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DETERMINATION OF GLYCERYL TRINITRATE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE ION CHEMICAL IONIZATION—SELECTED ION MONITORING

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SUMMARY

A specific, sensitive and accurate quantitation method for glyceryl trinitrate was developed using gas chromatography—negative ion chemical ionization—selected ion monitoring with dichloromethane as a reagent gas. [$^{15}N_3$] and [$^2H_5$, $^{15}N_3$] variants were synthesized from non-labelled or [$^2H_8$]glycerol and [^{15}N]nitric acid. The former variant was used for preventing adsorption of glyceryl trinitrate onto active sites on column materials and the latter was used as an internal standard for quantitation of glyceryl trinitrate in biological fluids by selected ion monitoring. The quantitation limit of this method is 0.1 ng/ml of human plasma. When glyceryl trinitrate was administered intravenously in the dose of 4 μg/kg to patients receiving hypotensive anesthesia for surgical operation, the plasma levels exhibited a biexponential decay. The mean and standard deviation of half-lives of the α and β phases were found to be about 0.41 ± 0.13 and 5.34 ± 1.60 min, respectively.

INTRODUCTION

Glyceryl trinitrate (GTN) has been used effectively to decrease peripheral vascular resistance in patients in heart failure and after acute myocardial infarction. Recently, it has been recognized that GTN is useful as a hypotensive drug during general anesthesia for surgical operation. However, there are no

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reports on the pharmacokinetic data of GTN in patients receiving hypotensive anesthesia with it. Previous studies on the metabolism of GTN in experimental animals using radioisotope technique [1, 2] indicated that the plasma level is extremely low because GTN is rapidly metabolized by endogenous esterases to glyceryl dinitrates, glyceryl mononitrates and glycerol. It is therefore essential to determine exactly plasma GTN levels in patients in order to establish a dose schedule which enables favourable blood pressure during hypotensive anesthesia.

Several analytical methods for the microdetermination of GTN in plasma have been published, in which gas chromatography (GC) with an electron-capture detector [3—5] was used. However, there are no methods that use an internal standard to compensate completely for the losses of GTN during sample preparation.

GC—mass spectrometry (MS) enables the use of an ideal internal standard labelled with stable isotopes. It is known that in the electron impact ionization mode GTN gives rise to NO_2^+ as a base peak [6]. With the positive ion chemical ionization (PICI) mode using various reagent gases, there were no prominent ions reflecting the molecular weight in the high mass region [7]. The use of the above fragment ions for monitoring GTN in biological fluids may be unsuitable because of poor specifity due to lack of information on its molecular weight. On the other hand, there has been only one report [8] that $[M+H]^+$ could be observed in the PICI mode when the temperature of the ion source in a direct inlet system was kept at less than 60°C by a special temperature-control device.

Negative ion chemical ionization (NICI)-MS provides not only enhancement of the sensitivity of the detection limit but also enough information to confirm the molecular weight of the sample in comparison with PICI-MS [9]. Consequently, this technique has been applied to the micro-analysis of compounds with high electron capture [10—12], suggesting that it may be applicable to the determination of trace amounts of GTN in biological fluids. This paper deals with a sensitive and specific method for the quantitation of GTN in human plasma by GC—NICI—SIM using the $[^2H_5, ^{15}N_3]$ variant as an internal standard.

EXPERIMENTAL

Materials

All reagents and solvents used in this study were of analytical grade and were used without further purification.

GTN used as the standard material was obtained by several washings of the medicinal grade with water until nitrate ions, glycerol, glyceryl mononitrates and glyceryl dinitrates were completely removed. Its purity was checked by the high-performance liquid chromatographic method of Crouthamel and Dorsch [13]. $[^{15}N_3]$ and $[^2H_5, ^{15}N_3]$ variants were synthesized in our Explosives Research Laboratory, using $[^2H_8]$glycerol (Merck Sharp & Dohme Canada Ltd., Quebec, Canada) and/or $[^{15}N]$nitric acid (Merck Sharp & Dohme Canada Ltd.). The concentration of GTN and the $[^2H_5, ^{15}N_3]$ variant in ethanol solution (50 ng/ml) was determined according to the procedure described in the Pharmacopeia Japonica (9th edition).

Sephadex LH-20 (25—100 μm) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and silica gel (70—230 mesh) from Merck (Darmstadt, G.F.R.). An Extube® No. 1003 was purchased from Analytichem International (CA, U.S.A.).

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 column was 1.0 m × 2.0 mm I.D. glass, packed with 3% OV-3 (Ohio Valley Speciality Chemical Co., Marietta, OH, U.S.A.) on Gas-Chrom Q 80—100 mesh (Applied Science Labs., State College, PA, U.S.A.). The temperature of the column oven was maintained at 130°C. The temperature of the injection port and separator was 180°C, and the ionization source was kept at 190°C. The flow-rate of helium gas was 30 ml/min. The accelerating voltage was —3.5 kV. The ionization energy and emission current were 500 eV and 500 μA, respectively.

Selected ion monitoring

Prior to the determination of GTN plasma levels, the column was loaded with three 1-μl injections of the [$^{15}N_3$] variant in ethanol solution (1 μg/ml) to saturate all binding sites: then the sample solutions were subjected to selected ion monitoring (SIM).

The peak heights of chlorinated molecular ions $[(M+Cl]^-$ of GTN and the [$^2H_5$, $^{15}N_3$] variant used as an internal standard were monitored at *m/z* 262 and 270. The ratio of the peak heights of these two ions was calculated and compared with a calibration curve to determine the plasma levels.

Administration of GTN

Six patients, average age 52 years (range 36—73 years), received hypotensive anesthesia for surgical operation under the supervision of Drs. K. Hanaoka and K. Nishitatsuno (Department of Anesthesiology, Medical School, Tokyo University, Tokyo, Japan). Hypotension was induced during the operation by bolus injection of GTN aqueous solution (0.5 mg/ml) in the dose of 4 μg/kg. Blood samples were obtained at 0.5, 1, 2, 5, 10 and 15 min after injection of the GTN solution, using a heparinized syringe.

Sample preparation

The blood sample was immediately centrifuged at 1910 *g* for 15 min at 0°C and the plasma was collected. The internal standard solution (40 ng/ml) was added to each 2.0-ml aliquot of plasma. This procedure was carried out in a ice-bath in order to avoid the enzymatic degradation of GTN. The plasma samples were transferred directly onto the Extube No. 1003 (solid-phase extraction tube). After the Extube had been allowed to stand for about 5 min, the GTN was eluted with 25 ml of *n*-hexane. This eluate was transferred to a column (50 × 5 mm) packed with silica gel in *n*-hexane. The column was 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 approximately 0.1—0.2 ml. The residue was redissolved with 0.5 ml of *n*-hexane—chloroform—methanol (10:10:1) and applied to a column (100 × 5 mm) packed with

Sephadex LH-20 in *n*-hexane—chloroform—methanol (10:10:1). The column was washed and eluted with the same solvent described above. The fraction of 4—8 ml was collected and concentrated to approximately 5—10 μl. The residue was redissolved with 30 μl of benzene and an aliquot of this solution was subjected to GC—NICI—SIM.

RESULTS AND DISCUSSION

NICI-MS provides the negative molecular ion yielded by compounds possessing electron-capture ability. Contrary to our expectations, however, when methane, isobutane or ammonia was used as a reagent gas in the NICI mode, GTN gave rise to the nitrate ion $[NO_3]^-$ as a base peak, and there were no negative ions in the high mass region, whereas, when dichloromethane was used as a reagent gas, it has been reported that the chlorinated molecular ion $[M+Cl]^-$ was often observed as a prominent ion [6]. Thus, NICI-MS of GTN using dichloromethane was examined.

Fig. 1A shows the NICI mass spectrum of GTN with dichloromethane as a reagent gas. The chlorinated molecular ion $[M+Cl]^-$ was confirmed by the presence of the doublet ion at *m/z* 262 and 264, due to the characteristic intensities of the chlorine atom. The adduct ion with nitrate ion yielded by the GTN molecule was observed at *m/z* 289. Although it has been reported that the NICI mass spectrum of dichloromethane is characterized by the appearance of Cl^-, HCl_2^- and $CH_2Cl_3^-$ ions [14], the expected $[M + HCl_2]^-$ and $[M + CH_2Cl_3]^-$ ions could not be observed in the spectrum of GTN. The fragment ions ap-

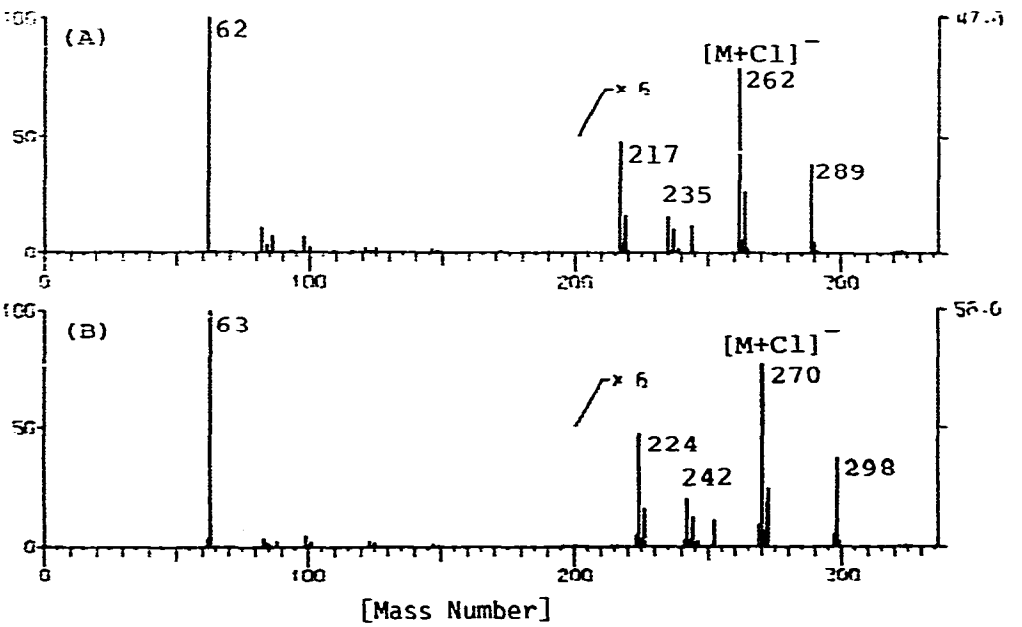

Fig. 1. NICI mass spectra of glyceryl trinitrate (A) and its $[^2H_5, ^{15}N_3]$ variant (B) using dichloromethane as a reagent gas.

pearing at *m/z* 217 and 235 were considered to be produced by the loss of $NOCl_2$ and HNO_3 from the unidentified ion $[M+HCl_2]^-$, because, when $C_2{}^2H_2Cl_2$ was used as a reagent gas, the ion at *m/z* 217 was shifted by one mass unit to *m/z* 218, whereas the ion at *m/z* 235 remained without any 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 the $[^2H_5, {}^{15}N_3]$ variant (Fig. 1B).

On the other hand, it has already been reported [4, 5, 15] that the GC column should be loaded with GTN by several injections prior to analysis, to prevent loss due to adsorption of GTN onto possible active sites on the column materials. However, if the GTN adsorbed to active sites on the column material was labile, it might be released gradually and lead to the appearance of a "memory" peak, resulting in the over-estimation of GTN. In order to clarify this problem, it was necessary to examine whether the adsorbed GTN was released by the subsequent injection of another sample solution containing GTN. Thus, 2 μl of the $[^{15}N_3]$ variant in ethanol (1 μg/ml) used as a tracer were injected three times to saturate all binding sites on the column materials, and then 1 μl of the $[^2H_5, {}^{15}N_3]$ variant in ethanol (50 μl/ml) was injected. Then the release of the $[^{15}N_3]$ variant coated onto the column materials was monitored using the $[M+Cl]^-$ ion of *m/z* 265 in the SIM mode, but there was no corresponding peak by this monitoring. When a mixture of GTN and the $[^2H_5, {}^{15}N_3]$ variant in the ratio 1:2 was injected ten times in succession, it was found that the initial ratio calculated by measuring the peak height of these two peaks was maintained for every subsequent injection, as shown in Table I, indicating that GTN adsorbed onto column materials was not replaced by the following injection of GTN. These results show that preinjection of the $[^{15}N_3]$

TABLE I

REPRODUCIBILITY OF THE PEAK HEIGHT RATIOS IN TEN SUCCESSIVE INJECTIONS OF A 1:2 MIXTURE OF GLYCERYL TRINITRATE AND ITS $[^2H_5, {}^{15}N_3]$ VARIANT

A 1-μl aliquot of the mixture of GTN and its $[^2H_5, {}^{15}N_3]$ variant in ethanol solution (0.5 and 1.0 μl/ml) was injected. Prior to the determination, ion intensities of chlorinated molecular ions $[M+Cl]^-$ of *m/z* 262 and 270, which appeared with an intensity ratio of about 1:2, were made equal by adjusting a gain controller in the multiple-ion detector.

Injection	Ratio of peak heights
1	1.09
2	0.98
3	0.99
4	1.01
5	1.06
6	1.04
7	0.98
8	0.99
9	0.96
10	1.04
Mean ± C.V. (%)	1.01 ± 3.27

variant in ethanol solution may be a useful means of covering the active sites on the column materials.

It is well known that GTN is rapidly degraded enzymatically in plasma [16]. Thus some enzyme inhibitors were added to prevent loss due to the enzymatic degradation of GTN. For instance, Yap et al. [17] reported that this enzymatic degradation of GTN could be inhibited for at least 1.5 h by the addition of silver nitrate in a 0.05% final concentration and the residual amount of GTN after 12 h was found to be 80%. When human blood with a suitable amount of GTN added was allowed to stand at room temperature, GTN was degraded with a half-life of 3.5 h, as shown in Fig. 2. Whereas the degradation of GTN could be inhibited significantly by cooling the blood sample immediately in a ice-bath, practically quantitative recovery of GTN was obtained if fresh plasma was kept below 0°C by immersion in a ice-bath and analyzed within 12 h. Thus, the loss of GTN before the addition of the internal standard solution could be eliminated completely by the above simple treatment.

Solid-phase extraction using an Extube and non-polar organic solvent made it possible to extract GTN from its polar metabolites such as denitration products and their glucuronide conjugates. The recovery of GTN from human plasma by extraction using Extube No. 1003 was more than 95%, whereas the polar metabolites were retained on this column. This method provides not only reproducible and excellent recovery but also simple and rapid extraction and purification of GTN from plasma. The fraction obtained was further purified in order to eliminate interfering substances and to prevent overloading the column with endogenous substances in the sample, since the signal-to-noise ratio may be affected by endogenous substances with physicochemical properties similar to those of the compounds of interest.

The [$^2H_5$, $^{15}N_3$] variant was synthesized for use as an internal standard in the quantitation of GTN in human plasma by GC—NICI—SIM. As shown in Fig. 1B, the NICI mass spectrum of the [$^2H_5$, $^{15}N_3$] variant was similar to that of the non-labelled compound except for the moderate mass unit shift in some ions, i.e. the chlorinated molecular ion was observed at *m/z* 270 with a shift of eight mass units from *m/z* 262 in the non-labelled compound. The relative abundance of the chlorinated molecular ion cluster agreed well with that

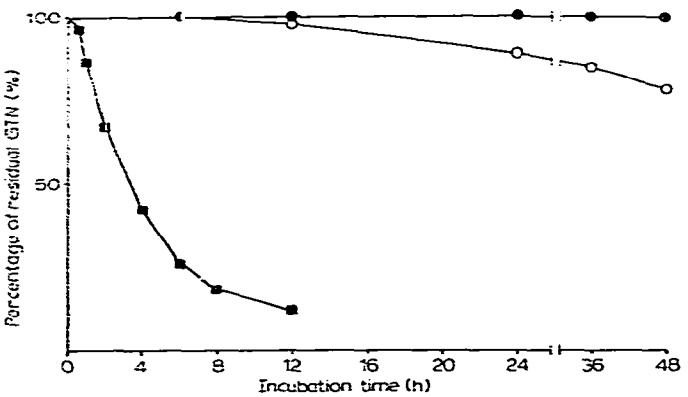

Fig. 2. Stability of glyceryl trinitrate in human plasma at −20°C (•), 0°C (○) and 37°C (▪).

calculated from the enrichment of [^{15}N]nitric acid (isotope purity ≈ 96%), suggesting that no loss of the deuterium atom from the carbon—deuterium bond in [$^{2}H_8$]glycerol occurred during nitration. MS analysis revealed that the labelled compound synthesized as an internal standard was a mixture of the [$^{2}H_5$, $^{15}N_2$] and [$^{2}H_5$, $^{15}N_3$] variants, and the isotopic purity of the [$^{2}H_5$, $^{15}N_3$] variant was estimated to be 89.6%.

To clarify the maximum permissible amount of internal standard that could be added to human plasma, the accurate ratio of non-labelled GTN to the [$^{2}H_5$, $^{15}N_3$] variant in the internal standard was determined by SIM. The recording of ion intensities at *m/z* 262 and 270 indicated that the ratio of non-labelled GTN to the [$^{2}H_5$, $^{15}N_3$] variant was less than 0.1%. This result confirms that it is permissible to add the internal standard to the extent of 10—100 times the amount of GTN in plasma.

The calibration curve constructed for GTN using the [$^{2}H_5$, $^{15}N_3$] variant as an internal standard gave a good linearity in the concentration range 1—20 ng/ml of plasma. Fig. 3 shows a representative selected ion recording obtained when a 1-μl aliquot of plasma extract containing approximately 300 pg of GTN and 1 ng of the [$^{2}H_5$, $^{15}N_3$] variant was analyzed. The retention times of these compounds were approximately 1 min.

Drug-free control plasma showed no interfering peaks at *m/z* 262 and 270, suggesting that the quantitation of GTN by the present method can be performed without interference from endogenous substances in plasma. A known amount of GTN was added to the drug-free plasma together with an aliquot of the internal standard solution and then extracted, purified and analyzed as described in Experimental. The analytical data and recovery are

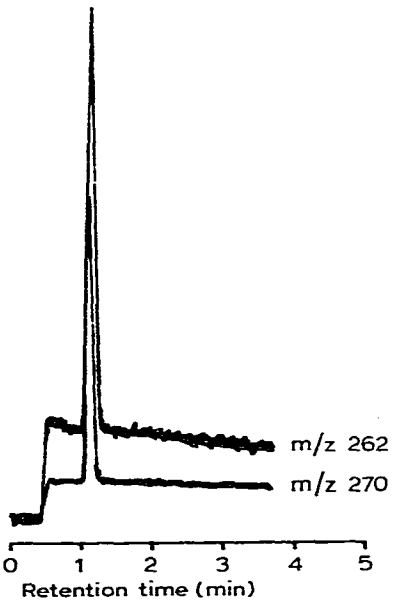

Fig. 3. Selected ion recording of glyceryl trinitrate and its [$^{2}H_5$, $^{15}N_3$] variant extracted from human plasma of a patient receiving hypotensive anesthesia for surgical operation. Apparatus: Shimadzu-LKB 9000A with multiple-ion detector. Column: 3% OV-3 on Chromosorb W HP. Column temperature: 130°C. Reagent gas: dichloromethane.

TABLE II

RECOVERY OF GLYCERYL TRINITRATE FROM DRUG-SUPPLEMENTED HUMAN PLASMA

Added (ng/ml)	Recovery (%)			Analysis of variance*	
	X_1	X_2	Mean ± S.D.	$\bar{\sigma}_E$	$\bar{\sigma}_S$
1.0	98.3	91.5			
	94.1	89.3			
	108.3	106.2	97.9 ± 6.9	3.07	6.34**
	104.1	101.5			
	95.2	91.5			
5.0	106.0	99.7			
	104.1	102.2			
	92.2	97.3	98.8 ± 4.4	3.01	3.42
	98.3	97.1			
	97.6	93.4			

*$\bar{\sigma}_E$ = estimated value of standard deviation for SIM process; $\bar{\sigma}_S$ = estimated value of standard deviation for sample preparation process.
**Statistically significant at the 5% level.

shown in Table II. The recoveries for the two series of drug-supplemented plasma were found to be 97.9 ± 6.9% and 98.8 ± 4.4%, and there were no significant differences in drug level. The analytical data Table II were submitted

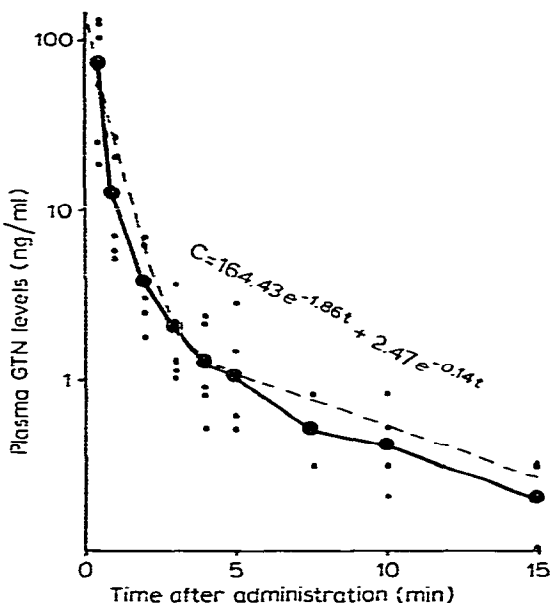

Fig. 4. Average plasma glyceryl trinitrate levels after intravenous administration of a single dose (4 μg/kg) in patients receiving hypotensive anesthesia for surgical operation. (•) Observed value; (•——•) curve for averaged value; (— — —) curve calculated by fitting a two-compartment model to the data.

TABLE III

TWO-COMPARTMENT DISPOSITION CONSTANTS* FOR GLYCERYL TRINITRATE FOLLOWING INTRAVENOUS ADMINISTRATION (4 μg/kg) IN SIX PATIENTS UNDERGOING HYPOTENSIVE ANESTHESIA FOR SURGICAL OPERATION

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^{-1})
A	3.09	0.22	0.58	0.28	2.46	0.60	1.48
B	1.76	0.16	0.23	0.19	1.50	4.68	7.02
C	1.54	0.16	0.18	0.18	1.33	1.65	2.19
D	1.47	0.11	0.43	0.16	0.99	8.44	8.36
E	2.14	0.10	0.10	0.11	2.03	0.62	1.26
F	1.16	0.10	0.22	0.13	0.91	6.78	6.17
Mean	1.86	0.14	0.29	0.18	1.54	3.80	4.41
S.D.	0.68	0.05	0.18	0.06	0.61	3.35	3.13

* α = elimination rate constant of the α phase.
β = elimination rate constant of the β phase.
k_{12} = apparent first-order intercompartment transfer rate constant from the central compartment to the peripheral compartment.
k_{21} = apparent first-order intercompartment 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 in the central compartment.
Cl = clearance.

to the statistical analysis of one-way lay out [18, 19] in order to divide the total variation in this experiment into the variations of the sample preparation and the SIM measurement. As shown in the last column of Table II, the estimated standard deviations for sample preparation and SIM process in this recovery experiment were calculated to be 6.34 and 3.07 at the 1 ng/ml GTN level and 3.42 and 3.01 at the 5 ng/ml GTN level, respectively. This result indicates that the loss of GTN through the process of sample preparation may be compensated for completely by the use of internal standard.

Fig. 4 shows the average of the plasma GTN concentration—time curve observed after intravenous bolus administration of a GTN aqueous solution in the dose of 4 μg/kg to six patients receiving hypotensive anesthesia with GTN for surgical operation. The plasma levels exhibited a biexponential decrease and then the decay curve was fitted in a two-compartment open model. The average and standard deviation of the half-lives of the α and β phases were 0.41 ± 0.13 and 5.34 ± 1.60 min, respectively. Table III lists the pharmacokinetic parameters in the two-compartment model calculated from the above data. These pharmacokinetic data may be useful for the estimation of GTN levels suitable for maintaining a favourable hypotension during hypotensive anesthesia.

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