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Improved gas chromatographic assay for the simultaneous determination of nitroglycerin and its mono- and dinitrate metabolites

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ABSTRACT

A sensitive, specific capillary gas chromatographic–electron-capture detection method for the simultaneous determination of nitroglycerin (GTN), 1,2- and 1,3-glyceryl dinitrate (1,2-GDN and 1,3-GDN, respectively) and 1- and 2-glyceryl mononitrate (1-GMN and 2-GMN, respectively) is reported. The minimum quantifiable concentration for GTN, GDNs and GMNs is 0.4 ng/ml in plasma, with extraction recoveries for GMNs >76% and for GTN and the GDNs >95%. Over the full range of quantifiable concentrations the inter-run assay precision and accuracy were less than 13 and 11%, respectively, for all five nitrates. Similar intra-run assay precision and accuracy values were found. The method was employed in the preliminary *in vitro* examination of GTN, GDN and GMN kinetics in human blood. Following addition of GTN to human blood, the ratio of 1,2-GDN to 1,3-GDN maximum concentrations (C_{max}) was *ca.* 7:1, reflecting preferential denitration of the GTN molecule at the primary positions, while the C_{max} ratio for 2-GMN to 1-GMN in this system was *ca.* 6:1, representing a highly selective if not specific primary denitration of the 1,2-GDN molecule. Following the intravenous administration of 1,2-GDN to five healthy male volunteers, 2-GMN/1-GMN C_{max} ratios averaged 8.8:1, representing a highly selective but not specific formation of 2-GMN from the 1,2-GDN molecule. The assay will find utility in *in vitro* studies attempting to address the molecular pharmacology of GTN and its metabolites, and in *in vivo* clinical pharmacology studies attempting to address the relationship between pharmacokinetics and pharmacodynamics of GTN and its metabolites.

INTRODUCTION

The organic nitrate nitroglycerin (GTN) is a commonly used anti-anginal agent. Following its administration to man, its elimination is exclusively via metabolism to its primary dinitrate metabolites, *i.e.*, 1,2- and 1,3-glyceryl dinitrate (1,2-GDN and 1,3-GDN, respectively). Based on pharmacokinetic and pharmacological studies of GTN and the GDNs in man [1–3], it is reasonable to hypothesize that the GDNs may make a significant contribution to the therapeutic efficacy observed following GTN administration. The GDNs undergo further *in vivo* denitration to form their respective 1- and/or 2-glyceryl mononitrate metabolites (1-GMN and 2-GMN, respectively). *In vivo* animal studies [4,5] have addressed the relative hemodynamic effects of the GDNs and GMNs, and have shown that although the GMNs are hemodynamically active, they are so at much higher doses than the GDNs. The full elucidation of the pharmacokinetic and pharmacodynamic relationships between GTN and its di- and mononitrate metabolites requires that appropriate studies be conducted utilizing the simultaneous determination of these five organic nitrate moieties.

The routine use of high-performance liquid

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chromatography (HPLC) with UV detection for the simultaneous determination of GTN and the specific GDN and GMN isomers is limited by detection sensitivity, although HPLC analysis with detection by thermal energy analyzer [6–8] and radiometric analysis [9,10] have been reported. Gas chromatographic–mass spectrometric (GC–MS) assays for GTN and the GDNs have been described [11,12], but the use of GC with electron-capture detection (ECD) has proved the most popular method for routine analysis. GC–ECD techniques provide sufficient detection sensitivity [13–16], although extraction procedures are generally complicated and require careful handling to ensure reproducibility. Work in this laboratory [17,18] has contributed assays for the simultaneous and specific determination of GTN and 1,2- and 1,3-GDN. A separate assay solely for the specific determination of the GMN isomers has been reported [19]. In 1988, a simultaneous, specific GC–ECD assay for the determination of nitroglycerin and its dinitrate and mononitrate metabolites was published by Carlin *et al.* [20]. However, the assay sensitivity was low (the limit of detection for all five nitrates was 10 ng/ml), and a very low extraction recovery for the GMNs (30–40%) was reported. Further, no information was provided relating to the precision and accuracy of the assay. In the same year, another method for the simultaneous determination of nitroglycerin and its metabolites using wide-bore capillary column chromatography was reported by Svobodova *et al.* [21]. Although the sensitivity reported was significantly increased, the ranges of the calibration points (for GTN 0.1–1.2 ng/ml, for GDN 0.05–0.5 ng/ml and for GMN 0.2–1.5 ng/ml) were too narrow to allow the convenient simultaneous determination of GTN and the di- and mononitrates in clinical studies. Further, the reported extraction recoveries exhibited a concentration dependence for GTN and the GMNs. In this paper we report an improved, specific, validated GC–ECD assay for the simultaneous determination of GTN and the GDNs and GMNs with a low detection limit for all five nitrate moieties (0.4 ng/ml), and high, concentration-independent extraction recoveries from plasma (*i.e.*, GMNs >76% and GDNs and GTN >95%).

EXPERIMENTAL

Materials

Analytical standards for GTN, 1,2-GDN, 1,3-GDN and 1- and 2-GMN were purchased (>99% purity; 1 mg/ml in ethanol) from Radian (Austin, TX, USA) and used as received. *o*-Iodobenzyl alcohol was available commercially at >99% purity from Aldrich (Milwaukee, WI, USA). Methyl *tert.*-butyl ether was purchased at the highest grade of purity (Omnisolve; EM Science, Gibbstown, NJ, USA) and before use was dried over anhydrous potassium carbonate (Fisher Scientific, Santa Clara, CA, USA). Absolute ethanol was purchased from Quantum Chemical (Tuscola, IL, USA). To prevent adsorption of the nitrates, all glassware was silanized prior to use with a 5% (v/v) solution of dichlorodimethylsilane (Aldrich, Milwaukee, WI, USA) in toluene. Nitrogen and hydrogen gases (Liquid Carbonic, Chicago, IL, USA) were of zero grade.

Gas chromatograph

The gas chromatograph (HP 5890; Hewlett-Packard, San Jose, CA, USA) was equipped with an on-column injector and a ^{63}Ni electron-capture detector. The chromatograms were recorded and peak-height ratios calculated on an HP3396 recording integrator (Hewlett-Packard). Separation of the nitrates was performed using a DB-1 fused-silica capillary column (30 m × 0.32 mm I.D.; 1 μm thick Durabond stationary phase) protected by a 1 m × 0.32 mm I.D. precolumn (DB-1, 1 μm stationary phase). Connections between the precolumn and main column were accomplished with the use of zero-dead-volume metal unions (J&W Scientific, Folsom, CA, USA). As reported previously [22], the length of insertion of the column into an electron-capture detector is particularly important when determining nitroglycerin and its metabolites; experiments showed that maximum sensitivity was achieved when the column was inserted to a

length of 7.0 cm from the tip of the column inside the detector to its point of entry into the detector. Zero-grade hydrogen was utilized for the carrier gas (70 kPa head pressure; flow-rate 3 ml/min at 120°C oven temperature) and nitrogen was used as the ECD make-up gas (flow-rate 120 ml/min); all gases were passed through in-line precolumn water, hydrocarbon and oxygen filters (Alltech, Deerfield, IL, USA). The GC oven temperature program was 120°C for 10 min, increased to 165°C at 10°C/min, then immediately increased to 280°C at 30°C/min, 280°C being maintained for 4 min, allowing a thermal "clean-up" of the column. The detector temperature was maintained at 260°C. The on-column injector was not thermostatically controlled.

Calibration, sample preparation and injection

Three primary stock solutions consisting of (a) GTN, (b) 1,2- and 1,3-GDN and (c) 1- and 2-GMN were prepared from the respective Radian standards as 1 μg/ml solutions in purified deionized water; these stock solutions were kept at 4°C for no more than four months, with frequent stability confirmation. Two secondary stock solutions, each including all five nitrates, were prepared daily or when required as 100 and 10 ng/ml solutions in purified deionized water. Appropriate aliquots of these secondary stock solutions were used in the preparation of spiked samples for measuring the calibration graphs. Calibration graphs for clinical blood samples were constructed with ten points ranging from 0.4 to 15 ng/ml by the addition (using silanized Hamilton glass microsyringes) of nitrate standards to 1-ml volumes of a subject's own predose plasma; 3 ng (6.0 μl) of internal standard (*o*-iodobenzyl alcohol) dissolved in ethanol was added to all standard calibration and post-dose samples. Because of concern for degradation of the nitrates in plasma at room temperature, the frozen plasma samples were placed on dry-ice during addition of organic solvent so that thawing occurred in the presence of the plasma–organic solvent mixture, which decreased the potential for nitrate degradation. Calibration graphs (peak-height ratio *versus* concentration) were constructed by use of weighted least-squares linear regression analysis (weighting Y^{-1}).

All extractions were performed using silanized glass tubes with PTFE-lined caps. For sample extraction, a 0.8-g amount of sodium chloride was added to each standard calibration or post-dose sample. This was followed by the addition of 2 ml of methyl *tert.*-butyl ether and gentle rotation for 5 min. To separate the organic and aqueous phases, the samples were first centrifuged for 10 min at 1000 *g*; the aqueous (plasma) phase was frozen by placing the extraction tubes in a solution of dry-ice–methanol, and the organic phase was transferred into another tube. This extraction procedure was repeated once, with the organics being pooled for evaporation under nitrogen to a final volume of *ca.* 0.5 ml, which was then transferred into a 1-ml glass micro-reaction vial and evaporated to dryness under nitrogen. The organic phase residue was reconstituted with 0.5 ml of methyl *tert.*-butyl ether and stored at −20°C until required; stability tests confirmed that storage conditions were appropriate for at least three months. Following additon of the organic phase to the biological samples, all procedures were conducted at room temperature.

A 0.5–1.0 μl aliquot of each extract was injected into the gas chromatograph. As observed with previous nitrate assays developed in this laboratory [17] and as described by Carlin *et al.* [20], injection of extracted plasma samples results in rapid deterioration of the column efficiency; therefore, it was necessary to replace the precolumn approximately every twenty injections. When more than ten post-dose samples were required to be analyzed (in single-dose clinical studies approximately 20–25 post-dose samples would be collected from each subject), reinjection of four calibration standards on consecutive precolumns was performed. All peak-height ratios for these four reinjected calibration standards were required to fall within the 95% confidence interval of the original ten-point calibration graph.

Precision, accuracy and recovery experiments

For the study of inter-run precision and accu-

racy, six calibration graphs with six sets of quality control samples (concentrations of 15.0, 8.0, 2.0 and 0.4 ng/ml) were prepared by spiking 1-ml plasma samples with standard solution. The calibration graphs and quality control samples were analyzed on six different precolumns over a number of days. For the assessment of intra-run precision and accuracy, a single calibration graph with six sets of quality control samples (concentrations as above) were prepared, with each of the four quality control concentrations being analyzed on a single precolumn. Precision is defined as % (standard deviation/mean), while accuracy (or error) is defined as % [(test concentration − observed concentration)/test concentration] for the observed quality control samples.

For the assessment of the recovery of the nitrates using the extraction procedure described above, samples of plasma (1 ml), from the same individual, were spiked to 0.5, 2, 8 and 15 ng/ml with GTN, 1,2- and 1,3-GDN and 1- and 2-GMN. Extraction of the samples was performed as described above except that internal standard was added after the extraction procedure was complete, *i.e.*, at the time of reconstitution of the organic phase residues. Control samples were made by spiking 1 ml of methyl *tert.*-butyl ether with both internal standard and the respective amounts of all five nitrates; relative recovery was determined by comparison of peak-height ratios obtained with extracted plasma samples to those of control samples.

Assay test experiments

Incubation of nitroglycerin with human blood. Blood was obtained from three healthy male volunteers in evacuated blood collection tubes (Becton Dickinson, Rutherford, NJ, USA), each tube containing 143 USP units of sodium heparin. Aliquots (15 ml) of blood were immediately added to silanized scintillation vials and shaken gently in a thermostatically controlled (37°C) waterbath. After 5 min of preincubation, an aqueous solution of GTN was added to each vial and mixed thoroughly; the amount of GTN added yielded an initial dilution concentration of 40 ng/ml. Serial blood samples (1–2.5 ml) were withdrawn from the scintillation vials at 0, 5, 30, 60, 120 and 180 min after GTN addition. The blood samples were immediately centrifuged (Eppendorf; Brinkman Instruments, Westbury, NY, USA) at 15 000 *g* for 30 s, and aliquots of plasma (0.3–1.0 ml) immediately frozen by addition to glass extraction tubes placed on solid carbon dioxide. Samples were stored at −20°C until required and the extraction was then performed as described above.

Administration of 1,2-GDN to man. The 1,2-GDN was prepared as described previously [2]. Formulation and clinical study procedures were performed under Investigational New Drug Application 32 278, with the approval of the University of California, San Franciso Committee on Human Reseach. Five healthy male volunteers between the ages of 21 and 35 years were recruited and, following informed consent, each received a 2.2-mg intravenous dose of 1,2-GDN (88.2 μg/min over 25 min). Serial blood samples were collected in chilled heparinized polyethylene syringes (Sarstedt, Hayward, CA, USA) over 3–4 h after initiation of infusion. Stability investigations prior to the study confirmed no GDN adsorption on the polyethylene syringes. Plasma was obtained by immediate centrifugation (15 000 *g* for 30 s), then aliquoted into silanized glass extraction tubes and frozen.

RESULTS AND DISCUSSION

Chromatographic separation

Typical chromatograms of blank, spiked plasma and the 1-h plasma sample for the incubation of GTN in blood from a human volunteer are shown in Fig. 1. The retention times for 1-GMN, 2-GMN, 1,3-GDN, 1,2-GDN, GTN and internal standard are 4.5, 5.2, 8.9, 9.5, 11.3 and 14.1 min, respectively; 1- and 2-GMN and 1,2- and 1,3-GDN are well separated and easily determined.

Previous investigations in this laboratory have shown that nitroglycerin and its dinitrate metabolites are adsorbed on the active surfaces of exposed silica in the capillary columns and on components of the electron-capture detector [22]; we observed that this adsorption is more prominent

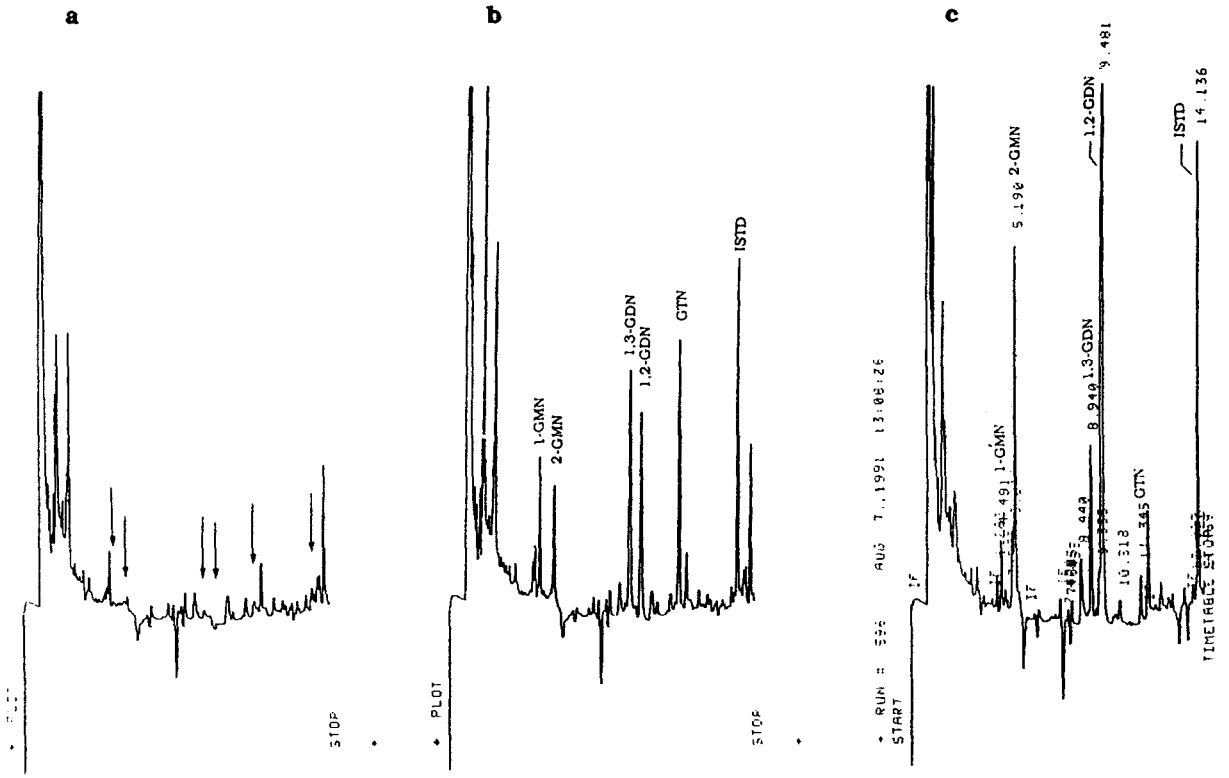

Fig. 1. Chromatograms of blank and spiked plasma. (a) Blank plasma. Arrows indicate retention times of compounds of interest. (b) 1 ml of blank plasma spiked with 4 ng of GMNs, GDNs, GTN and 3 ng of internal standard (*o*-iodobenzyl alcohol). (c) Plasma sample obtained 1 h following incubation in blood from a human volunteer at 37°C of 40 ng/ml GTN. The retention times are indicated on the peaks in (c).

with the GMNs. The adsorption phenomenon can be significant, resulting in a low assay precision and low detection sensitivity; therefore, the choice of capillary column and GC conditions is very important in minimizing adsorption. In the development of this assay three types of fused-silica capillary columns were examined: HP-1 (Hewlett-Packard) (25 m × 0.32 mm I.D., 1 μm thick methylsilicone stationary phase), DB-1 (J & W Scientific) (30 m × 0.32 mm I.D., 1 μm thick methylsilicone stationary phase) and DB-5 (30 m × 0.32 mm I.D., 1 μm thick 5% phenylsilicone stationary phase) (J & W Scientific). The DB-1 column performed best with regard to minimizing adsorption of the nitrates. The use of higher oven temperatures also helps to minimize the adsorption of nitrates on the exposed surfaces of the capillary column, although high temperatures may also result in the loss of chromatographic resolution. Our choice of the DB-1 column for assay development also reflected the fact that greater peak tailing was observed with the HP-1 and DB-5 columns. To minimize adsorption of the nitrates within an electron-capture detector, thereby maximizing sensitivity, requires the optimization of the position of the column within the detector [22]; the optimum length of column inside each detector was experimentally determined with respect to the observed peak-height ratio relative to internal standard. In addition to column length within the detector, nitrate adsorption can be reduced by using a high make-up gas flow-rate of 120 ml/min.

Extraction recovery and validation

Extraction recoveries from plasma, as listed in

TABLE I

RECOVERIES OF GMNs, GDNs, GTN, AND INTERNAL STANDARD (I.S.) FROM 1 ml OF PLASMA

Concentration (ng/ml)	Recovery (mean ± S.D., n = 6) (%)					
	1-GMN	2-GMN	1,3-GDN	1,2-GDN	GTN	I.S.[a]
0.5	83.4 ± 4.5	85.8 ± 8.6	98.5 ± 7.0	104.3 ± 3.4	102.9 ± 4.6	–
2.0	76.4 ± 3.0	79.6 ± 3.3	106.7 ± 2.8	103.7 ± 2.9	103.5 ± 5.7	–
8.0	77.0 ± 9.5	80.3 ± 7.6	98.5 ± 7.5	95.3 ± 6.6	95.9 ± 10.0	–
15.0	81.7 ± 4.4	85.8 ± 5.4	101.3 ± 4.8	99.6 ± 5.4	96.5 ± 4.7	–
3.0	–	–	–	–	–	85.7 ± 4.0

[a] Calculations of recovery were made using 2 ng of nitroglycerin in methyl *tert.*-butyl ether as the internal standard.

Table I, were greater than 76% for the GMNs, whereas for the GDNs and GTN the recovery was greater than 95%. No concentration dependence of the efficiency of extraction was apparent. The extraction of the GMNs from the aqueous plasma phase provides the most problematic aspect to achieving a high efficiency of extraction. These polar compounds require a more polar extraction solvent to achieve high recovery, with the resultant problem of increasing baseline noise due to concomitant extraction of polar material in plasma. Methyl *tert.*-butyl ether has previously been used in the extraction of GTN and its metabolites from plasma [18,20], with variation of the ionic strength to improve the extraction efficiency (*e.g.*, the GMN extraction efficiency is approximately doubled following addition of sodium chloride). In the report of Carlin *et al.* [20], a GMN extraction recovery of only 30–40% was attained. In our laboratory we have found that significant (30–40%) adsorption of GMNs occurs on the drying agent (*i.e.*, potassium carbonate) used in the assay of Carlin *et al.* In addition other potential drying agents such as 3Å and 4Å molecular sieves, silica gel, calcium chloride and sodium sulfate will also adsorb GMN and GDN to an extent that precludes their use in this assay. Using the approach eventually adopted in this work (*i.e.*, sequential nitrogen evaporations), residual water can be efficiently removed without a decrease in nitrate recovery and with the maintenance of a high assay precision.

Using non-linear weighted least-squares regression analysis (weighting Y^{-1}), the calibration graphs were linear ($r^2 > 0.995$) from the limit of detection (0.4 ng/ml) to our chosen maximum working concentration of 15.0 ng/ml. Intra-run assay precision and accuracy data are shown in Table II. Table III shows the inter-run assay precision and accuracy; these results are comparable to those given in Table II. The limit of detection for all five nitrates was 0.4 ng/ml in plasma. Over the full range of quantifiable concentrations the inter-run assay precision and accuracy (*i.e.*, error) for 1-GMN were within 8.8 and 7%, respectively, for 2-GMN 12.1 and 4%, respectively, for 1,3-GDN 12.8 and 10%, respectively, for 1,2-GDN 10.1 and 9%, respectively, and for GTN 11.6 and 8.8%, respectively. Similarly, the intra-run assay precision and accuracy for 1-GMN were within 12.5 and 6.5%, respectively, for 2-GMN 7.2 and 8%, respectively, for 1,3-GDN 11.9 and 7%, respectivley, for 1,2-GDN 9.6 and 10.5%, respectively, and for GTN 7.2 and 5.3%, respectively.

Applications

We believe that the assay of Carlin *et al.* [20], with a relatively high limit of detection (10 ng/ml), is not satisfactory for pharmacokinetic studies designed to evaluate metabolite levels following therapeutic doses of GTN or in drug trials following GDN administration, especially where the metabolite ratios (*i.e.*, 1,2-GDN/1,3-GDN)

TABLE II

INTRA-RUN PRECISION AND ACCURACY FOR GMNs, GDNs AND GTN ASSAY

Nitrate	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D., n = 6) (ng/ml)	C.V. (%)	Error (%)
1-GMN	0.4	0.407 ± 0.051	12.5	1.77
	2.0	2.13 ± 0.08	3.5	6.5
	8.0	7.97 ± 0.30	3.8	−0.4
	15.0	14.80 ± 0.98	6.6	−1.37
2-GMN	0.4	0.407 ± 0.025	6.1	1.81
	2.0	1.84 ± 0.11	6.2	−8.0
	8.0	7.47 ± 0.39	5.2	−6.6
	15.0	14.81 ± 1.07	7.2	−1.3
1,3-GDN	0.4	0.418 ± 0.050	11.9	4.5
	2.0	2.14 ± 0.10	4.7	7.0
	8.0	8.20 ± 0.08	1.0	2.5
	15.0	15.05 ± 0.44	2.9	0.3
1,2-GDN	0.4	0.399 ± 0.038	9.6	−0.30
	2.0	2.21 ± 0.08	3.4	10.5
	8.0	8.20 ± 0.09	1.1	2.5
	15.0	14.62 ± 0.34	2.3	−2.6
GTN	0.4	0.421 ± 0.025	6.0	5.25
	2.0	2.10 ± 0.15	7.2	5.0
	8.0	8.13 ± 0.22	2.7	1.6
	15.0	15.51 ± 0.47	3.0	3.4

TABLE III

INTER-RUN PRECISION AND ACCURACY FOR GMNs, GDNs AND GTN ASSAY

Nitrate	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D., n = 6) (ng/ml)	C.V. (%)	Error (%)
1-GMN	0.4	0.400 ± 0.035	8.8	0.0
	2.0	2.14 ± 0.05	2.4	7.0
	8.0	8.31 ± 0.60	7.2	3.9
	15.0	15.21 ± 0.96	6.3	1.4
2-GMN	0.4	0.386 ± 0.039	10.0	−3.5
	2.0	2.08 ± 0.25	12.1	4.0
	8.0	8.17 ± 0.88	10.8	2.1
	15.0	15.45 ± 1.39	9.0	3.0
1,3-GDN	0.4	0.422 ± 0.054	12.8	5.5
	2.0	2.20 ± 0.18	8.0	10.0
	8.0	8.39 ± 0.29	3.5	4.9
	15.0	15.92 ± 0.84	5.3	6.1
1,2-GDN	0.4	0.387 ± 0.039	10.1	−1.9
	2.0	2.18 ± 0.03	1.6	9.0
	8.0	8.29 ± 0.18	2.2	3.6
	15.0	15.17 ± 1.27	8.4	1.1
GTN	0.4	0.396 ± 0.046	11.6	−1.0
	2.0	2.04 ± 0.20	9.8	2.0
	8.0	8.18 ± 0.25	3.1	2.3
	15.0	16.31 ± 1.11	6.8	8.8

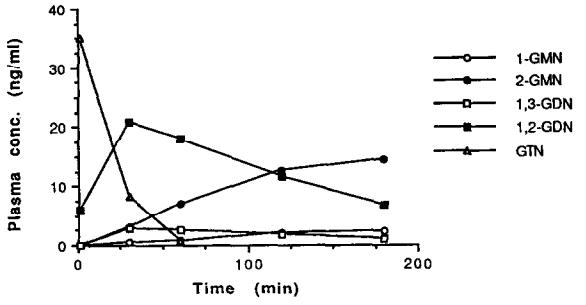

Fig. 2. Plasma concentration of GTN and its metabolites following incubation of GTN in blood at an initial concentration of 40 ng/ml at 37°C (n = 3).

may show a high degree of selectivity, *e.g.*, in man 1,2-GDN/1,3-GDN ratios > 7 have been reported following intravenous GTN administration [23]. Similarly, recent human studies in our laboratory [24] have shown a highly selective but not exclusive metabolism of 1,2-GDN to 2-GMN, *e.g.*, 2-GMN/1-GMN ratio ≈ 8–9 following 1,2-GDN intravenous dosing. It is possible that in a recent dog study by Carlin *et al.* [25] the exclusive metabolism of intravenously administered 1,2-GDN to form *only* the 2-GMN metabolite may reflect the influences of species differences between dog and man and/or the lack of a sufficiently sensitive analytical technique for 1-GMN measurement.

Using this improved GC assay, we have been able to determine the plasma concentrations of GTN, 1,3- and 1,2-GDN and 1- and 2-GMN following incubation of GTN in human blood and to determine the plasma concentrations of 1,2-GDN and 1- and 2-GMN following intravenous administration of 1,2-GDN to healthy volunteers. Following addition of GTN to human blood, the GTN concentrations were above the limits of detection for up to 60 min; 1,2-GDN concentrations were detectable as early as 5 min (see Fig. 2), while the GMNs and 1,3-GDN could be detected beginning at 30 min. The percentage of all five species, normalized to molar concentration, at different times are presented in Table IV. The mass balance for GTN and its metabolites is also presented in Table IV, normalized to the first measurement at 0.5 min. In agreement with previous data from our laboratory [9], a very rapid metabolism of GTN in blood, with a degradation half-life of *ca.* 20 min, was observed. The ratios of 1,2-GDN to 1,3-GDN from 30 to 180 min were in the range 6–7:1, suggesting preferential denitration of the GTN molecule at the primary position, with comparable degradation kinetics for 1,2- and 1,3-GDN. A similar ratio range is observed for 1-GMN to 2-GMN. It should be noted that denitration of 1,2-GDN has the potential to result in the formation of both 2- and 1-GMN, whereas denitration of 1,3-GDN can only result in the formation of 1-GMN. The comparable 1,2-GDN to 1,3-GDN and 2-GMN to 1-GMN ratios in blood further support a marked preference for denitration at the primary position. To examine the selectivity of 2-GMN formation from 1,2-GDN, we utilized this assay to measure plasma concentrations following the intravenous administration of 1,2-GDN to man (see Fig. 3). The ratios of 2-GMN to 1-GMN at

TABLE IV

PERCENTAGE OF GTN AND ITS IN VITRO METABOLITES IN HUMAN BLOOD

The percentages are normalized by molar concentration. Expressed as mean ± S.D. (n = 3).

Time (min)	1-GMN	2-GMN	1,3-GDN	1,2-GDN	GTN	Total[a]
0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	17.3 ± 4.3	82.7 ± 6.9	100.0
30	1.8 ± 0.6	11.7 ± 3.5	8.7 ± 0.7	61.0 ± 3.4	19.4 ± 2.8	102.6
60	3.8 ± 0.7	27.1 ± 5.0	7.9 ± 1.1	53.2 ± 3.0	1.9 ± 1.3	93.6
120	7.6 ± 1.1	49.7 ± 6.8	5.6 ± 1.4	33.9 ± 2.5	0.0 ± 0.0	96.8
180	9.1 ± 1.2	56.8 ± 5.3	3.0 ± 0.8	19.8 ± 3.5	0.0 ± 0.0	88.7

[a] Calculated using the 0.5-min value as 100%.

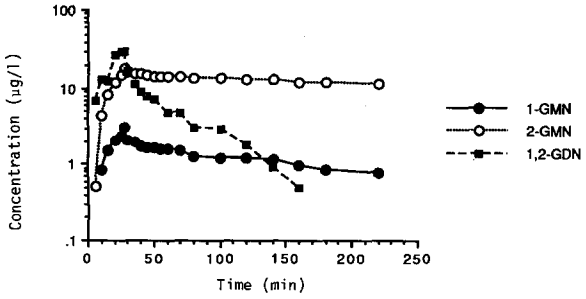

Fig. 3. Plasma concentrations of 1,2-GDN, 1-GMN and 2-GMN following an intravenous infusion of 2.2 mg of 1,2-GDN (88.2 µg/min for 25 min) to a healthy male volunteer.

C_{max} in the five subjects studied were 6.0, 9.7, 7.9, 9.6 and 10.9, average 8.8. This represents a highly selective, but not specific formation of 2-GMN from 1,2-GDN in humans. Carlin *et al.* [25] reported exclusive metabolism of intravenously administered 1,2-GDN to the 2-GMN metabolite. However, the ratio of 2-GMN to 1-GMN could have been as high as >15:1 and the investigators would not have observed any 1-GMN owing to the high detection limit of their assay (10 ng/ml). Hence, their report may indeed only reflect the primary but not exclusive denitration of intravenously administered 1,2-GDN in beagle dogs.

In conclusion, a specific and sensitive assay for GTN and all five of its organic nitrate metabolites has been reported. In previous reports of simultaneous assays for GTN and its di- and mononitrate metabolites [20,21], the assay sensitivity was low or the calibration ranges were narrow and no information was provided relating to the precision and accuracy of the assay. Here an improved, specific, validated GC–ECD assay for the simultaneous determination of GTN and the GDNs and GMNs with low detection limits for all five nitrate moities and high precision and accuracy has been developed. The assay will find utility in *in vitro* studies attempting to address the molecular pharmacology of GTN and its metabolites, and in *in vivo* clinical pharmacology studies attempting to address the relationship between pharmacokinetics and pharmacodynamics of GTN and its metabolites.

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