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1,2- and 1,3-dinitrate metabolites of nitroglycerin: Spectroscopic characterization and initial administration to man

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Summary

The purification and spectral characterization (IR, MS, NMR) of the 1,2- and 1,3-glyceryl dinitrate metabolites (1,2- and 1,3-GDN) of the anti-anginal agent nitroglycerin (GTN) were undertaken, prior to preliminary phase I administration of these compounds to healthy volunteers. Purification was accomplished using a combination of gravitational column chromatography and high-performance liquid chromatography. Spectral characterization of the 1,2- and 1,3-GDN molecules is discussed in relation to the chemical structure of the glyceryl dinitrate isomers. Preliminary pharmacokinetic evaluation following the oral administration of a single dose of 1,2- and 1,3-GDN to healthy volunteers demonstrated that these structural isomers possess comparable pharmacokinetic characteristics; area under the venous plasma concentration time curve normalized to dose (AUC; ng ml^{-1} min/kg body weight per mg dose) was 5.41 ± 1.67 for 1,2-GDN, and 4.52 ± 1.76 for 1,3-GDN; oral clearances (CL/F; ml/min per kg body weight) were 35.5 ± 2.1 (1,2-GDN), and 42.8 ± 10.4 (1,3-GDN); apparent volumes of distribution (V_{area}/F; l/kg body weight) were 2.17 ± 0.39 (1,2-GDN), and 1.92 ± 0.20 (1,3-GDN); elimination half-life ($t_{1/2}$; min) was 40.4 ± 2.80 (1,2-GDN), and 33.6 ± 6.1 (1,3-GDN). The GDNs may significantly contribute to the therapeutic efficacy of GTN. The purification and spectroscopic procedures described here will allow the further manufacture and administration of 1,2- and 1,3-GDN to man, enabling examination of the relationship between the pharmacokinetics and pharmacodynamics of GTN and its dinitrate metabolites.

Introduction

The primary dinitrate metabolites of the anti-anginal agent nitroglycerin (GTN) i.e., 1,2-glyceryl dinitrate (1,2-GDN) and 1,3-glyceryl dinitrate (1,3-GDN), may possess pharmacological activity, and contribute to the therapeutic efficacy of the parent compound. In vitro and in vivo studies examining the comparative doses of GTN and GDNs required to achieve a single predetermined effect, e.g., a specified decrease in blood pressure or relaxation in aortic strips, (Bogaert et al., 1968; Needleman et al., 1969) suggest that the GDNs are approx. 10–40 times less 'potent' than GTN, leading to the conclusion that the GDNs make little contribution to GTN therapeutic efficacy.

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However, an assessment of the contribution that the GDNs may make to GTN therapeutic efficacy requires consideration of comparative GTN and GDN pharmacokinetics; GTN undergoes almost complete first pass metabolism following oral administration, with less than 1% of the dose systemically available, but with considerable systemic concentrations of 1,2-GDN and 1,3-GDN observed (Noonan and Benet, 1986; Nakashima et al., 1990). Similarly, following transdermal GTN administration, significant amounts of the less rapidly eliminated GDNs accumulate, resulting in combined 1,2- and 1,3-GDN steady-state plasma concentrations that can be 6–7 fold higher than the steady-state levels of GTN (Noonan and Benet, 1987). Thus, the 1,2- and 1,3-GDNs may significantly contribute to the therapeutic efficacy of GTN on the basis of pharmacokinetic considerations, even if the GDNs possess less pharmacological 'potency' compared to GTN. The administration of the 1,2- and 1,3-dinitrate metabolites of GTN to man is a necessary requirement in the examination of the relationship between the pharmacokinetics and pharmacodynamics of GTN and its GDN metabolites.

The 1,2- and 1,3-GDNs are not available as licensed pharmaceutical products. Therefore, the purity and chemical characterization of the GDNs must be established prior to administration to man. This communication reports the purification and spectroscopic characterization of the 1,2- and 1,3-GDNs prior to their phase I administration to healthy volunteers under IND 32,278. Literature reports referring to the spectroscopic characterization of the GDNs are extremely limited (Capellos et al., 1984), with technical details of the chemical characterization of the GDNs available only within the internal reports of the armament industry (Capellos et al., 1979). The purification and spectroscopic procedures detailed in this manuscript will allow the eventual administration of GTN and 1,2- and 1,3-GDN to a study group of stable angina subjects, enabling evaluation of the comparative pharmacodynamics of these compounds. Preliminary pharmacokinetic data is presented in this report, following the phase I oral administration of a single dose of 1,2- and 1,3-GDN to a small number of healthy volunteers.

Materials and Methods

Chemicals and solvents used in this study were of the highest purity available and were purchased, unless otherwise stated, from either Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A. or Fisher Scientific, Santa Clara, CA, U.S.A. All procedures were performed at room temperature.

A mixture of 'Dilute Nitrate Esters' (11.5%, GTN; 25.4%, 1,2-GDN; 63.1%, 1,3-GDN) (ICI Americas Inc., Specialty Chemicals, Wilmington, DE, U.S.A.) was manufactured in conformity with Good Manufacturing Practices (GMP) for Bulk Pharmaceutical Products. A 5.0 g aliquot of nitrate ester oil was loaded onto a gravitational column consisting of 20 g silica, (particle size 63–200 μm, 70–230 mesh; Universal Absorbants Inc., Atlanta GA, U.S.A.). Column chromatography was performed in a stepwise gradient manner using an eluent of ethyl acetate/hexane (1 : 99 to 40 : 60, v/v) (Fisher Scientific), with a column flow rate of $\approx$ 1.5 ml/min. The elution of the nitrate material from the gravitational column was semi-quantitatively monitored by thin-layer chromatography (TLC), using silica gel G TLC plates (250 μm thickness; Analtech, Newark DE, U.S.A.), and a TLC solvent of ethyl acetate/hexane (20 : 80, v/v). After separation by TLC the nitrates were visualized as previously described (Carlson and Thompson, 1986), with a chromogenic agent consisting of potassium permanganate. Employing this system retention values (R_f) for GTN (0.75), 1,3-GDN (0.52), and 1,2-GDN (0.32) were observed. The purified fractions, comprising 1,2-GDN and/or 1,3-GDN, arising from the column chromatography, were pooled and further purified by preparative HPLC.

Mono- and dual GDN component fractions that were essentially free of GTN, were loaded (approximately 140 mg of nitrate ester), onto a preparative silica HPLC column (Dynamax Macro, 10 μm particle size, 25 cm $\times$ 23 mm i.d.; Rainin Instrument Co. Inc, Emeryville CA, U.S.A.). Detection of the dinitrates was achieved with UV absorbance at 215 nm (Kratos Spectroflow 783 UV detector; Applied Biosystems, Ramsey NJ, U.S.A.); the mobile phase was composed of isopropanol/hexane (3 : 97, v/v) (Fisher Scientific),

pumped isocratically at 8.3 ml/min (1108 solvent delivery system; Beckman Instruments Inc., San Ramon, CA, U.S.A.). At retention volumes corresponding to elution of the organic nitrates, (GTN, 301 ml; 1,3-GDN, 350 ml; 1,2-GDN, 688 ml) the HPLC eluent was collected, the organic solvent removed under reduced pressure (approx. 10 mmHg at 35–40°C), and the resulting oils were stored at −20°C.

Assessment of purity and spectral characterization of the glyceryl dinitrates

HPLC. Aliquots (≈ 300 ng) of highly purified 1,2-GDN and 1,3-GDN, were reconstituted with isopropanol/hexane (3 : 97, v/v) (Fisher Scientific), and loaded onto an analytical silica gel HPLC column (5 μm particle size, Ultrasphere, 25 cm × 4.6 mm i.d.; Beckman Instruments Inc., Altex Division, San Ramon, CA, U.S.A.). The mobile phase was composed of isopropanol/hexane (3 : 97, v/v) (Fisher Scientific), pumped isocratically at 1.0 ml/min. The column eluent was monitored for 100 min post injection, with detection using UV absorbance at 215 nm (absorbance units full scale 0.005) (Kratos Spectroflow 783 UV detector; Applied Biosystems, NJ, U.S.A.). Standards of analytically pure dinitrates (Marion Laboratories Inc., Kansas City, MO, U.S.A.), that were reconstituted in the mobile phase and analyzed by HPLC resulted in retention volumes of 7.9 ml for GTN, 13.9 ml for 1,3-GDN and 22.0 ml for 1,2-GDN. The purified 1,2- and 1,3-GDNs co-eluted with their respective standards. Purity of the 1,2- and 1,3-GDNs was based upon the area under the respective peak relative to the total area recorded upon completion of the HPLC run.

Gas chromatography. Aliquots (≈ 20 pg) of the purified 1,2-GDN and 1,3-GDN samples were reconstituted with *n*-butyl acetate (Burdick and Jackson Inc., Muskegon MI, U.S.A.), and introduced into a gas chromatograph (Varian 6000; Varian Instruments, Sugarland TX, U.S.A.) employing a ^{63}Ni electron capture detector (attentuation 10, range 1), and a fused silica capillary column (HP-1, 25 m × 0.32 mm; Hewlett Packard, Avondale PA, U.S.A.). The temperature program consisted of an isocratic period from 0 to 11 min at 95°C, followed by a 9 min temperature increase to 120°C at 3°C/min. GC analytical standards (Radian Co., Austin TX, U.S.A.) reconstituted in *n*-butyl acetate and injected into the system resulted in retention times of 7.8 min for 1,3 -GDN, 8.4 min for 1,2-GDN, and 10.4 min for GTN. The purified 1,2- and 1,3-GDNs co-eluted with their respective standards. Purity of the 1,2- and 1,3-GDNs was based upon the area under the respective peak relative to the total area recorded upon completion of the GC run.

Infrared spectroscopy. Aliquots of the purified 1,2-GDN and 1,3-GDN samples were reconstituted with chloroform (Aldrich Chemical Co. Inc.) and scanned (800–4000 cm^{-1}) as liquid films between sodium chloride crystal windows (25 mm × 4 mm; Aldrich Chemical Co. Inc.) on a Nicolet FT-IR 5DX (Nicolet Analytical Instruments, Madison WI, U.S.A.).

High-resolution and GC-mass spectrometry. Samples of the purified 1,2- and 1,3-GDNs were analyzed with direct probe sample introduction by electron ionization mass spectrometry using an MS-25 mass spectrometer (Kratos Analytical, Manchester, U.K) with an accelerating voltage of 6 kV. High-resolution electron ionization mass spectrometry was performed using an MS-9 mass spectrometer (Kratos Analytical), with an accelerating voltage of 8 kV. Elemental composition of 1,2-GDN and 1,3-GDN was determined on the mass spectral peak of molecular ion + 1 ($M^+ + 1$). GC-mass spectrometry analysis of the samples was performed by electron ionization, and accomplished with a Kratos MS-25 mass spectrometer (Kratos Analytical), interfaced to a Varian 3700 gas chromatograph (Varian Instruments). The gas chromatograph used a fused silica capillary column (DB-1 column, 30 m × 0.32 mm; J&W Scientific, Folsom CA, U.S.A.), and a programmed temperature increase of 100°C to 270°C at 6°C/min.

^{1}H-NMR. The 1,2-GDN and 1,3 -GDN were prepared for NMR analysis by the addition of a small volume (2–3 ml) of carbon tetrachloride (Aldrich Chemical Co. Inc.), added to 2.0 mg quantities of purified 1,2- and 1,3-GDN, after which the organic solvent was removed by evaporation under reduced pressure. This procedure was repeated, but with the addition of deu-

terated chloroform (99.8 atom% D, Aldrich Chemical Co. Inc.) in place of carbon tetrachloride. Finally, the nitrate oils were reconstituted with 400 μl deuterated chloroform containing the internal standard tetramethylsilane (Aldrich Chemical Co. Inc.). The reconstituted solutions were filtered through a bed of cotton into high-resolution NMR tubes (Gold label, 5 mm; Aldrich Chemical Co. Inc.). ^{1}H-NMR spectra were recorded with a General Electric spectrometer (General Electric Co., Fremont CA, U.S.A.) which was operated at an electromagnetic radiation frequency of 500 MHz and a temperature of 18–21°C. ^{1}H chemical shifts were expressed in ppm downfield from tetramethylsilane.

Preliminary phase I administration of 1,2-GDN and 1,3-GDN to man

The 1,2- and 1,3-GDN manufacturing, formulation and clinical study procedures were performed under Investigational New Drug application 32,278. Clinical study procedures were performed with volunteer 'informed consent' and with the approval of the University of California, San Francisco Committee on Human Research. Six healthy male volunteers, between the ages 21 and 35 years, were recruited into the study. Following an overnight fast, three subjects each received a 2.1 mg oral dose of 1,2-GDN, while an additional three subjects each received a 1.2 mg oral dose of 1,3-GDN. The oral GDN doses were administered as solutions in 50 ml of purified water; each subject drank a further 50 ml of purified water immediately following GDN administration. The GDN dose selection was based upon GDN metabolite kinetics following oral administration of GTN, and were chosen to achieve approx. 33% of the systemic delivery of 1,2- and 1,3-GDN previously observed following administration of a 13 mg oral solution of GTN to healthy volunteers (Nakashima et al., 1990). Based upon pharmacokinetic studies in the dog (Lee et al., 1990), an oral bioavailability (F) in man of 0.7 was assumed for both 1,2- and 1,3-GDN.

During a 6 h period post-dose, serial blood samples were collected into chilled heparinized polyethylene syringes (Sarstedt, Hayward, CA, U.S.A.). Stability investigations prior to the pharmacokinetic study confirmed no GDN adsorption to the polyethylene syringes. Processing of the blood samples and the analysis of 1,2- and 1,3-GDN plasma concentrations were as previously described (Nakashima et al., 1990), using a sensitive and specific GC assay (Lee et al., 1988), with published modifications (Yu et al., 1988). The chemical interconversion of 1,2-GDN to 1,3-GDN in aqueous solutions of alkaline pH has been reported (Aburawi et al., 1984; Capellos et al., 1984). In this study the in vivo isomerization of 1,2- to 1,3-GDN was not observed.

Pharmacokinetic parameters for the 1,2- and 1,3-GDN were calculated using standard non-compartmental techniques (Nakashima and Benet, 1988), with area under the plasma concentration-time curve from zero to infinity (AUC) calculated using both the trapezoidal method for increasing concentrations, and the log trapezoidal method for decreasing concentrations; area under the corresponding first moment curve (AUMC) was calculated using the trapezoidal method. The maximum % extrapolated AUC and AUMC i.e., t_{last} to infinity, were 5.2 and 20.3%, respectively. Absorption and elimination rate constants were obtained by nonlinear least-squares regression analysis (PCNONLIN 2.0, Statistical Consultants Inc., Lexington, KY, U.S.A.) (weighting function – $1/y^2_{predicted}$), with plasma concentration vs time profiles described by first order absorption, and by either mono- or biexponential disposition.

Results and Discussion

Following gravitational silica gel column chromatography, approx. 97% of the applied 1,2-GDN, and approx. 70% of the applied 1,3-GDN existed as dual component mixtures with the respective dinitrate isomer. Removal of GTN by silica gel column chromatography improved the HPLC loading capacity and allowed 1,2- and 1,3-GDN purification without the need to utilize the technique of peak shaving with repeat injections. Approx. 140 mg of the dual component dinitrate mixtures could be loaded onto the preparative HPLC system at one time, without loss of baseline resolution between the 1,2- and 1,3-GDNs. Load-

ing approx. 70 mg of the original mixture of Dilute Nitrate Esters at one time, containing 11.5% GTN, resulted in the loss of baseline separation between GTN and 1,3-GDN.

FT-IR spectral analysis of purified 1,2-GDN and 1,3-GDN showed asymmetrical stretching vibrations of the organo nitro bond with a strong absorption at 1650 cm^{-1}. Symmetrical stretching vibrations of the organo nitro bonds, were observed at 1287 and 1269 cm^{-1} for 1,2-GDN, and at 1275 cm^{-1} for 1,3-GDN. Stretching of the single O-NO_2 bonds was observed by absorption at 843 cm^{-1} for 1,2-GDN, and at 850 cm^{-1} for 1,3-GDN. The spectra for the 1,2-GDN and 1,3-GDN demonstrate absorption characteristics consistant with organic nitrate compounds.

HPLC and GC chromatograms of the purified 1,2-GDN and 1,3-GDN samples were free of impurities such as GTN, and the glyceryl mononitrates, and were regarded as > 99% pure with respect to the described assays. Following GC-MS analysis, the chromatograms of 1,2- and 1,3-GDN were characterized by single broad peaks of ionization occurring at 80–110 scans and 90–130 scans, respectively. The single peaks represented greater than 99% of the total ionization intensity of the respective chromatograms, and were broad presumably due to overloading of the GC column, which was required if minor impurities were to be detected. From the GC-MS chromatogram the GDN material was regarded to be > 99% pure. Examination of the mass spectra under these single peaks of ionization intensity revealed spectra that were consistent with the proposed structures, although in no case could a molecular ion be obtained by GC-MS. The spectra obtained with GC-MS differed from those obtained following direct probe sample introduction (see below), and this undoubtedly reflects the sample quantities analyzed by these methods, i.e., approx. 1.0 mg direct probe and approx. 5.0 μg by GC-MS. The distribution pattern of the low mass hydrocarbon fragment ions observed with GC-MS was similar to that observed following direct probe sample introduction.

Following direct probe sample introduction of 1,2-GDN and 1,3-GDN, the electron-impact mass spectra (Figs 1 and 2, respectively) were observed to be without a molecular ion at mass 182, although a peak at 183 ($M^+ + 1$ peak) was observed. For the 1,2-GDN spectra (Fig. 1), ions above *m/e* 160 are shown with *Y*-scale expansion (×3). The

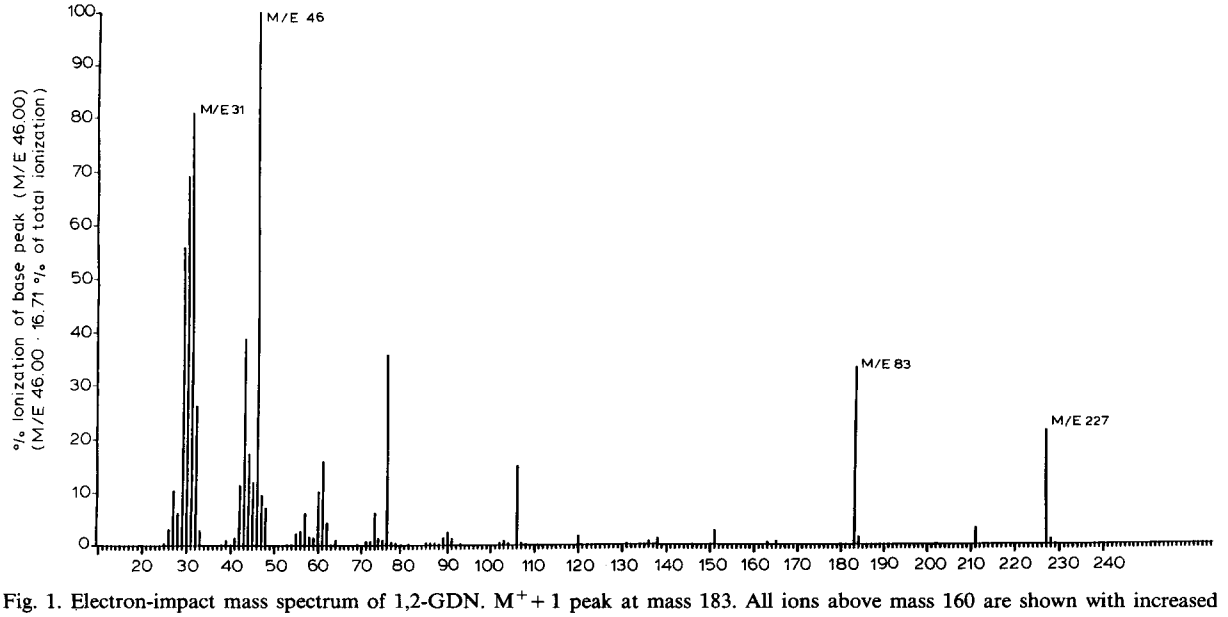

Fig. 1. Electron-impact mass spectrum of 1,2-GDN. $M^+ + 1$ peak at mass 183. All ions above mass 160 are shown with increased (×3) *Y*-scale expansion.

$M^+ + 1$ peak for 1,2-GDN (Fig. 1) comprised 1.83% of the total ionization, while for 1,3-GDN (Fig. 2) the $M^+ + 1$ peak comprised 4.48% of the total ionization. The lack of a molecular ion with these compounds is consistent with the fact that aliphatic nitrates often have a very weak or absent molecular ion as a result of parent compound fragmentation during ionization (Silverstein et al., 1974). The $M^+ + 1$ peak probably arises from chemical ionization phenomena occurring within the electron-impact MS. With aliphatic nitrate compounds a peak equivalent to elemental mass of NO_2 (*m/e* 46) is often prominant. Indeed, the assigned base peak in the mass spectra of 1,2-GDN and 1,3-GDN is at *m/e* 46, the intensity of which represents 19.04 and 16.70% of the total ionization, respectively. A peak at *m/e* 30, equivalent to an elemental composition of NO, was prominent, and was observed to represent 11.55 and 12.99% of the total ionization in Figs 1 and 2, respectively. For the 1,2-GDN molecule the other significant (> 2% total intensity) contributing peaks were at *m/e* 29, 31, 32, 44, 45, 61, 76 and 106. For the 1,3-GDN molecule significant contributing peaks other than at *m/e* 46 and 30, were observed at *m/e* 29, 31, 43, 76 and 106. These contributing peaks represented masses equivalent to hydrocarbon fragment ions which could be derived from fragmentation of the parent molecule, e.g., *m/e* 76, CH_2NO_3 and *m/e* 106, $C_2H_4NO_4$. An *m/e* peak at 227 in the 1,2-GDN spectrum (1.17% of total ionization) and in the 1,3-GDN spectrum (7.62% of total ionization) may result from chemical ionization occurring within the ionization chamber resulting in the interaction between fragment ions. Electron-impact MS of an alternative source of GDN material (analytical standards purity > 99% confirmed by HPLC; Marion Laboratories Inc.) demonstrated comparative features such as the absence of a molecular ion peak at *m/e* 182, the presence of an $M^+ + 1$ peak at *m/e* 183, and the presence of multiple lower mass hydrocarbon fragment ions, and the generation of an *m/e* peak at a mass of 227.

The GDNs have an elemental composition of $C_3H_6N_2O_7$ and a molecular weight based on the mass of each of the commonest isotopes of 182.01748. High resolution mass spectral analysis of the $M^+ + 1$ peak resulted in a determined elemental mass for 1,2-GDN of 183.02531 which is consistent with the elemental composition of $C_3H_6N_2O_7$. Based on the $M^+ + 1$ peak the de-

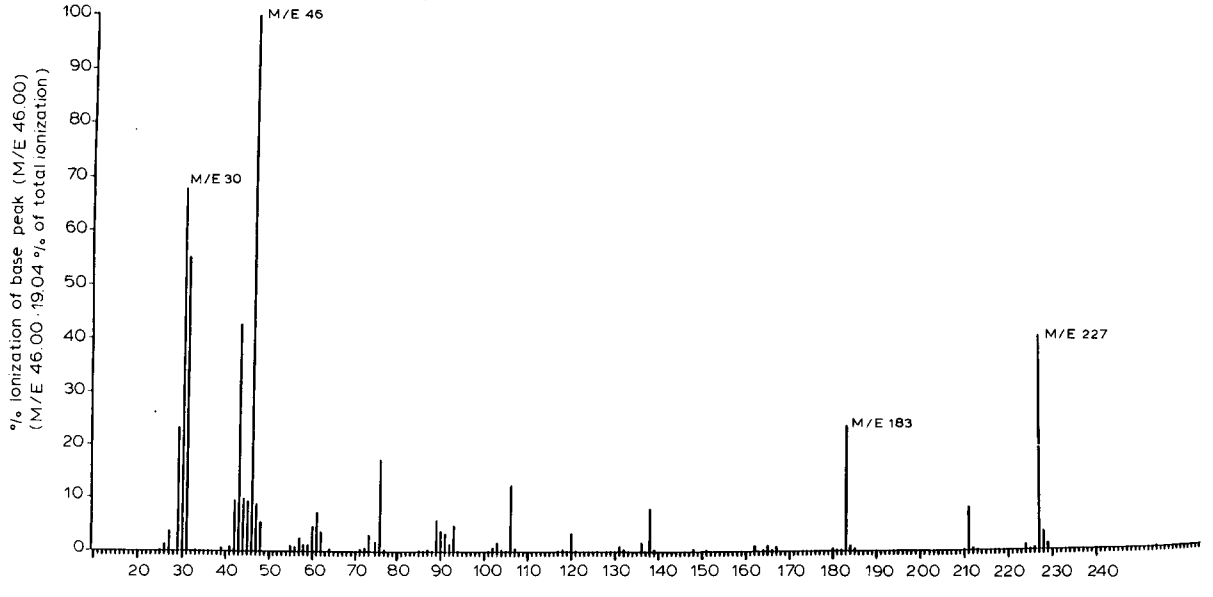

Fig. 2. Electron-impact mass spectrum of 1,3-GDN. $M^+ + 1$ peak at mass 183.

termined 1,3-GDN elemental mass was 229.01821, and exceeded the calculated prediction by a mass equivalent to the elements of NO_2. A probable explanation, as discussed above, may be that chemical ionization and interaction occurs within the ionization chamber between the generated fragment ions, resulting in an inaccurate determination of elemental composition. Why such an interaction should occur solely with the 1,3-GDN molecule and not the 1,2-GDN molecule is not readily apparent. Mass spectral examination of the glyceryl dinitrates has previously been undertaken (Capellos et al., 1979), with the resulting spectra demonstrating parent compound fragmentation, and being devoid of a molecular ion. Interpretation of the mass spectral data for the GDNs is complicated by the fragmentation of the molecules, and the lack of a molecular ion. The elemental composition determined for the 1,3-GDN molecule may be explainable in terms of a chemical reaction between the fragment ions within the mass analyzer.

The NMR spectra for 1,2-GDN, and 1,3-GDN are shown in Figs 3 and 4, respectively. The figures contain the structural formula for 1,2- and 1,3-GDN, together with the assignment of protons. The 1,2-GDN molecule contained a pair of asymmetric diastereotopic protons (protons H_a-H_b and H_d-H_e). The signals at 4.66–4.88 ppm (integration – two protons), comprised two quartets from the splitting of the geminal coupling between the diastereotopic protons H_a and H_b and their independent vicinal coupling with the alpha proton H_c. In contrast to the splitting pattern observed with the H_a and H_b protons, a low chemical shift difference for the geminal coupling of H_d to H_e is evident, resulting in coupled multiplets merging into a complex nine peak multiplet (3.90–4.01 ppm; integration assignment – two protons). A more complex second-order splitting pattern is

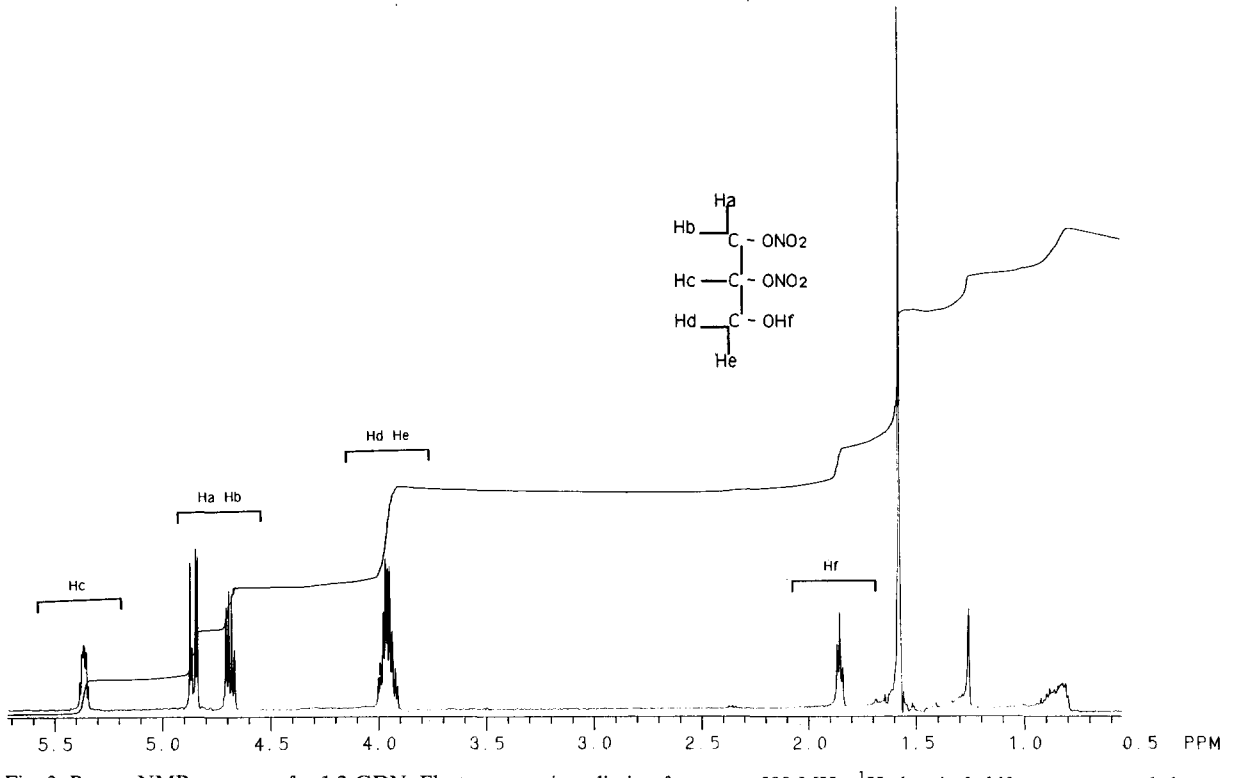

Fig. 3. Proton NMR spectrum for 1,2-GDN. Electromagnetic radiation frequency 500 MHz. ^1H chemical shifts are expressed shown in ppm downfield from tetramethylsilane (0 ppm). Structural formula for 1,2-GDN and ^1H assignments are shown in the inset. Signals at 0.8–1.4 ppm are contaminants present in the deuterated chloroform/tetramethylsilane solution. The signal at 1.55–1.65 ppm is from water.

evident and peak spacings may not correspond to coupling constants. The protons H_d and/or H_e may couple with the hydroxyl proton H_f. The presence within this multiplet of H_d and H_e proton signals with geminal coupling may be inferred by the asymmetry in the multiplet signal with the inner peaks being larger than the outer peaks. The signal at 5.33–5.39 ppm (integration assignment – one proton), comprised a quintet, and the splitting pattern was generated from the vicinal coupling of four inequivalent protons, Ha, Hb, H_d and H_e with the alpha proton H_c. The quintet may be rationalized on the basis that the chemical shift difference between the vicinal splitting protons H_a and H_b, and between the protons H_c and H_d can be expected to be relatively small. An expected eight peak splitting pattern for H_c degenerated into a quintet consisting of overlapping peaks. The protons H_a, H_b and H_c were downfield from the protons H_d and H_e as a result of the strong deshielding effect of the nitrate groups at positions 1 and 2. Although the H_a, H_b and H_c protons are all subject to the deshielding effect of the neighboring nitrate groups, the tertiary proton H_c was further deshielded by the environment of two carbon-carbon single bonds. A hydroxyl proton can vary in position depending upon the temperature of the spinning NMR probe, the NMR solvent utilized, and the degree of hydrogen bonding present in the system. The signal at 1.83–1.88

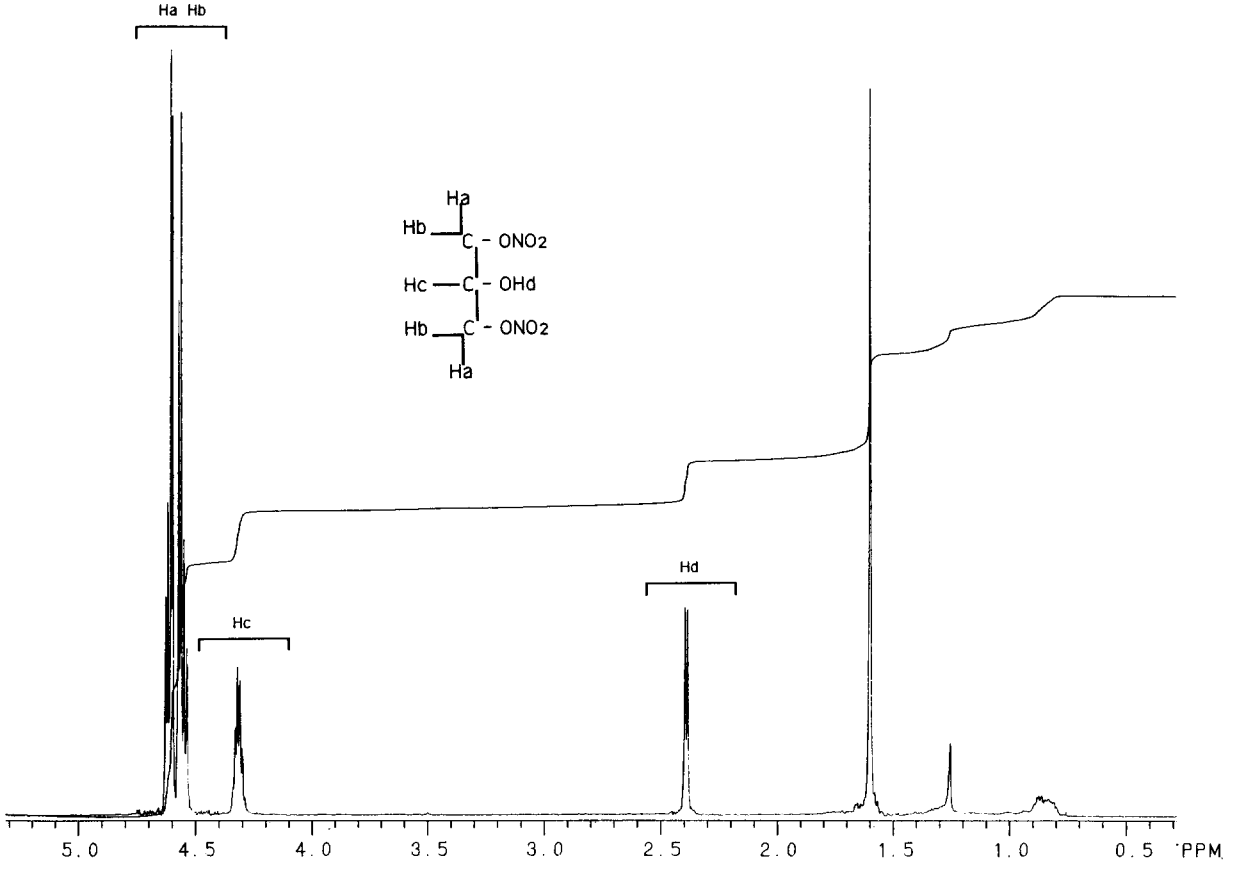

Fig. 4. Proton NMR spectrum for 1,3-GDN. Electromagnetic radiation frequency 500 MHz. ^1H chemical shifts are expressed shown in ppm downfield from tetramethylsilane (0 ppm). Structural formula for 1,3-GDN and ^1H assignments are shown in the inset. Signals at 0.8–1.4 ppm are contaminants present in the deuterated chloroform/tetramethylsilane solution. The signal at 1.55–1.65 ppm is from water.

ppm (integration assignment – one proton), comprising a triplet is confirmed to be the hydroxyl proton H_f by NMR spectral analysis following the addition of deuterium oxide. The relative peak height intensities indicated that the overlapping triplet splitting pattern were derived from the coupling with two inequivalent protons rather than from a first-order splitting pattern with two equivalent protons.

Fig. 4 shows the NMR spectra for 1,3-GDN, a molecule containing a pair of symmetric diastereotopic protons (H_a-H_b). The signals at 4.52–4.63 ppm (integration assigment – four protons) comprised two quartets, which represented the pair of symmetric diastereotopic protons H_a and H_b at carbon positions 1 and 3, and is the splitting pattern expected from the geminal coupling between a symetrical pair of diastereotopic protons and their vicinal coupling with an alpha proton. In contrast to the 1,2-GDN molecule, the equivalency of the pair of diastereotopic protons in the 1,3-GDN molecule results in equivalent chemical shifts with each quartet representing two protons. The signal at 4.28–4.35 ppm (integration assignment – one proton), comprising a multiplet of six peaks, was consistent with the splitting pattern expected from the 1st order vicinal coupling of the H_c proton with two equivalent protons i.e., H_a and H_b, in addition to the coupling with the hydroxyl proton H_d, which may occur. The protons H_a and H_b were downfield from proton H_c, as a result of the strong deshielding effect of the nitrate groups. The signal at 2.37–2.41 ppm (integration assignment – one proton), comprised a doublet, which was confirmed to be the hydroxyl proton H_d, by NMR spectral analysis following the addition of deuterium oxide. The relative peak height intensities indicated that the doublet splitting pattern was derived from coupling with one proton. The NMR spectra for the 1,2-GDN and 1,3-GDN molecules was consistent with the NMR signal predicted for these molecules in terms of the chemical shift pattern, proton counting and the proton splitting patterns.

Figs 5 and 6 show the individual log plasma concentration-time profiles for 1,2-GDN and 1,3-GDN, respectively. The pharmacokinetic characteristics of 1,2- and 1,3-GDN in man, has thus

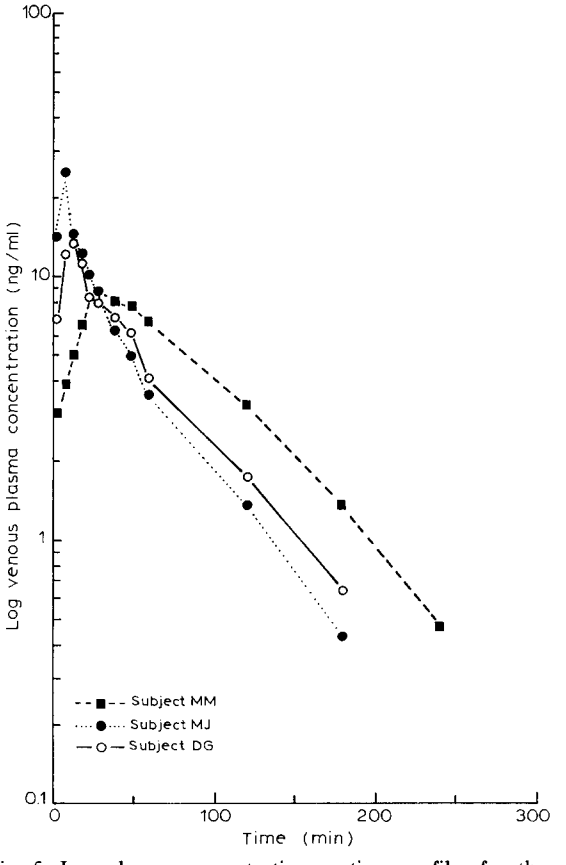

Fig. 5. Log plasma concentration vs time profiles for three subjects (MJ, MM, DG) after receiving a single 2.1 mg dose of 1,2-GDN administered as an oral solution.

far been limited to secondary GDN pharmacokinetic parameters that are based upon GDN metabolite kinetics (Noonan et al. 1985; Noonan and Benet, 1986, 1987; Yu et al. 1988; Nakashima et al. 1990). This preliminary study allows the estimation of some primary GDN pharmacokinetic parameters in man and, although the study did not follow a cross-over design, it allows initial 1,2- and 1,3-GDN pharmacokinetic comparisons to be made. The area under the plasma concentration time curve normalized to the dose administered was, for 1,2 GDN (mean $\pm$ S.D.) 5.41 $\pm$ 1.67 ng ml^{-1} min/kg per mg dose, while for 1,3-GDN it was 4.52 $\pm$ 1.76 ng ml^{-1} min/kg per mg dose, with an average oral clearance (CL/F) for 1,2-GDN calculated to be 35.5 $\pm$ 2.1 ml/min per kg body weight, and for 1,3-GDN 42.8 $\pm$ 10.4 ml/min

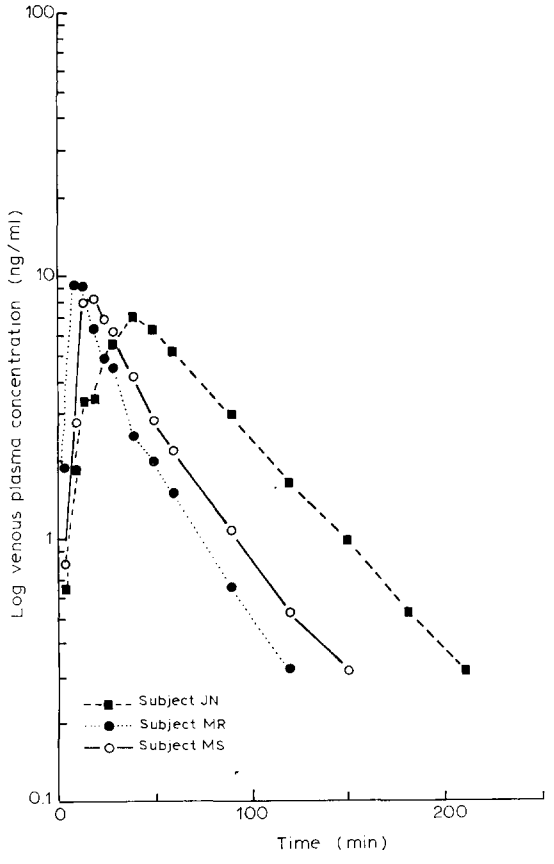

Fig. 6. Log plasma concentration vs time profiles for three subjects (JN, MR, MS) after receiving a single 1.2 mg dose of 1,3-GDN administered as an oral solution.

per kg body weight. The average apparent volume of distribution (V_{area}/F) for 1,2-GDN was 2.17 ± 0.39 l/kg body weight, while for 1,3-GDN the V_{area}/F was 1.92 ± 0.20 l/kg body weight. The disposition of other organic nitrates, such as GTN and isosorbide dinitrate (ISDN) is characterized by large CL and distribution volume parameters. Venous plasma CL values reported for GTN in healthy volunteers have been observed to approach or even exceed cardiac output (approx. 5–71 l/min; Noonan et al., 1985). Similarly, the CL of ISDN in man is recognized to be high (approx. 4 l/min; Fung, 1987), while the primary mononitrate metabolites of ISDN, i.e., isosorbide 5-mononitrate (ISMN-5) and isosorbide 2-mononitrate (ISMN-2) have reported CL values of 0.14 and 0.39 l/min, respectively (Straehl et al., 1984). The CL in healthy volunteers of the 1-glyceryl mononitrate metabolite (GMN-1), has recently been reported to be 0.28 l/min (Laufen and Leitold, 1987). The work of Fung et al., (1984), demonstrating extensive vascular uptake/metabolism of GTN and ISDN, appears to provide partial explanation for the large systemic CL observed with some organic nitrates. The lipophillic GTN and ISDN share the property of a high volume of distribution, in the range 3–4 l/kg body weight (Fung, 1987), indicating that they are mainly located outside plasma water. The more polar ISMN-5 and ISMN-2 having distribution volumes of approx. 0.80 l/kg body weight (Straehl et al., 1984), with GMN-1 having a reported value of 0.76 l/kg body weight (Laufen and Leitold, 1987).

The terminal half-life for 1,2-GDN was on average 40.4 ± 2.80 min, while for 1,3-GDN it was 33.6 ± 6.1 min, with the fractional area under plasma concentration-time curves attributable to the terminal half-lives being greater than 40% of the total AUC. These elimination half-lives are in close agreement with the values previously reported for the 1,2- and 1,3-GDN metabolites in man, following administration of glyceryl trinitrate by a variety of routes (Noonan and Benet, 1987). The observed AUMC/AUC ratio for 1,2-GDN was 65.2 ± 19.8 min, while for 1,3-GDN the ratio was 54.7 ± 16.7 min, values comparable with those obtained for 1,2- and 1,3-GDN following oral solution administration of GTN (Nakashima et al., 1990). If GDN and GTN oral absorption rates can be considered similar, the comparable AUMC/AUC ratios discussed above are consistent with almost complete first pass metabolism of orally administered GTN. The data in Figs 5 and 6 yielded average absorption rate constants of 0.274 ± 0.246 and 0.166 ± 0.115 min^{-1} for 1,2- and 1,3-GDN, respectively; with calculated mean residence times (Watari and Benet, 1989) of 54.6 ± 7.9 min for 1,2-GDN, and 46.8 ± 13.0 min for 1,3-GDN.

This report details the purification and spectroscopic characterization of the 1,2- and 1,3-dinitrate metabolites of GTN, which will be of importance to investigators utilizing the GDNs for analytical, pharmacokinetic or pharmacological

purposes. The efficiency of 1,2- and 1,3-GDN purification, using a preparative HPLC system, was improved by utilization of an initial purification step with gravitational column chromatography, removing contaminating GTN that was present. Consistent with the mass spectral examination of many aliphatic nitrates, the mass spectra of 1,2- and 1,3-GDN is complicated by the lack of a molecular ion, resulting from GDN fragmentation during chemical ionization. The NMR spectra for the 1,2-GDN and 1,3-GDN molecules is consistent with that predicted for the molecules on the basis of chemical shift patterns, proton counting and the proton splitting patterns. The essential features of the two NMR spectra differ as a result of the pair of asymmetric diastereotopic protons present in the 1,2-GDN molecule, and the pair of symmetric diastereotopic protons present in the 1,3-GDN molecule. Preliminary phase I oral administration of 1,2- and 1,3-GDN to healthy volunteers, demonstrated that the 1,2- and 1,3-GDN molecules possess comparable pharmacokinetic characteristics, with GDN distribution volume and clearance parameters that are considerably less than that of both GTN and ISDN.

The purification and spectroscopic procedures described in this article will allow further manufacture of 1,2- and 1,3-GDN, and the conduct of further pharmacokinetic studies in healthy volunteers. The eventual administration of GTN and 1,2- and 1,3-GDN to a study group of stable angina subjects will enable evaluation of the comparative pharmacodynamics of these compounds, clarify the relationship between GTN pharmacokinetics and pharmacodynamics, and allow evaluation of therapeutic potential of the GDNs.

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