strips, indicating an important relationship between the metabolism of and the vasorelaxation induced by organic nitrates. When the full concentration-response relationships of rabbit aortic strips in the presence of SBP or ECA were examined, ECA caused a more prominent change in the concentration-response relationships by changing both the E_{max} and the slope factor. However, a similar 10% change in the E_{max} of SBP-treated strips was also observed. ECA was known to be a pi-class selective substrate, but has also been shown to best inhibit mu-class GST isozymes. These results suggest that the crucial enzyme which is important for nitrate metabolism and the action of these compounds possess activities towards ECA, and is likely to be an isozyme in the GST family.

5. In vitro pharmacodynamics of GTN and its metabolites

The concentration-response relationships for GTN and its metabolites in rabbit aortic strips were compared. The ratios of EC_{50}'s for GTN versus its 1,2-GDN, 1,3-GDN, 1-GMN, and 2-GMN metabolites were observed to be 1:44:40:7400:59000. Incorporating the results from this potency study to published in vivo pharmacokinetic data following GTN administration, it can be rationalized that the GDNs contribute significantly to the observed effects following GTN administration. The GMNs, however, would not

be expected to exert any significant effects, due to their relatively low potencies. After inducing GTN-tolerance to aortic strips in vitro, the responses to all the metabolites were found to be diminished. The observation that 1-GMN exerts a significantly higher potency than 2-GMN was interesting. Due to the fact that the two GMNs are similar in their lipophilicity, the differences in their concentration-response relationships may be due to the more rapid C-1 as compared to C-2 denitration, leading to a faster rate of generation of the vasoactive species. In GTN-tolerant strips, the response to 1-GMN was more affected than that of 2-GMN. These results suggest that there may be a mechanistic relationship between the regioselectivity of denitration and the pharmacological effects of glyceryl nitrates, and that the crucial enzymatic pathway for denitrating GTN may possess a higher preference for C-1 than C-2 denitration.

6. Future directions of the project

The identity of the crucial enzyme in vascular tissues which is responsible for the metabolism of GTN and the generation of its effects is still not clear at this point. From the results in this thesis, we believe that some GST isozyme(s) may be the enzyme(s) responsible for these processes. Recent literature reports also indicate that certain mu-class isozymes may be important for GTN metabolism in blood vessels. Therefore, isolation, purification, and characterization of these GST isozymes needs to be carried out. Arteries and veins may consist of different GST isozymes, and should be studied separately. The regioselectivity of GTN denitration in these purified enzymes should be examined, in order to explain in vitro studies which suggest that the crucial enzyme may be a primarily C-1 denitrating enzyme. Moreover, more specific GST inhibitors should be investigated with respect to their effects on GTN metabolism, which can then be correlated to the effects of these inhibitors on the generation of effects such as cGMP elevation. The consequence of tolerance development on the activity of the enzyme should be investigated as well, in light

of the tightly coupled relationship between tolerance development and attenuation in GTN metabolism.

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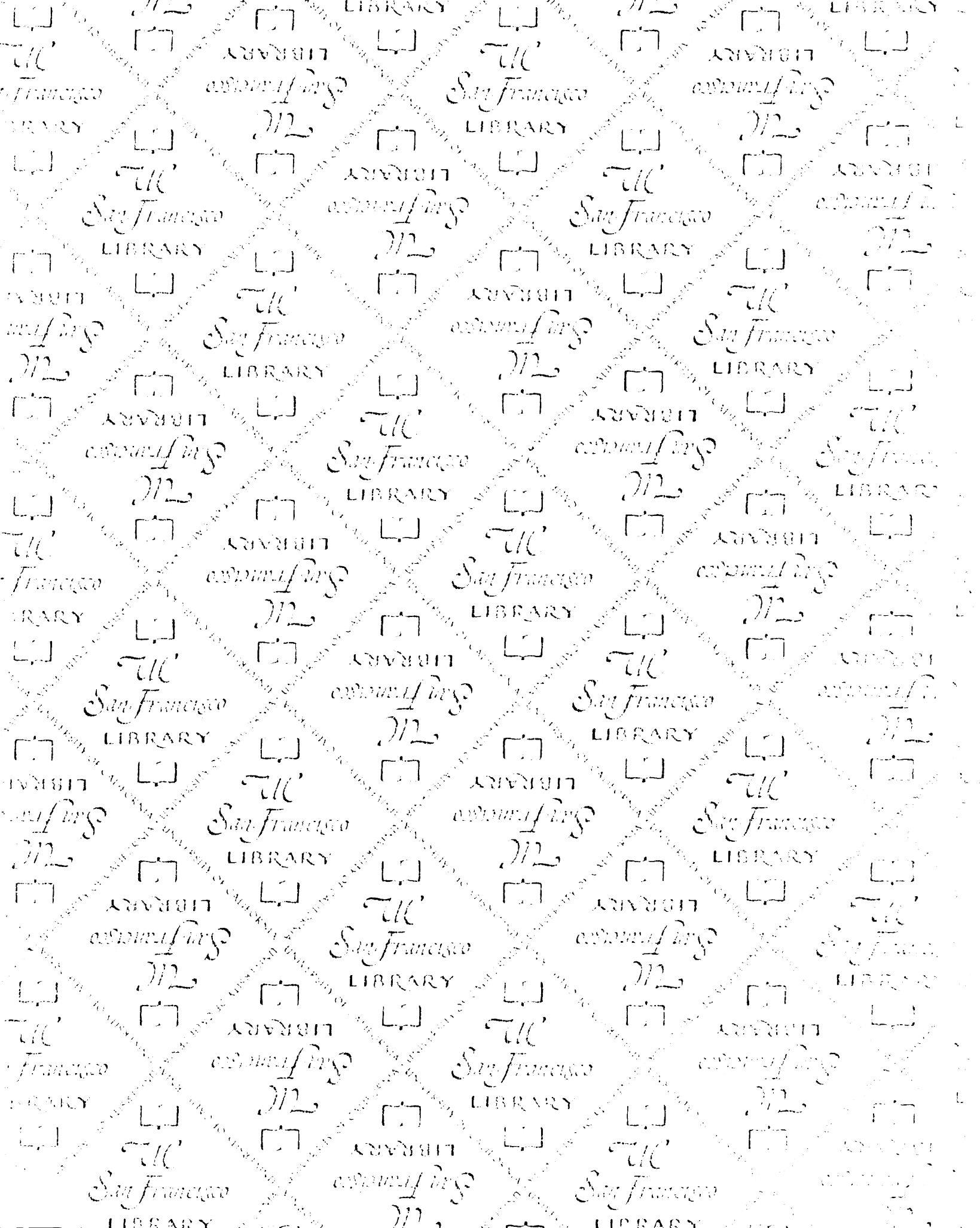
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