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SIMULTANEOUS DETERMINATION OF GLYCERYL TRINITRATE AND ITS PRINCIPAL METABOLITES, 1,2- AND 1,3-GLYCERYL DINITRATE, IN PLASMA BY GAS CHROMATOGRAPHY–NEGATIVE ION CHEMICAL IONIZATION–SELECTED ION MONITORING

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SUMMARY

A specific and sensitive method for the quantitation of glyceryl trinitrate (GTN) and its principal metabolites, 1,2- and 1,3-glyceryl dinitrate (GDN) in dog plasma by capillary gas chromatography–negative ion chemical ionization–selected ion monitoring using dichloromethane as a reagent gas and the corresponding compounds labelled with stable isotopes as internal standards. The quantitation limits of the method for GTN and the GDNs were 0.1 and 1.0 ng/ml in plasma, respectively. When GTN was administered intravenously to four anaesthetized beagle dogs at a dose of 6 μg/kg · min for 30 min, the plasma levels of GTN and 1,2- and 1,3-GDN reached a maximum at the end-point of infusion and decreased with bi-exponential decay. The half-lives of the α- and β-phases were 0.50 and 4.95 for GTN, 8.10 and 40.6 for 1,2-GDN and 8.50 and 48.5 for 1,3-GDN, respectively.

INTRODUCTION

GTN has been widely used as one of the most effective agents for decreasing peripheral vascular resistance in patients with heart failure and after myoinfarction. Recently, GTN has been tried clinically for the production of controlled hypotension during general anaesthesia in surgical operations because of the rapid appearance of its hypotensive action and its short duration, resulting in easy control of blood pressure. GTN is easily metabolized with endogenous esterase in man, and much attention has been focused on the relationship between arterial blood pressure and the plasma levels of GTN in patients receiving controlled hypotension with GTN.

We developed a specific and precise method for the quantitation of GTN in plasma by GC–NICI–SIM using [$^2H_5$,$^{15}N_3$]GTN as an internal standard, and this method was applied to the investigation of the pharmacokinetics of GTN in patients receiving it during general anaesthesia[1]. Contrary to our expectations, the study indicated that the recovery of the blood pressure to the initial value after the

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withdrawal of the infusion of GTN was relatively slow in some patients although its plasma levels had decreased to non-effective levels. This result led to speculation that this sustained hypotension might be caused by the action of GDNs yielded from GTN, because 1,2- and 1,3-GDNs exhibited weak hypotensive action[2].

Hence it was considered necessary to develop a specific and precise method for the quantitation of 1,2- and 1,3-GDNs in addition to GTN in plasma. This paper deals with the simultaneous determination of GTN, 1,2-GDN and 1,3-GDN in dog plasma by capillary GC–NICI–SIM using the corresponding compounds labelled with stable isotopes as internal standards.

EXPERIMENTAL

Materials

All reagents and solvents were of analytical-reagent grade and were used without further purification.

The GTN used as a standard material was obtained by several washings of the medicinal-grade product with water until nitrate ion, G, GMNs and GDNs were completely removed. GDNs were prepared as follows. GTN was moderately hydrolysed by treatment with 1 *N* sodium hydroxide solution–ethanol (9:1) for 30 min at 15°C. The reaction product was extracted with diethyl ether and the residue was concentrated under reduced pressure. The residue was dissolved in benzene–ethyl acetate (4:1) and chromatographed over a silica gel column with benzene–ethyl acetate (4:1). Each of the GDNs fractions was collected carefully and re-chromatographed over the silica gel column using the same solvent system. The purities of GTN, 1,2-GDN and 1,3-GDN were checked by TLC and HPLC[3,4]. The concentrations of working standard solutions prepared from the above standard materials were determined according to the colorimetric procedure described in Pharmacopoeia Japonica (Editio Nona).

[$^2H_5$,$^{15}N_3$]GTN was synthesized in our Explosives Research Laboratory, using [$^2H_8$]G (Merck Sharp & Dohme Canada, Quebec, Canada) and [^{15}N]nitric acid (Merck Sharp & Dohme Canada)[1]. 1,2- and 1,3-[$^2H_5$,$^{15}N_2$]GDNs were prepared from [$^2H_5$,$^{15}N_3$]GTN according to the method described above.

Sephadex LH-20 (25–100 μm) was obtained from Pharmacia (Uppsala, Sweden) and Kieselgel (70–230 mesh) from Merck (Darmstadt, G.F.R.). Extube 1003 was purchased from Analytichem International (CA, U.S.A.), BSTFA from Tokyo Kasei Kogyo (Tokyo, Japan) and CD_2Cl_2 from Merck Sharp & Dohme Canada.

Gas chromatography–mass spectrometry

A Shimadzu LKB-9000A GC–MS system modified for detection of negative ions and equipped with a data processing system was employed. The columns used were a WCOT glass capillary column coated with SE-30 (25 m × 0.35 mm I.D.; LKB, Stockholm, Sweden) for GTN and a SCOT glass capillary column coated with XE-60 (30 m × 0.30 mm I.D.; Shinwa Kako, Kyoto, Japan) for GDNs. The temperatures of the column oven were maintained at 110°C for GTN and at 150°C for GDNs. The temperature of the injection port and the separator was 180°C and the ionization source was kept at 160°C. The flow-rate of the carrier gas (helium) was 1.5 ml/min. The accelerating voltage was −3.5 kV. The ionization energy and emission current were 500 eV and 170 μA, respectively.

Selected ion monitoring

The following ions were used for monitoring GTN and 1,2- and 1,3-GDNs: *m/z* 262 (GTN), *m/z* 270 ([$^2H_5$,$^{15}N_3$]GTN), *m/z* 289 (1,2- and 1,3-GDN TMS ethers) and *m/z* 296 (1,2- and 1,3-[$^2H_5$,$^{15}N_2$]GDN TMS ethers). The ratios of the peak heights in SIR for ions of *m/z* 262 and 270 and of *m/z* 280 and 296 were calculated and compared with a calibration graph to determine the plasma levels.

Administration of GTN

Intravenous bolus injection. GTN was administrated to three conscious beagle dogs by bolus injection in a dose of 150 μg each. Blood samples were obtained 0.5, 1, 2, 3, 4, 5, 10, 15 and 30 min after the injection.

Intravenous infusion. Four beagle dogs were anaesthetized using pentobarbital and received GTN for 30 min by intravenous infusion at a dose of 6 μg/kg·min. Blood samples were obtained 15 and 30 min after the start-point of infusion and 1, 2, 5, 10, 20, 30, 45, 60 and 90 min after the withdrawal of infusion.

Sample preparation procedure

The blood samples were immediately centrifuged at 1900 *g* for 15 min at 0–4°C and the plasma was collected. An internal standard solution containing 50 ng/ml of [$^2H_5$, $^{15}N_3$]GTN and 100 ng/ml each of 1,2- and 1,3-[$^2H_5$, $^{15}N_2$]GDNs was added to 2 ml of plasma. This procedure was carried out in an ice-bath in order to avoid the enzymatic degradation of GTN in plasma[1].

The plasma sample was transferred directly on to Extube 1003 (solid-phase extraction tube). After standing for 5 min, GTN was eluted with 30 ml of *n*-hexane and subsequently GDNs were eluted with 25 ml of benzene.

GTN fraction. The eluate was transferred to a column (50 × 5 mm I.D.) packed with silica gel in *n*-hexane, washed with 15 ml of *n*-hexane and eluted with benzene–*n*-hexane (1:6). The fraction of 20–50 ml was collected and concentrated to about 0.1–0.2 ml. The residue was dissolved in 0.5 ml of *n*-hexane–chloroform–methanol (10:10:1) and applied to a column (100 × 5 mm I.D.) packed with Sephadex LH-20 in *n*-hexane–chloroform–methanol (10:10:1). The column was washed and eluted with the solvent described above. The fraction of 4–8 ml was collected and concentrated to approximately 5–10 μl. The residue was dissolved in 30 μl of benzene and an aliquot of this solution was subjected to GC–NICI–SIM using a glass capillary column coated with SE-30.

GDN fraction. The benzene eluate was evaporated at 20–25°C in a water-bath under reduced pressure. The residue was dissolved in 5 ml of *n*-hexane in an ultrasonic generator, and the solution was transferred to a column (20 × 6 mm I.D.) packed with silica gel in *n*-hexane. This step was repeated twice. The column was washed with 15 ml of *n*-hexane and eluted with a further 15 ml of *n*-hexane. The eluate was concentrated under reduced pressure and the concentrate was silylated with 20 μl of BSTFA at room temperature. An aliquot of this solution was subjected to GC–NICI–SIM using a glass capillary column coated with XE-60.

RESULTS AND DISCUSSION

GTN is metabolized to 1,2- and 1,3-GDNs; these dinitrates are further metabolized to their GMNs and ultimately to G, and a significant proportion of the GDNs

and GMNs formed is conjugated to glucuronic acid[5]. In order to separate 1,2- and 1,3-GDNs, these two isomers were silylated with BSTFA to convert them into their TMS ether derivatives without degradation. However, both TMS ether derivatives were eluted with almost the same retention times when analysed on packed columns using OV-101, OV-3 and XE-60 as the liquid stationary phase. Subsequently capillary columns coated with SE-30, OV-101, OV-17 and XE-60 were examined to achieve the separation of GDN TMS ether isomers. A baseline separation could be obtained by the use of a capillary column coated with XE-60.

NICI–MS provides negative molecular ions from compounds that possess electron-capturing ability. Contrary to our expectations, however, when methane, isobutane or ammonia was used as a reagent gas in the NICI mode, GTN and GDN TMS ethers gave rise to the negative anion (NO_3^-) as the base peak, and there were no negative ions in the high-mass regions. However, when CH_2Cl_2 was used as the reagent gas, it has been reported that the chlorinated molecular ion $[M + Cl]^-$ was often observed as a prominent ion[6]. Then, NICI–MS of GTN and 1,2- and 1,3-GDN TMS ethers using CH_2Cl_2 was examined.

Fig. 1A shows the NICI mass spectrum of GTN with CH_2Cl_2 as the reagent gas. The chlorinated molecular ion $[M + Cl]^-$ was confirmed by the presence of the doublet ion due to the characteristic intensities of the chlorine atom at *m/z* 262 and 264. The ion due to the addition of nitrate ion produced from the GTN molecule was observed at *m/z* 289. Although the NICI mass spectrum of CH_2Cl_2 was characterized by Cl^-, HCl_2^- and $CH_2Cl_2^-$ ions[7], the expected $[M + HCl_2]^-$ and $[M + CH_2Cl_3]^-$ ions could not be observed. The fragment ions appearing at *m/z* 217 and 235 were considered to be produced by the losses of $NOCl_2$ and HNO_3 from an unidentified ion of $[M + HCl_2]^-$, because when CD_2Cl_2 was used as the reagent gas, the ion of *m/z* 217 was shifted by one mass unit to *m/z* 218, whereas the ion at *m/z* 235 did not shift, and the ions corresponding to those of *m/z* 217 and 235 were observed at *m/z* 224 and 242 in the mass spectrum of $[^2H_5,^{15}N_3]$GTN (Fig. 1B).

Figs. 2A and 3A show the NICI mass spectra of the 1,2- and 1,3-GDN TMS ether derivatives. The mass spectral fragmentation of these derivatives were very

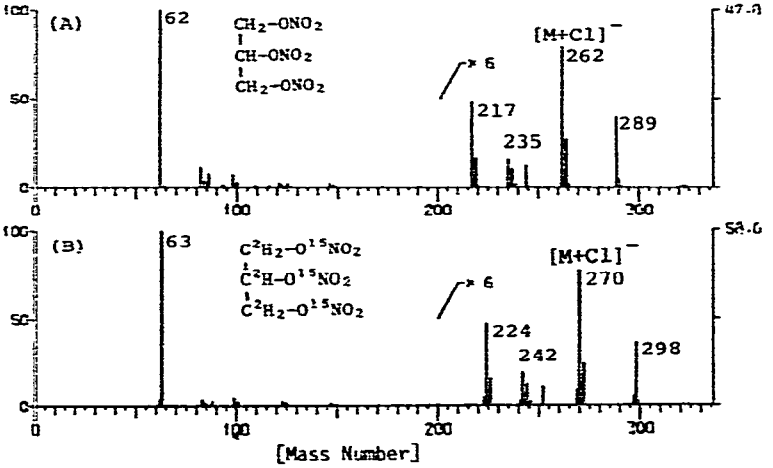

Fig. 1. NICI mass spectra of (A) GTN and (B) $[^2H_5,^{15}N_3]$GTN with dichloromethane as reagent gas.

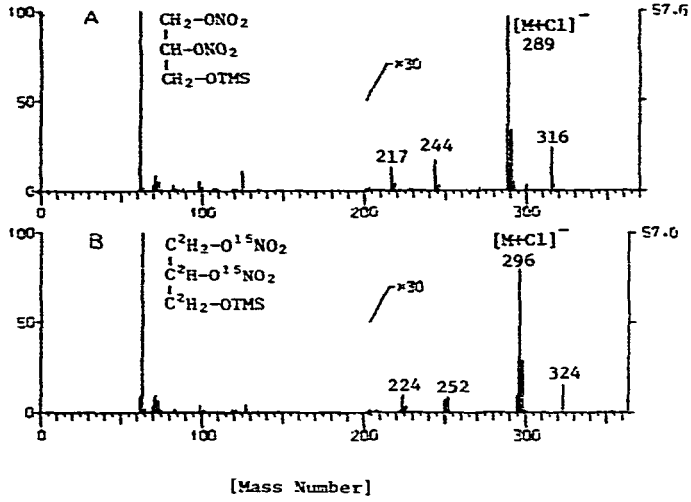

Fig. 2. NICI mass spectra of (A) 1,2-GDN and (B) 1,2-[$^2H_5$, $^{15}N_2$]GDN with dichloromethane as reagent gas.

similar to that of GTN except for a 27 mass unit shift in the ions containing the TMS ether group instead of the NO_3 group in GTN, *i.e.*, the $[M + Cl]^-$ ion was shifted from *m/z* 262 to 289 for GTN and the $[M + NO_3]^-$ ion from *m/z* 289 to 316 for GDNs.

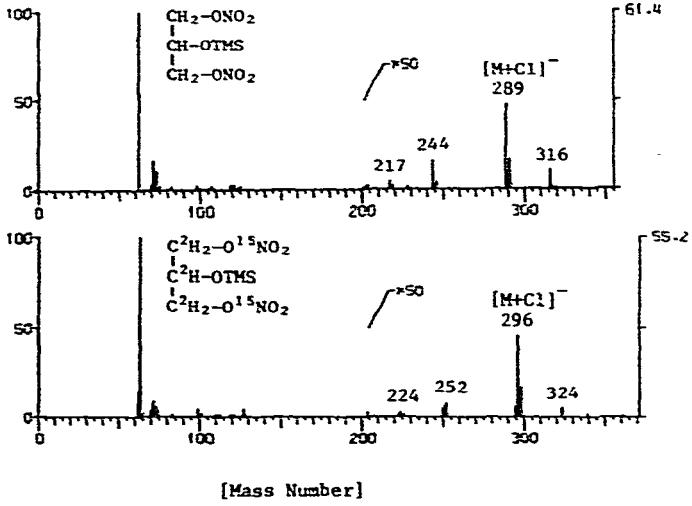

Fig. 3. NICI mass spectra of (A) 1,3-GDN and (B) 1,3-[$^2H_5$,$^{15}N_2$]GDN with dichloromethane as reagent gas.

[$^2H_5$,$^{15}N_3$]GTN and 1,2- and 1,3-[$^2H_5$,$^{15}N_2$]GDNs were synthesized as internal standards for quantitation of GTN and GDNs in plasma by GC–NICI–SIM. As shown in Figs. 1B, 2B and 3B, the NICI mass spectra of these compounds were similar to those of the corresponding non-labelled compounds except for the reasonable mass unit shift in some ions, *i.e.*, the chlorinated molecular ion $[M + Cl]^-$ of [$^2H_5$,$^{15}N_3$]GTN was observed at *m/z* 270 with an 8 mass unit shift from *m/z* 262 in

the non-labelled compound and that of $[^2H_5,^{15}N_2]$GDNs at *m/z* 296 shifted 7 mass units from that of the non-labelled compound at *m/z* 289. The relative abundance of the chlorinated molecular ion cluster agreed well with that calculated from the enrichment of [^{15}N]nitric acid (isotopic purity: 96%), suggesting that no loss of deuterium atom from the carbon–deuterium bond in $[^2H_8]$G took place during nitration. Therefore, the mass spectrometric analysis revealed that the labelled GTN synthesized as an internal standard was a mixture of $[^2H_5,^{15}N_2]$- and $[^2H_5,^{15}N_3]$GTN. Each of labelled 1,2- and 1,3-GDNs was a mixture of $[^2H_5,^{15}N]$- and $[^2H_5,^{15}N_2]$GDN. The isotopical purities of $[^2H_5,^{15}N_3]$GTN and $[^2H_5,^{15}N_2]$GDNs were estimated to be 89.6 and 91.7%, respectively.

To establish the maximum permissible amount of the internal standards added to plasma, an accurate ratio of non-labelled GTN to $[^2H_5,^{15}N_3]$GTN and that of non-labelled GDNs to $[^2H_5,^{15}N_2]$GDNs in them were determined by SIM. The recordings of ion intensities at *m/z* 262 to 270 for GTN and at *m/z* 289 to 296 for GDNs indicated that each of the ratios of non-labelled to labelled compounds was less than 0.1%. This result confirms that it is permissible to add the internal standards to plasma to the extent of 10–50 times the amounts of GTN and GDNs.

When packed columns are used for GTN analysis, GTN should be loaded prior to the analysis in order to prevent losses due to adsorption of GTN on to active sites on the column material[8–10]. On the other hand, the use of capillary columns gave a constant response with ten successive injections of 0.1 ng of GTN without preloading of GTN. This result indicates that capillary columns may have no active sites for adsorption of GTN.

A solid-phase extraction using Extube 1003 made it possible to fractionate effectively GTN and GDNs from the polar metabolites of GTN[1] such as GMNs and glucuronic acid conjugates of GDNs and GMNs. GTN and GDNs adsorbed on the Extube 1003 were eluted with an increase in the polarity of the eluents. Each of the fractions obtained was further purified in order to eliminate interfering substances and to prevent overloading of the capillary column with sample, because the signal-to-noise ratio of quantitation may be affected by coexisting amounts of endogenous

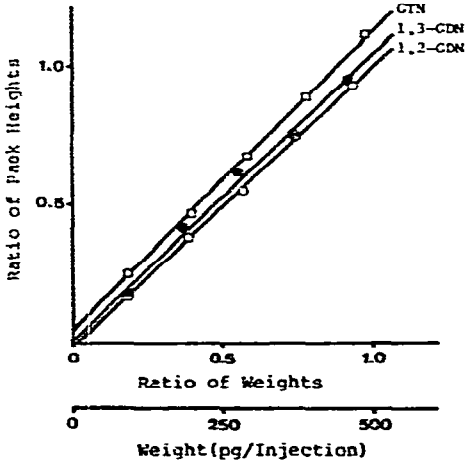

Fig. 4. Calibration graphs for GTN, 1,2-GDN and 1,3-GDN.

substances that have physico-chemical properties similar to those of the compounds of interest.

Fig. 4 shows the calibration graphs for GTN and GDNs using [$^2H_5$,$^{15}N_3$]GTN and [$^2H_5$, $^{15}N_2$]GDNs as internal standards. There were linear relationships between the peak-area ratio and the amount of GTN and GDNs in the range 50–500 pg.

Fig. 5A and B show typical SIRs obtained when 1-μl aliquots of plasma extract containing about (A) 150 pg of GTN and 1 ng of [$^2H_5$,$^{15}N_3$]GTN and (B) 500 pg each of 1,2- and 1,3-GDNs and 2.5 ng each of 1,2- and 1,3-[$^2H_5$, $^{15}N_2$]GDNs were analysed.

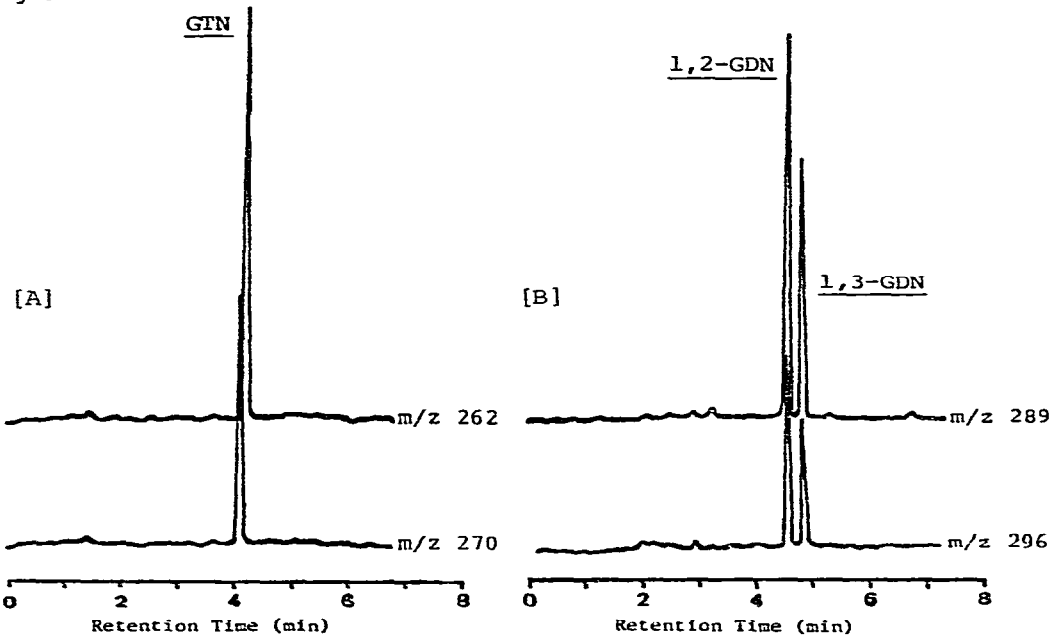

Fig. 5. Selected ion recordings of (A) GTN and (B) GDNs in extract obtained from beagle dog plasma.

Drug-free control plasma gave no interfering peaks at *m/z* 262 and 270 for GTN or at *m/z* 289 and 296 for GDNs, suggesting that the quantitation of GTN and GDNs by this method can be performed without interference from endogenous substances in plasma. Then, known amounts of GTN, 1,2-GDN and 1,3-GDN were added to the drug-free plasma together with the internal standard solution, and then extracted, purified and analysed as described. The analytical data and recoveries are shown in Table I. The recoveries of GTN and GDNs from drug-supplemented plasma were 100.4 ± 0.8% for GTN, 103.1 ± 7.2% for 1,2-GDN and 102.2 ± 8.2% for 1,3-GDN. The data in Table I were submitted to statistical analysis of one-way layout[11,12] in order to divide the total variation into those of sample preparation and measurement of SIM. As shown in the last column in Table I, the estimated standard deviations for sample preparation and SIM in this recovery experiment were calculated to be 0.48 and 0.67 for GTN, 5.73 and 4.60 for 1,2-GDN and 4.96 and 6.90 for 1,3-GDN, respectively. This result indicates that the loss of GTN and GDNs through the process of sample preparation may be compensated for completely by the use of these internal standards.

Fig. 6 shows the plasma level *vs.* time curve observed after intravenous bolus injection of GTN at a dose of 150 μg/kg to three conscious beagle dogs. The plasma

TABLE I

RECOVERY OF GTN AND GDNs FROM DRUG-SUPPLEMENTED DOG PLASMA

Compound	*Recovery (%)*			*Analysis of Variance*	
	X_1	X_2	*Mean ± S.D.*	$\hat{\sigma}_E$*	$\hat{\sigma}_S$**
GTN	101.1	101.9	100.4 ± 0.8	0.48	0.67
	100.2	99.0			
	99.7	100.0			
	100.4	100.6			
	100.9	100.6			
1,2-GDN	113.1	107.9	103.1 ± 7.2	5.73	4.60
	102.2	105.9			
	90.6	97.5			
	98.2	113.6			
	101.8	100.0			
1,3-GDN	109.6	118.9	102.2 ± 8.2	4.96	6.90
	92.0	93.9			
	106.3	97.1			
	98.6	107.0			
	99.8	98.7			

* $\hat{\sigma}_E$ = Estimated value of the standard deviation for the SIM process.
** $\hat{\sigma}_S$ = Estimated value of the standard deviation for the sample preparation process.

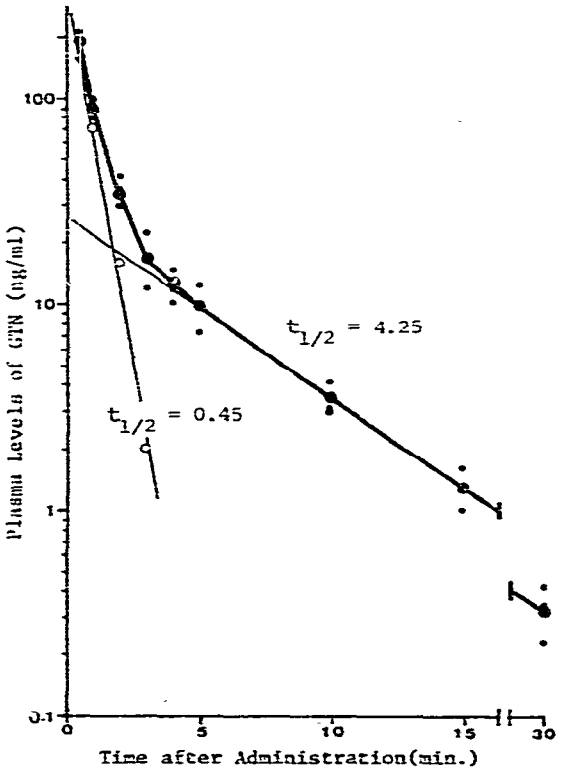

Fig. 6. Plasma GTN levels after intravenous bolus administration of GTN to three conscious beagle dogs at a dose of 150 μg each.

TABLE II

PHARMACOKINETIC PARAMETERS FOR GTN AFTER INTRAVENOUS ADMINISTRATION OF GTN TO THREE CONSCIOUS BEAGLE DOGS AT A DOSE OF 150 μg EACH

α = Elimination rate constant of the alpha phase; β = elimination rate constant of the beta phase; k_{12} = apparent first-order intercompartmental transfer rate constant from the central compartment to the peripheral compartment; k_{21} = apparent first-order intercompartmental transfer rate constant from the peripheral compartment to the central compartment; k_{10} = apparent first-order elimination rate constant from the central compartment; V_1 = apparent volume of the distribution of the central compartment; Cl = clearance.

Patient	α (min^{-1})	β (min^{-1})	k_{12} (min^{-1})	k_{21} (min^{-1})	k_{10} (min^{-1})	V_1 (*l*)	Cl (*l/min*)
A	1.40	0.14	0.53	0.26	0.75	3.63	2.72
B	1.74	0.20	0.46	0.29	1.18	3.37	3.37
C	1.55	0.16	0.60	0.24	0.88	2.99	2.63
Mean	1.56	0.16	0.53	0.26	0.94	3.33	3.11
S.D.	0.17	0.03	0.07	0.03	0.22	0.32	0.75

levels exhibited a bi-exponential decrease and this decay curve was fitted to a two-compartment open model. The pharmacokinetic parameters are listed in Table II. The average and standard deviation of the half-lives of α- and β-phases were 0.45 $\pm$ 0.05 and 4.25 $\pm$ 0.74, respectively. The half-lives of GTN in this study were in good agreement with the results of a pharmacokinetic study on patients receiving controlled hypotension during anaesthesia for surgical operation[1].

Fig. 7 shows the plasma levels of GTN, 1,2-GDN and 1,3-GDN during and after intravenous infusion of GTN (6 μg/kg · min) for 30 min into four anaesthetized beagle dogs. The plasma levels of GTN reached a maximum (57 ng/ml) at the end-point of infusion and decreased rapidly with a bi-exponential decay. The half-lives of the α- and β-phases were 0.50 and 4.95 min, respectively. Constant plasma levels were observed for 45–90 min after withdrawal of the infusion. The half-lives of GTN in intravenous infusion for 30 min were in good agreement with those in intravenous

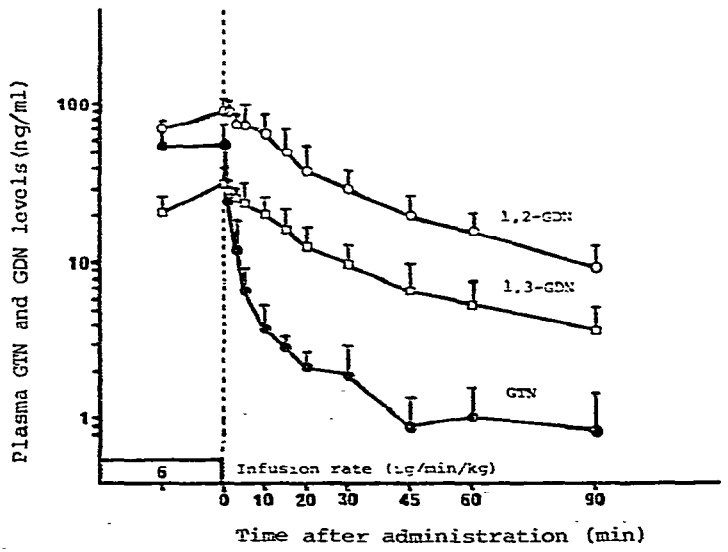

Fig. 7. Plasma levels of GTN and GDNs after intravenous infusion of GTN to four anaesthetized beagle dogs at a dose of 6 μg/kg · min.

bolus injection, suggesting that the GTN plasma levels may decrease at a definite rate regardless of the velocity of administration and/or the administered dose of GTN. The plasma levels of 1,2-GDN and 1,3-GDN also reached a maximum at the end-point of infusion, and the plasma GDN level *vs.* time curves exhibited a bi-exponential decrease. The half-lives of the α- and β-phases were 8.10 and 40.6 min for 1,2-GDN and 8.50 and 48.5 min for 1,3-GDN, respectively.

The relationship between the pharmacokinetics of GTN and GDNs and blood pressure is being investigated, and the results will be reported elsewhere.

ABBREVIATIONS

GTN	Glyceryl trinitrate
1,2-GDN	1,2-Glyceryl dinitrate
1,3-GDN	1,3-Glyceryl dinitrate
GMN	Glyceryl mononitrate
G	Glycerol
TMS	Trimethylsilyl
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CH_2Cl_2	Dichloromethane
CD_2Cl_2	[$^2H_2$]Dichloromethane
GC	Gas chromatography
MS	Mass spectrometry
NI	Negative ion
CI	Chemical ionization
SIM	Selected ion monitoring
SIR	Selected ion recording
WCOT	Wall-coated open tubular
SCOT	Support-coated open tubular
TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography

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* *Editor's Note:* For similar work using HPLC and GC see C. C. Wu, T. D. Sokoloski, A. M. Burkman and L. S. Wu, *J. Chromatogr.*, 216 (1981) 239, and C. C. Wu, T. D. Sokoloski, A. M. Burkman, M. F. Blanford and L. S. Wu, *J. Chromatogr.*, 229 (1982) 327.