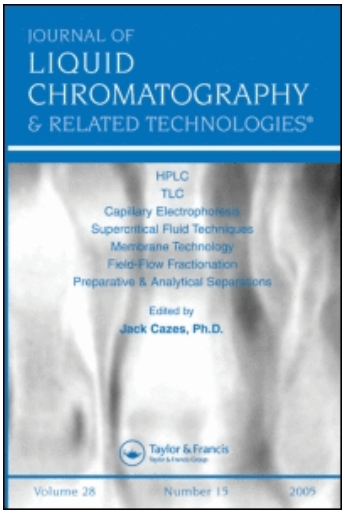


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# Determination of GLG-V-13, a Novel Antiarrhythmic Agent, in Plasma and Urine by High-Performance Liquid Chromatography

C. L. Chen[a]; S. Sangiah[a]; J. D. Roder[a]; H. Chen[a]; K. D. Berlin[b]; G. L. Garrison[b]; B. J. Scherlag[c]; R. Lazzara[c]
[a] Department of Physiological Sciences, Oklahoma State University, Stillwater, Oklahoma [b] Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma [c] Department of Medicine, OHSC, Oklahoma City, Oklahoma

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# DETERMINATION OF GLG-V-13, A NOVEL ANTIARRHYTHMIC AGENT, IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. L. CHEN[1], S. SANGIAH[1], J. D. RODER[1],
H. CHEN[1], K. D. BERLIN[2], G. L. GARRISON[2],
B. J. SCHERLAG[3], AND R. LAZZARA[3]
[1]*Department of Physiological Sciences and* [2]*Department of Chemistry*
*Oklahoma State University*
*Stillwater, Oklahoma 74078*
[3]*Department of Medicine*
*OHSC*
*921 N.E. 13th Street*
*Oklahoma City, Oklahoma 73104*



## ABSTRACT

A sensitive reversed-phase HPLC technique with UV detection has been developed to determine the concentration of GLG-V-13 (3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane dihydroperchlorate) (I), a novel combined class I and class III antiarrhythmic agent, in dog plasma and urine. Alkalinized plasma and urine samples were extracted with chloroform, and the extracts were reconstituted in methanol. An aliquot was injected on to a Waters HPLC system with a 250 x 4.6 mm Ultramex 5 $C_6$ analytical column (5 $\mu$m) and 30 x 4.6 mm Ultramex 5 $C_6$ guard column (5 $\mu$m). The elute was detected by a UV detector at 256 nm. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH6.8 (27:27:46 v/v)

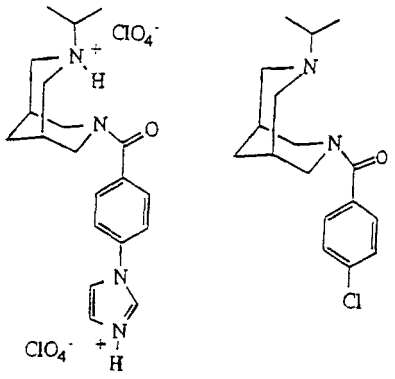
containing 3.6 mM triethylamine was used as the mobile phase. The average extraction recovery was 89% from plasma and 93% from urine. Good lineary ($r>0.999$) was observed throughout the range of 8 - 8000 ng/ml in plasma and in urine with the quantitation limit of 8 ng/ml. Intra- and inter-assay variabilities were less than 4%. HPLC analysis of plasma and urine samples from a dog treated with I has demonstrated that the method was accurate and reproducible. Preliminary pharmacokinetic results showed that the plasma concentration-time curves fitted a two compartment open model with slow elimination ($t_{1/2\beta}$ 3.0827 $h^{-1}$); wide distribution ($V_c$ 2.389 L/kg and $V_{d(ss)}$ 3.6808 L/h·kg); and longer mean residual time (MRT 4.7632 h), respectively. It seems that there is a difference in disposition of this compound in pathological dogs compared to normal one.



## INTRODUCTION

3,7-Diheterobicyclo[3.3.1]nonane (DHBCN) derivatives have been found to show potential antiarrhythmic properties.[1-5] Several DHBCN derivatives exhibited antiarrhythmic activity in animal models and therefore are viable candidates for the treatment of life-threatening disorders in humans who experence sudden heart attacks or major infarctions of the heart.[6-8] GLG-V-13,3-[4-(1H-imidazol-1-yl)benzoyl]-7-isopropyl-3,7-diazabicyclo[3.3.1] nonane dihydroperchlorate (I) (Figure 1), has been demonstrated to increase the ventricle effective refractory period, to prolong QT duration (increase atria Hispurkinje and Hispurkinje-ventricle interval), to prevent sustained ventricular tachycardia induced by programmed electrical stimulation of infarcted dog heart.[9-10] Compound I was found to have combined class I and class III antiarrhythmic activity without hemodynamic depressant effect.[9-10]

The longer duration of pharmacological effects, low proarrhythmic activity, apparent lack of cardiodepressent effects, and combined class Ib and class III antiarrhythmic actions of I make this compound a very promising candidate as an antiarrhythmic agent. Thus, there is merit in characterizing the pharmacokinetics of I in animals. To date, there are no analytical




A B

Figure 1. Chemical Structures of GLG-V-13 (A) and the I.S. (B)



methods currently available for analyzing compound I in biological fluids. We now describe a rapid, selective, and sensitive HPLC technique for the determination of this compound in biological fluids, including plasma and urine. Using this method, preliminary pharmacokinetic profiles of I in dogs were characterized.

## EXPERIMENTAL MATERIALS AND METHODS

### Chemicals

All the reagents used in this study were HPLC grade, and deionized distilled water was used throughout [Milli-Q™ Water system (Millipore Corp., Marlborough, MA)]. Acetonitrile, methanol, chloroform, and potassium phosphate monobasic were obtained from Fisher Chemicals (Fair Lawn, NJ), and Ionate™ triethylamine was purchased from Pierce Chemical Co. (Rockford, IL). Compound I and SAZ-VII-22 [3-(4-chlorobenzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane, II], as an internal standard (I.S.) were synthesized by established methods.[11-12]

**Standard Solution and Internal Standard**

A stock solution of compound I was prepared in methanol at a concentration of 0.4 mg/ml. This stock solution was then diluted further to yield appropriate working solutions for the preparation of the calibration standards.

A stock solution of the I.S. was prepared in methanol (10 μg/ml) for addition to plasma and urine samples.

**Extraction Procedures**

For the determination of I, II was used as I.S. To 250 μl of dog plasma or urine, 25μl of 10 μg/ml of the I.S. was added. After alkalinization with 100 μl of 5 *M* NaOH, five ml of chloroform was added, and the mixture was mixed for 3 min. Following centrifugation (1000 x g 10 min), the organic phase was transferred into a clean test tube. The supernatant was re-extracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream of $N_2$. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution was injected for HPLC analysis.



**Apparatus and Chromatographic Conditions**

The HPLC system consisted of a Waters 501 HPLC pump, Waters U6K universal liquid chromatography injector with a 2 ml injection loop, a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 Chromatography Work Station with a NEC Powermate Sx plus a computer, and an NEC Pinwriter P5200 (Millipore, Milford, MA).

The analytical column used was a 250 x 4.6 mm Ultramex 5 $C_6$ (5 μm) and the guard column was a 30 x 4.6 mm Ultramex 5 $C_6$ (5 μm). Both columns were purchased from Phenomenex (Torrance, CA).

The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH6.8-triethylamine (27:27:46 v/v) containing 3.6 mM triethylamine. The mobile phase was filtered through a 0.5 μm Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow

rate of 1.2 ml/min at ambient temperature. The detection wavelength was 256 nm.

**Extraction Recovery**

The samples (n=5) were prepared to give final concentrations of 100 ng/ml and 4000 ng/ml of compound I and 3 μg/ml of the I.S. in plasma and in urine, respectively. Using the extraction procedure described previously, the samples were extracted in the absence of the I.S. The organic layer was evaporated, and the residues were reconstituted in methanol. The ratio of the peak area of I extracted over that of unextracted equivalent concentrations of agent under identical chromatographic conditions was calculated as extraction recovery.



**Calibration Curves**

A calibration curve was generated to confirm the linear relationship between the peak-area ratio and the concentration of the agent in the samples. Twenty five μl of a suitable standard solution of compound I and 25 μl of the I.S. working solution were added to drug-free plasma and urine to give compound I concentrations at 8, 40, 80, 200, 400, 800, 2000, 4000, and 8000 ng/ml. Plasma and urine samples with known concentrations of I and the I.S. were extracted as previously described, and standard curves were generated by plotting peak-area ratios (agent/I.S.) against drug concentrations tested. Each standard curve was replicated five times. Linear regression analysis of standard curve was performed using the computer program PHARM/PCS. [13] The concentrations of I in unknown plasma and urine samples were calculated by interpolating the peak-height ratios with the calibration curve.

**Intra- and Inter-assay Accuracy and Precision**

To evaluate the intra-assay accuracy and precision, I and the I.S. were added to drug-free plasma and urine samples (n=6) at concentrations of 80

and 4000 ng/ml. These standard samples were prepared and stored at -20°C, and analyzed with the unknown samples. The concentrations were calculated using a standard curve. The percentage of the mean concentration determined over the mean concentration added was taken as the accuracy of the method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined similarly over 6 consecutive days. Precision was estimated by determing the inter-assay coefficient of variations (C.V.).

**Stability of Compound I**

Compound I was added to free-drug dog plasma and urine to a final concentration of 100 and 4000 ng/ml, respectively. An aliquot of plasma or urine was extracted immediately as described above for the determination of compound I, while other aliquots of plasma or urine were stored frozen at -20°C or exposure to light at room temperature (25±2°C). Each month, an aliquot of frozen plasma and urine sample was thawed, extracted and analyzed as described above to evaluate the stability. For the determination of stability of I exposed to light at room temperature, a one day experimental duration was performed (one sample /two hour interval).



**Drug Administration and Sampling**

One experiment was carried out 24-96 hours after two-stage ligation of the left anterior descending coronary artery in an anesthetized beagle dog. Programmed premature stimuli or rapid intermittent 3-beat-bursts (240-420/min) were delivered to the right ventricle to induce sustained monomorphic ventricular tachycardia. After administration of 3 mg/kg of compound I via an i.v. bolus injection, blood samples (± 5 ml) were collected via vein puncture at 5, 10, 15, 30, 45, 60, 75, 90 and 120 min. Another experiment was carried out on a normal unanesthetized beagle dog with an intravenous bolus injection of 6 mg/kg of I with blood samples (± 5 ml) being

collected up to 12 hr. The samples were heparinized and centrifuged at 20000 x g for 10 min. The plasma fractions were stored at -20°C until analyzed. Urine was collected by catheter and stored at -20°C until analyzed.

## RESULTS AND DISCUSSION

### Extraction Efficiency

Alkalinization of plasma and urine samples increased extraction efficiency with the pH value for the solution at 12 showing the best extraction recovery. Most of compound I was in a nonionized state at pH value of 12. The use of chloroform to precipitate proteins and to extract compound I directly from plasma offered marked advantage since fewer peaks were found for contaminants. Using trichloroacetic acid (TCA) to precipitate proteins decreased the absolute recovery. This may be due to decomposition of I. This situation was similar to that of some other DHBCN derivatives. [14-16] Anticoagulators, such as EDTA and heparin, did not affect the extraction recovery. Extraction recoveries of compound I were 87.5-91.2% from plasma and 92.2-93.6% from urine, respectively (Table 1). The recoveries of the I.S. were 85% from plasma and 95% from urine.

### Chromatographic Separation

Several combinations of acetonitrile, methanol, buffer (with different pH) and triethylamine were evaluated as possible mobile phases. It was determined that the combination described herein was found to be the most suitable for separating I. Under the described chromatographic conditions, a good separation of compound I and its I.S. was achived. The retention times were 11.40±0.44 and 19.07±0.73 min, respectively. At the retention time, the compound I and its I.S. were eluted without an interference peak from the blank plasma and urine (Figures 2 and 3). Varying proportions of

TABLE 1.
Recovery of Compound 1 added to dog plasma and urine (Mean±SD, n = 5)

| | Added (ng/ml) | Found (ng/ml) | Recovery (%) |
|---|---|---|---|
| Plasma | | | |
| | 100 | 87.3±4.5 | 87.3±4.5 |
| | 4000 | 36480±1320 | 91.2±3.6 |
| Urine | | | |
| | 100 | 92.2±5.3 | 92.2±5.3 |
| | 4000 | 37420±1800 | 93.6±4.5 |



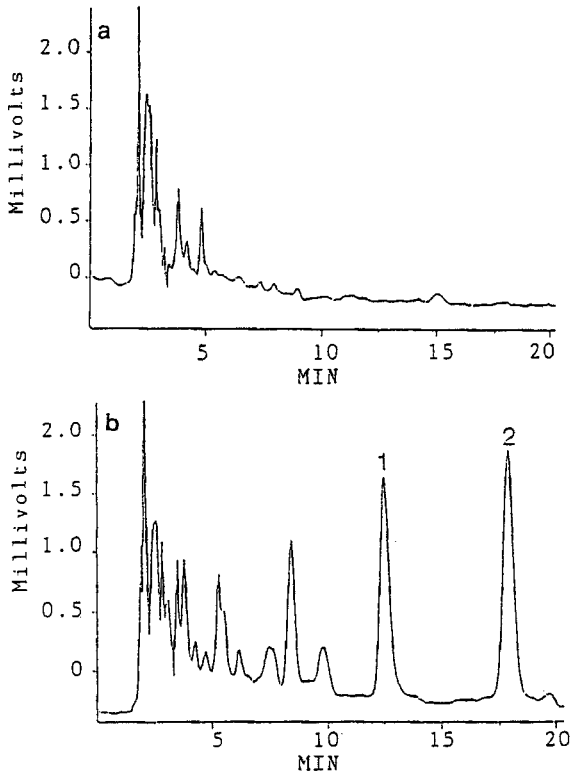



Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 8 hr after i.v. dose of 6 mg/kg of I. See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 0.27 μg/ml.

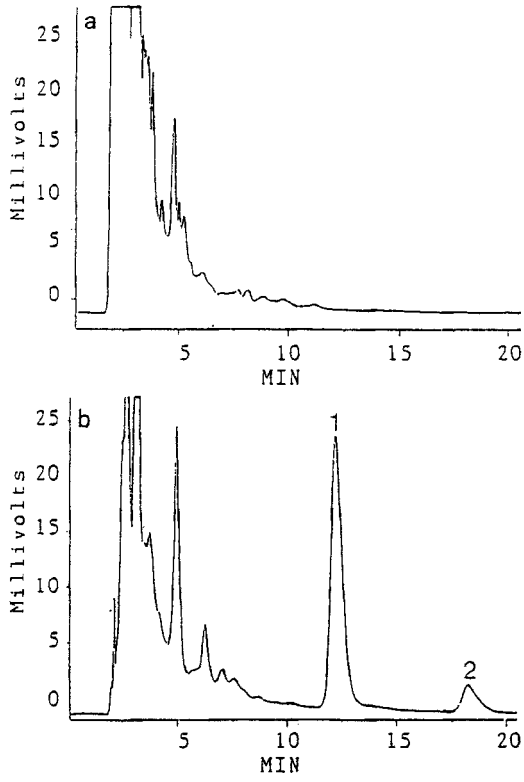



Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after i.v. dose of 6 mg/kg of I (b). See experimental for chromatographic conditions.
Peaks: 1 = I, 2 = I.S.
The estimated concentration of I was 5.1 μg/ml.

triethylamine in the mobile phase changed both retention time and sharpness of the peak for compound I. The pH of the mobile phase is a very important factor influencing the elution of I. If the mobile phase pH was below 6.8, the retention time for I was shorter. There was a concomitant decrease in sensitivity. Neither acetonitrile nor methanol alone was suitable as the strong solvent. These observations were very similar to that with other DHBCN

derivatives. [14-16] Therefore, varying ratio of methanol-acetonitrile-triethylamine-phosphate buffer may be the best combination for separating and analyzing DHBCN derivatives in biological fluids.

## Assay Validation

**Linearity.** Five consecutive standard curves for pure I analyzed on separate days demonstrated a good linear relationship between concentration and peak area. The standard curves obtained from extraction of both dog plasma and urine containing known amounts of I were linear over the concentration ranges tested (8-8000 ng/ml). The range of coefficient of variations was between 1-17%. The calibration curves were found to be linear and could be described by the regression equations: $Y = -0.01492 + 0.5127X$ ($r = 0.9996$) for plasma and $Y = -0.04057 + 0.6557X$ ($r = 0.9995$) for urine, respectively, in which Y was the agent recovered in $\mu$g/ml, and X was peak area ratio (agent/internal standard). The limits of quantitation of I were 8 ng/ml in plasma and in urine, respectively. This sensitivity has proved useful in the analysis of pharmacokinetic data of dog plasma and urine after administration of compound I.

**Precision and Accuracy.** The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine were less than 4%. The accuracy of this method was 94-99% (Table 2). These results suggest that the procedures described as above are satisfactory with respect to both accuracy and precision.

## Stability of Compound I

It was found that the free amine of compound I turned yellow when exposed to light for 2-3 hr, while compound I (disalt) was not sensitive to light. This experimental observation showed that I was very stable in dog plasma and urine at -20°C for at least 6 months and was also stable when exposed to light at room temperature (25±2°C) for one day. Possibly compound I may be present as the disalt or monosalt in the biological fluid.

TABLE 2.
Intra-assay (Within-day) and Inter-assay (Between-day) Precision and Accuracy of the Determination of Compound I in Dog Plasma and Urine

| Concentration (ng/ml) | | Accuracy (%) | C.V. (%) |
|---|---|---|---|
| Added | Found | | |
| Plasma | | | |
| Intra-assay (n = 6) | | | |
| 80 | 76.5 | 95.6 | 1.65 |
| 4000 | 3728 | 93.2 | 2.26 |
| Inter-assay (n = 6) | | | |
| 80 | 79.2 | 96.8 | 3.00 |
| 4000 | 3810 | 95.2 | 2.29 |
| Urine | | | |
| Intra-assay (n = 6) | | | |
| 80 | 76.0 | 95.0 | 3.14 |
| 4000 | 3780 | 94.5 | 1.52 |
| Inter-assay (n = 6) | | | |
| 80 | 77.6 | 97.0 | 3.53 |
| 4000 | 3960 | 99.0 | 1.38 |

* C.V. = coefficient of variance



**Preliminary Pharmacokinetic Studies**

The plasma and urine samples were extracted and analyzed as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (6 mg/kg) intravenously are shown in Figure 2 and 3.

The plasma concentration-time profiles of I in dogs given intravenous dose of 3 and 6 mg/kg are shown in Figure 4. Data fitting and

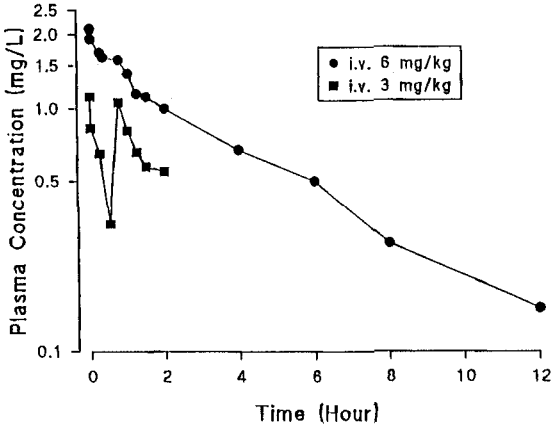



Figure 4. Plasma concentration profiles after i.v. dose of 6 and 3 mg/kg of compound I in a normal dog and in a pathological dog, respectively.



pharmacokinetic parameters calculations were carried out using the Boomer program. [17] The plasma concentration change can be best described as two compartment model with equation: $C = A_1e^{-\alpha t} + A_2e^{-\beta t}$. The calculated pharmacokinetic parameters after iv bolus injection of 6 mg/kg was showed in Table 3. The results show that the compound I has very low elimination rate with $t_{1/2\beta}$ 3.0827 $h^{-1}$ and longer mean residual time (MRT 4.7632 h), which is consistent with the duration of pharmacological effects. It was interesting that the disposition of I was different in the beagle dog under normal and pathological conditions. In a pathological dog, plasma concentration of I durimg 45-60 min was higher than that of 20-30 min. This may be due to the enterohepatic circulation. The exact mechanism is unknown.

The results showed that the HPLC method described above has a lower quantitation limit of 8 ng/ml using a 250 $\mu$l sample. As shown in this report, this method is suitable for pharmacokinetic studies of this novel antiarrhythmic agent. Studies of the pharmacokinetic and metabolite(s) profiles of I in animals are in progress.

TABLE 3.
Pharmacokinetic Parameters Following iv Bolus 6 mg/kg of I to One Dog

| Parameters | Value |
|---|---|
| iv bolus (6 mg/kg) | (n = 1) |
| A1 (mg/L) | 0.844 |
| A2 (mg/L) | 1.669 |
| $\alpha$ ($h^{-1}$) | 6.754 |
| ß ($h^{-1}$) | 0.2248 |
| $K_{10}$ ($h^{-1}$) | 0.3326 |
| $K_{12}$ ($h^{-1}$) | 2.086 |
| $K_{21}$ ($h^{-1}$) | 4.575 |
| $t_{1/2\alpha}$ (h) | 0.1026 |
| $t_{1/2ß}$ (h) | 3.083 |
| $V_c$ (L/kg) | 2.389 |
| $V_{d(area)}$ (L/kg) | 3.437 |
| $V_{d(ss)}$ (L/kg) | 3.681 |
| $Cl_B$ (ml/h/kg) | 0.7727 |
| $AUC_{iv}$ (mg·h/L) | 7.765 |
| $AUMC_{iv}$ (mg/L) | 36.984 |
| $MRT_{iv}$ (h) | 4.763 |

*Abbriviations:* $K_{10}$-first-order elimination rate constant; $K_{12}$, $K_{21}$ are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissues); $t_{1/2\alpha}$ is distribution half-life after iv; $t_{1/2ß}$ is elimination half-life after iv; $V_c$ = volume of the central compartment; $V_{d(area)}$ is apparent volume distribution calculated using AUC; Vd(ss) is apparant volume of distribution at stead state; $Cl_B$ is body clearance of the drug.



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