showing that the elimination half-life ($t_{1/2}$) of GTN is 4.2 ± 1.5 minutes. These studies also showed that GTN has a relatively large volume of distribution, $V_d$ of 3.1 ± 0.9 l/kg in the rat.

Before the role of chronic nitrate therapy can be adequately defined, the optimal mode of delivery, dose-response relationships, tolerance, dependence and subsequent withdrawal sequellae must be investigated. Our study describes a method for the analysis of both GTN and ISDN which should be helpful in answering some of these critical questions.

Acknowledgments

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References


Quantitative Determination of Trinitroglycerin in Human Plasma

JEANNE Y. WEI, M.D., PH.D. AND PHILIP R. REID, M.D.

SUMMARY We developed a simplified method for quantitative measurement of trinitroglycerin in human plasma using hexane extraction and analysis by gas-liquid chromatography with electron-capture detection. This assay was linear from 0.5–60 ng/ml. Sensitivity and reproducibility were ± 0.5 ng/ml.

We used this assay to evaluate the pharmacodynamics of trinitroglycerin in 14 patients. Maximum plasma levels were similar with trinitroglycerin given by constant intravenous infusion (1.6 ± 0.4 ng/ml (SEM)), transcutaneously (2.3 ± 0.6 ng/ml), or sublingually (1.6 ± 0.6 ng/ml). Despite similar levels and hemodynamic responses after intravenous trinitroglycerin, the dose range was wide (37.5–175 µg/min, $n=5$), emphasizing the need to individualize therapy. In normal volunteers on no other drugs, the plasma level time course followed changes in heart rate better than blood pressure changes. Use of the trinitroglycerin assay may enhance optimization of trinitroglycerin therapy when administered by different methods.

TRINITROGLYCERIN, OR NITROGLYCERIN (TNG), has been used to alleviate symptoms of angina pectoris for over 100 years.1 Recently, it has also become an important therapeutic agent for chronic congestive heart failure2 and for heart failure associated with acute myocardial infarction.3 In addition, it has been found to reduce acute myocardial ischemia,4 enhance electrical stability of ischemic myocardium,5–6, and improve localized ventricular asynergy.7 Despite its widespread use, little is known about the bioavailability of TNG administered by different methods. Due partly to the lack of convenient analytical methods, there has been little data to examine the relationship between plasma concentrations and hemodynamic or clinical effectiveness.8–11

This report describes a simplified method for quantitative measurement of TNG in human plasma using gas-liquid chromatography with electron-capture detection.

We have applied this technique to evaluate the plasma TNG levels and hemodynamic responses obtained in normal volunteers and patients when TNG was administered by sublingual, transcutaneous, and intravenous methods.

Methods

Blood samples were obtained in iced heparinized glass tubes. The plasma was immediately separated and frozen (–20°C) until assayed. Five milliliters of chromatography-grade hexane were added to 5 ml of plasma containing metadinitrobenzene (DNB) as the internal standard in a screw-top glass tube. The tube

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was mechanically shaken for 8 minutes and centrifuged at 2000 rpm for 8 minutes at 4°C. After the organic layer was transferred to a glass conical tube on ice, the above extraction was repeated twice and all three organic portions were combined. The combined fractions were air dried to near dryness; 2–5 µl of the remaining 20 µl of concentrated hexane solution was used for injection with a 10 µl Hamilton syringe after the conical tube was shaken using a centrifugal shaker (Vortex). A gas chromatograph (Hewlett-Packard Series 5700) with a tritium electron-capture detector (Analog Technology Corp) was used. The glass column, 1.83 m × 2 mm internal diameter, treated with Sylon-CT (Supelco, Inc.) was packed with 30% SE30 on Chromosorb WHP, 80/100 mesh (Supelco, Inc). The injection port, column, and detector temperatures were maintained at 150°, 130°, and 210°C, respectively. The argon/methane carrier-gas flow-rate was maintained at 90 ml/min. A heated oxygen and water filter trap was used to decrease contamination of the carrier gas.

Calibration and quantitation were accomplished using DNB as the internal standard. Known amounts of nitroglycerin (from a 9.1% ethanol solution) and DNB (40–60 ng) were added to blank plasma. The chromatographic peak areas were computed using the base-height method. The gas chromatographic peak of the trinitroglycerin was confirmed by mass spectrometry. Standard curves were obtained from the peak area ratios of TNG to DNB.

Using spiked plasma samples, the lower limits of sensitivity was found to be slightly less than 0.5 ng/ml. The TNG standard curves using 35 spiked plasma samples on different days were linear (r = 0.997 ± 0.003, sp) with an intercept on the x-axis (concentration) which was not significantly different from zero (intercept = -0.01 ± 0.15 ng/ml, SD, p > 0.3). Linearity was also observed when the TNG concentration was constant and the DNB concentration varied. Day-to-day changes in the slope of the regression line were observed (mean ± 3.76 ± 1.27, sp) and therefore a new standard curve was obtained with each set of patient samples to be analyzed. All patient samples were run in duplicate and the mean difference was found to be ± 0.35 ± 0.27 ng/ml (sp). For the same samples analyzed on different days, the mean difference was ± 0.34 ± 0.17 ng/ml (sd).

The TNG assay was used in a preliminary study to evaluate plasma levels in three groups of subjects. Six normal volunteers who had been receiving no medication (ages 25–32 years) received 0.6 mg TNG sublingually in the sitting position; heart rate and auscultatory cuff blood pressure were measured and venous blood collected at 0, 2, 5, 10, 20, and 30 minutes after TNG. Three patients (ages 59, 63, and 74 years) with unstable angina pectoris received 3 inches of 2% TNG ointment (for relief of chest pain) applied to the skin over approximately 40 cm² and had venous blood samples collected 1 hour after application. Five patients with acute myocardial infarction received a constant intravenous infusion of TNG. All patients were in Killip class I or II and were studied within 12 hours of pain onset. None had been receiving organic nitrates or propranolol. The TNG infusion rate was gradually increased to lower the mean arterial blood pressure by 10% as measured by the Doppler technique (Arteriosonde). One hour after the infusion rate and blood pressure were stabilized, blood samples were collected for measurement of plasma TNG.

The paired t test was used where appropriate with the significance level set at p < 0.05.

Results

Typical gas chromatograms are shown in figure 1. The TNG peak appeared approximately 12 minutes after injection, while the DNB peak appeared at 20 minutes, with little or no interference from endogenous compounds. The retention time of TNG relative to DNB was 0.58.

Figure 1. Gas chromatograms obtained from plasma samples extracted with hexane. Left) Extract of a blank plasma sample. Right) Extract of the same subject's plasma after sublingual administration of nitroglycerin (300 µg), to which dinitrobenzene (40 ng) was added. The retention time of nitroglycerin relative to dinitrobenzene is 0.58.
Figure 2. Standard calibration curves determined by the method outlined in the text for plasma concentrations ranging from 5-50 ng/ml (top) and from 1-16 ng/ml (bottom). Less dinitrobenzene was used for the standard curves in the bottom panel. The four curves were obtained on four different days.

Standard calibration curves obtained on different days are shown in figure 2. There is a linear relationship between the peak area ratios and the nitroglycerin levels over the entire TNG range examined. Since our initial studies indicated that therapeutic levels of TNG may be less than 20 ng/ml, standard curves were obtained using TNG standards in the lower range (fig. 2, bottom).

To evaluate the use of this assay in subjects receiving nitroglycerin, plasma concentrations were determined from forearm venous blood in 14 patients or normal volunteers who received TNG by various methods. Table 1 shows the plasma TNG levels and hemodynamic responses that were observed in six normal subjects over a 30-minute period after sublingual administration of one 0.6 mg TNG tablet. All subjects were maintained in the sitting position throughout the study and measurements were made from cuff blood pressure and heart rate. There was marked individual variability in the heart rate and blood pressure responses as indicated by the large SEM. When compared with control, the mean increase in heart rate was significant (p < 0.05) at 2, 3, and 10 minutes. Mean plasma TNG levels also demonstrated much individual variability but were maximal for the group at 2 or 5 minutes. However, the mean fall in systolic blood pressure was delayed in these patients and did not become significant until 10 and 20 minutes after sublingual TNG administration. No significant changes in the mean diastolic pressure were noted throughout the 30-minute observation period. Although the diastolic blood pressure showed an initial increase followed by a steady decrease, these changes were not statistically significant.

Less than 12 hours after an acute myocardial infarction, five supine patients in Killip class I or II received TNG as a constant intravenous infusion at a rate titrated to lower the mean arterial pressure by 10%. The plasma concentrations were measured after the blood pressure changes were stable. The TNG infusion rate ranged from 37.5 to 175 μg/min. The plasma TNG levels varied from ≤ 0.5-2.7 ng/ml, with an average of 1.6 ng/ml (table 2). There appeared to be no correlation between rate of TNG infusion and the plasma TNG concentration. The fall in systolic, diastolic, and mean arterial pressures were statistically significant, as would be expected since the end point was approximately a 10% drop in the mean blood pressure in each patient. However, no definite pattern was noted in the heart rate changes, and the mean heart rates were not significantly different.

TNG levels were also determined in three patients with unstable angina in the supine position who were receiving 3 inches of 2% TNG ointment every 4 hours with symptomatic pain relief. Samples for analysis were collected 1 hour after application of the TNG ointment. The TNG concentrations found were 1.2, 2.6, and 3.0 ng/ml (mean 2.3 ng/ml). No significant changes in blood pressure or heart rate were noted in this group. All patients had been on chronic nitrate therapy and all were receiving oral propranolol.

Discussion

The assay presented in this study may have more advantages than previously reported methods.9-11 The extraction procedure is simple, relatively rapid, and requires no elaborate solvent cleanup procedure. Furthermore, other reported methods which use isosorbide dinitrate as the internal standard could preclude TNG determination in any patient using isosorbide.9-11 Using meta-dinitrobenzene as the internal standard offers the potential for quantitative analysis even if a patient is receiving other organic nitrates because it has a different relative retention time than isosorbide dinitrate or erythritol tetranitrate.11,14 By using a high percentage of liquid phase in
packing the chromatographic column, possible active sites on the solid support which may interact irreversibly with nitroglycerin are reduced, and attainment of the desired sensitivity may thereby be facilitated.

Some investigators who used an ethyl acetate extraction have observed the metabolites of TNG. De-esterification of TNG results in a marked increase in water solubility: The least water-soluble metabolite (1,2-dinitroglycerin) is over 60 times more water soluble than its parent compound, and the mononitrates have even greater water solubility. The use of a non-polar solvent such as hexane in the present system probably permits preferential extraction of TNG over its di- or mononitrated metabolites.

In normal subjects who received sublingual TNG (Table 1), the significant rise in heart rate soon after administration of TNG when the plasma levels are maximal suggests that the heart rate changes correlate with TNG concentrations. Although the observed tachycardia is thought to reflect a baroreceptor reflex response to blood pressure change, the finding that the mean as well as individual increase in heart rate actually preceded the blood pressure fall raises the possibility that the tachycardia did not occur solely as a reflex effect secondary to a drop in blood pressure.

In patients who had recent acute myocardial infarctions and received TNG intravenously (Table 2), the fall in blood pressure was significant since that was the end point for titration of the infusion rate. However, in these patients, the heart rate changes were not significant. These results reflect steady-state TNG effects after slow intravenous introduction. It is possible that the change in heart rate reflects the abrupt changes in plasma levels observed in the volunteers who received sublingual TNG, while gradual variations in the plasma TNG level may permit countering reflex changes. For example, a decrease in sympathetic tone due to a progressive reduction of left ventricular dysfunction and congestive heart failure with TNG could reflexly attenuate any heart rate increase that might otherwise have been observed. In addition, the control heart rate was 99 beats/min in patients with myocardial infarction, compared with a control of 70 beats/min in the normal volunteers.

Although only a few patients received topical TNG, these preliminary results suggest that plasma TNG

### Table 1. Plasma Trinitroglycerin Levels After 600 µg Sublingual Administration in Normal Volunteers (n = 8)

<table>
<thead>
<tr>
<th>Time after administration (min)</th>
<th>Δ SBP (mm Hg)</th>
<th>Δ DBP (mm Hg)</th>
<th>Δ HR (beats/min)</th>
<th>Plasma TNG level (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-1.0 ± 1.5</td>
<td>+3.2 ± 3.0</td>
<td>+19.8 ± 10.6</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>-3.7 ± 2.1</td>
<td>-0.3 ± 1.7</td>
<td>+13.0 ± 2.8</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>-4.3 ± 1.2</td>
<td>-0.7 ± 2.0</td>
<td>+6.5 ± 1.7</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>20</td>
<td>-4.7 ± 1.5</td>
<td>-3.2 ± 2.9</td>
<td>+3.5 ± 3.3</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>-2.2 ± 2.1</td>
<td>-3.3 ± 2.4</td>
<td>-0.3 ± 1.7</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM.

Abbreviations: Δ SBP = change in systolic blood pressure from control (109.2 ± 4.7 mm Hg); Δ DBP = change in diastolic blood pressure from control (71 ± 3.8 mm Hg); Δ HR = change in heart rate from control (70.3 ± 3.8 beats/min); TNG = trinitroglycerin.

### Table 2. Plasma Trinitroglycerin Concentration During Intravenous Administration (n = 5)

<table>
<thead>
<tr>
<th>Pt</th>
<th>Intravenous infusion (µg/min)</th>
<th>Blood pressure (mm Hg) systolic/diastolic (mean)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control During TNG infusion</td>
<td>Control During TNG infusion</td>
<td>Plasma TNG level (ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>37.5</td>
<td>115/90 (98)</td>
<td>110/80 (88)</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>124/102 (110)</td>
<td>106/90 (99)</td>
</tr>
<tr>
<td>3</td>
<td>123</td>
<td>100/68 (79)</td>
<td>94/64 (74)</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>154/100 (118)</td>
<td>142/90 (106)</td>
</tr>
<tr>
<td>5</td>
<td>175</td>
<td>102/78 (86)</td>
<td>90/72 (78)</td>
</tr>
</tbody>
</table>

98 ± 24.6 SBP 119 ± 9.8 DBP 108 ± 9.2* MBP 99 ± 6.9

All values are expressed as the mean ± SEM.

*P ≤ 0.05.
†Not significant.

Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; MBP = mean blood pressure; TNG = trinitroglycerin.
levels can be achieved (2.3 ± 0.6 ng/ml) which are similar to those found intravenously (1.6 ± 0.4 ng/ml). While no significant changes in heart rate or blood pressure were noted after TNG application, the supine position, hemodynamic improvement produced by TNG, and the use of β-adrenergic blockade might explain these responses.

In all three groups of patients the blood pressure responses did not correlate with the TNG plasma concentration. However, the time course of heart rate change did parallel the plasma TNG changes in the normal volunteers who received sublingual TNG. Thus, intergroup hemodynamic responses may be quite variable. This variability is further emphasized by examination of the TNG infusion rates used in the group of patients with acute myocardial infarction. Similar hemodynamic responses and plasma levels were achieved by the intravenous TNG infusion, but the rates of infusion ranged from 37.5–175 µg/min.

In summary, a method using gas-liquid chromatography with electron-capture detection has been described for quantitative determination of TNG in human plasma. This method has been used successfully to determine blood levels after intravenous, transcutaneous, and sublingual administration of TNG. A major use of this assay may be to improve patient-to-patient TNG dosing, since this study suggests that marked individual differences exist for hemodynamic responses to a fixed dose. Future studies should examine the relationship of plasma TNG levels to other known pharmacologic effects such as changes in venous tone, left ventricular preload and coronary vasodilation.

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