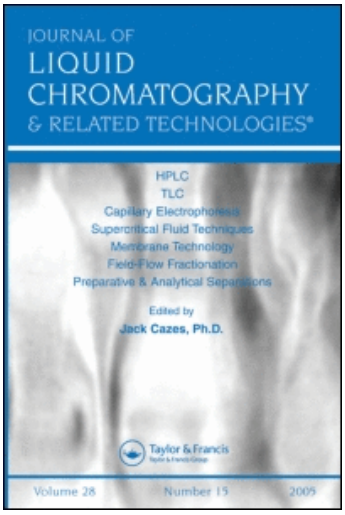


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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(17), 3681-3694 (1994)

# SIMULTANEOUS DETERMINATION OF A NOVEL ANTIARRHYTHMIC AGENT, 7-BENZYL-3-THIA-7-AZABICYCLO[3.3.1]NONANE AND ITS SULFOXIDATION METABOLITE IN PLASMA AND URINE BY HPLC

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## ABSTRACT

A HPLC method was developed for the simultaneous determination of the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) as the hydrochloride and the corresponding sulfoxide (II), the major metabolite, in dog plasma and urine. Plasma and urine samples were alkalinized and extracted with chloroform. An aliquot was injected on to a HPLC system with a C6 reversed-phase column and an UV detector. Acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine was used as a mobile phase. The compounds I and II were detected at 261 nm. The extraction recovery for I and II was 85% and 94% from plasma and 89% and 91% from urine, respectively. Good linearity ($r>0.994$) was

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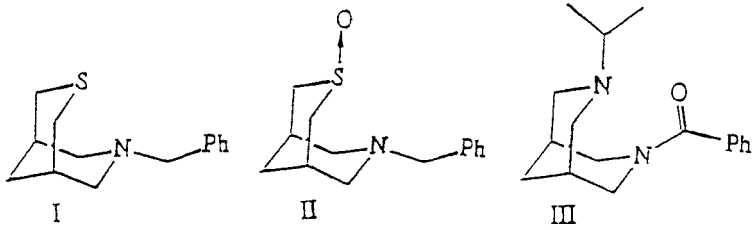
observed throughout the range of 0.1-10.0 μg/ml for I and 0.04-10 μg/ml for II in plasma and in urine. Intra- and inter-assay variabilities were less than 8%. The accuracy of this method was > 95% for both compounds, and the limits of quantitation were 0.08 μg/ml for I and 0.03 μg/ml for II in plasma, and in urine, respectively. This method was applied to determine plasma and urine concentrations of I and II simultaneously in a dog treated with I.

## INTRODUCTION

7-Benzyl-3-thia-7-azabicyclo[3.3.1]nonane (I) (Figure 1) has been demonstrated to have effective antiarrhythmic properties[1-4] and less proarrhythmic activity than those of lidocaine in dog models.[5] This drug could be classified as class Ib antiarrhythmic drug.[6,7] The significant elevation of systemic arterial blood pressure at the effective antiarrhythmic dose of I during sinus rhythm in dogs[2,4,5] could possibly be due to its inhibitory effects on myocardial $Na^+$,$K^+$-ATPase and $Mg^{2+}$-ATPase activities and its positive inotropic effects on atrial and papillary muscles.[8] Reduced proarrhythmic effects and little cardiac depressant actions make this compound a very promising candidate as an antiarrhythmic agent.

A HPLC method for determining the concentrations of 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane·$HClO_4$ in biological fluids has been developed.[9] The pharmacokinetics and plasma protein binding of I·$HClO_4$ in rats[10] and of I·HCl in dogs[11] have been characterized. In dogs, parent compound I·HCl was found to be extensively metabolized to form a major metabolite, 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide (II)[12] (Figure 1). However, no analytical methods are currently available to analyze II in biological fluids.




I II III

Figure 1. Chemical structure of I, II and III
I = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane
II = 7-benzyl-3-thia-7-azabicyclo[3.3.1]nonane-3-oxide
III = 3-benzyol-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane



This paper describes a reversed-phase HPLC method for simultaneous determination of I and its major metabolite II in dog plasma and urine.

## EXPERIMENTAL MATERIALS AND METHODS

### Chemicals

The compounds I and II were synthesized by established methods.[1,13] All solvents used in this study were HPLC grade. All water used was purified through a Milli-Q™ water system (Millipore Corp., Marlborough, MA). Acetonitrile, methanol, chloroform, potassium phosphate monobasic (Fisher Chemicals, Fair Lawn, NJ), and triethylamine (Pierce Chemical Co. Rockford, IL) were used in this study. ß-Glucuronidase crude solution (G 0876) from *Helix pomatia* was purchased from Sigma Co. (St. Louis, MO). 3-Benzoyl-7-isopropyl-3,7-diazabicyclo[3.3.1]nonane (III) was synthesized via a type of Mannich reaction starting from *N*-benzyl-4-piperidinone[14] and used as internal standard (I.S.).

## Stock Solutions and Standards

Methanol stock solutions of I, II (1 mg/ml) and the I.S. (10 μg/ml) were stable for at least 2 months at 2-4°C. Known amounts of the solution were added to 0.25 ml of drug-free plasma and 0.50 ml of drug-free urine to obtain calibration curves in the range of 0.1-10 μg/ml for I and 0.04-10 μg/ml for II. Several standards were prepared for each curve.

## HPLC Analysis

The HPLC system consisted of a Waters 501 HPLC pump, a 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 a NEC Pinwriter P5200 (Millipore, Milford, MA). A 250 x 4.6 mm Ultramex 5 $C_6$ (5 μm) and 30 x 4.6 mm Ultramex 5 $C_6$ guard column (5 μm) were purchased from Phenomenex (Torrance, CA). The mobile phase was acetonitrile-methanol-37.5 m*M* phosphate buffer, pH 6.8 (28:28:44 v/v) containing 4.0 m*M* triethylamine. The mobile phase was filtered through a 0.5 um 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 261 nm for I and II.

## Animal Study

An adult, male mongrel dog (10.5 kg) was pre-conditioned for 7 days and was housed in a controlled environment (12-hour light/12-hour dark

photoperiod, 22±1°C, 60±10% relative humidity). The dog was allowed free access to food and water. Twenty-four hours before the experiments, food was withdrawn. The dog had free access to water before and during the experiments. The dog had polyethylene cannulas implanted in the right and left cephalic veins for collection of blood samples. The experiment was initiated between 0900 and 1100 h. Compound I was dissolved in water immediately before each experiment, and a dose of 20 mg/kg was administrated orally. Blood samples (ca. 5 ml) were collected via the cephalic vein catheter at 0, 5, 10, 30, 45 min and at 1, 2, 4, 6, 8, 12 hr after administration of I. After each sampling, the lost blood was replaced with an equal volume of sterile heparinized saline. Blood samples were heparinized, and centrifuged at 2000 x g for 10 min. Urine samples were collected from the dogs housed in a stainless steel metabolism cage at designated times, 0-2, 2-24, 24-48, 48-72 hr, via a urinary catheter. Both plasma and urine samples were stored at -20°C. Aliquots of 0.25 ml of plasma and of 0.5 ml urine were used for HPLC assay.



**Extraction of I and II from Dog Plasma**

Twenty five μl of 10 μg/ml of internal standard (III) were added to 250 μl of dog plasma. After alkalinization with 100 μl of 5 *M* NaOH, 5 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 reextracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream

of $N_2$. The solid residue was reconstituted in 50 $\mu$l of methanol, and 35 $\mu$l of this solution was utilized for HPLC analysis.

**Extraction of Total (Free + Conjugated) I and II from Dog Urine**

A crude solution of ß-glucuronidase (100,000 units of ß-glucuronidase and 1000-5000 units of sulfatase per ml) was used to hydrolyze the glucuronidate and sulfate of I and II. For the determination of total (free plus conjugated) I and II, aliquots of 0.5 ml of dog urine samples were subjected to enzymatic hydrolysis prior to extraction. The urine sample were adjusted to pH 5 with acetic acid, to which 0.1 ml of ß-glucuronidase crude solution was added, and then the solution was incubated at 37°C for 24 hr. The hydrolysate solution was adjusted to pH ±12 with NaOH (2 *M*) and then extracted with chloroform to yield total I and II.



**Extraction Recovery**

The samples (n = 5) were prepared to give final concentrations of 0.2 and 4 $\mu$g/ml in plasma and urine, respectively. Using the extraction procedure cited above, the samples were extracted in the absence of III (I.S). The ratio of the peak area of I and II extracted to that of unextracted equivalent concentrations of drugs under identical chromatographic conditions was calculated as extraction recovery.

**Pharmacokinetic Analysis**

Pharmacokinetic modelling and parameters were performed by PharmK program.[15]

## RESULTS AND DISCUSSION

### Extraction Efficiency

The same procedures described previously for extraction of I from biological fluids (9) was used to extract simultaneously I and II from plasma and urine. Alkalinization of plasma and urine samples increases extraction efficiency. The use of chloroform to precipitate proteins and to extract compounds I and II directly from plasma offered great advantage in that fewer pollutant peaks were found. Anticoagulants, such as EDTA and heparin, do not affect the extraction recovery. Extraction recoveries of compound I were 79-92% from plasma and 78-95% from urine and of compound II, 80-103% from plasma and 85-101% from urine, respectively (Table 1). The recoveries of I.S. were 85% from plasma and 90% from urine.



### Chromatographic Separation

A modified chromatographic separation conditions[9] for I was used to separate I, II, and III (I.S). A minor change in the combinations of acetonitrile, methanol, buffer and triethylamine were evaluated as possible mobile phases. It was determined that the combination described in the method was found to be the most suitable for separating I and II. Under the described chromatographic conditions, a good separation of compound I, II and its internal standard was achieved. The retention times were $5.1 \pm 0.3$ and $10.3 \pm 0.6$ min for I and I.S., respectively. We were still unable to get a stable retention time for I,[9] but the retention time for I was determined to be 14-19 min. With these retention times, the drug I, metabolite II, and its I.S. were

TABLE 1
Linearity and recovery of compound I and II added to plasma and urine

| Samples | Range ($\mu$g/ml) | n | Linear Correlation[a] | r | CV[b] (%) | m[c] |
|---|---|---|---|---|---|---|
| I in plasma | 0.1-10 | 35 | Y= 0.05484+11.461X | 0.995 | 1.2-10.1 | 85 |
| I in urine | 0.1-10 | 35 | Y= 0.11655+5.5174X | 0.994 | 3.5-9.7 | 89 |
| II in plasma | 0.04-10 | 35 | Y=-0.19556+3.1716X | 0.997 | 2.8-11.2 | 94 |
| II in urine | 0.04-10 | 35 | Y=-0.10697+3.0815X | 0.995 | 1.8-9.7 | 91 |

[a] Y was drug recovered in $\mu$g/ml, X was peak area ratio (drug/I.S.).
[b] CV = coefficient of variance
[c] m = mean recovery



eluted without interference peak originating in blank plasma and urine (Figure 2 and 3). The effects of varying proportions of triethylamine and pH value in the mobile phase on separation were similar to that in the analysis of I and other 3,7-diheterobicyclo[3.3.1]nonane analogues. [9,16-18] The effect of these variables were not only on the retention time but also on the sharpness of the peaks for compounds I and II. Like other DHBCN (3,7-diheterobicyclo-[3.3.1]nonane) analogues,[9,16-18] neither acetonitrile nor methanol alone is suitable as a strong solvent for the separation of I and II. It was found that minor changes in the combination of acetonitrile, methonal, triethylamine and buffer (pH 6.8) could provide a very good mobile phase for analyzing several DHBCN analogues.[9,16-18]

## Assay Validation

**Linearity.** Five consecutive standard curves for pure I and II analyzed on separate days demonstrated a good linear relationship between

concentration and peak area. The standard curves obtained from extraction of dog plasma and urine containing known amounts of I were linear ($r>0.996$) over the concentration ranges tested. The range of coefficient of variations was between 1-12%. The calibration curves were found to be linear (Table 1). The limits of quantitation of I and II were 80 ng/ml and 30 ng/ml for plasma and for urine, respectively. This sensitivity is adequate for use in the analysis of pharmacokinetics data of dog plasma and urine after administration of compound I or II.

**Precision and accuracy**. The results obtained indicate that intra- and inter-assay coefficient of variance (C.V.) in plasma and urine was less than 8%. The accuracy of this method was 89-97%. These results suggest that the proposed procedure is satisfactory with respect to both accuracy and precision.



## Applications to Dog Samples

The internal standard was added to the dog plasma and urine samples, and samples were extracted as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (20 mg/kg) orally are shown in Figures 2 and 3.

The plasma concentration-time profiles of I and II in one dog given an oral dose of 20 mg/kg are shown in Figure 4 and are fitted to a one compartment open model. There were 3.22% and 15.6% of I and II excreted into urine with respect to the oral dose. The area under the curve for II is 1.77 times of that of I, indicating that I undergoes extensive metabolism. A recent drug metabolism study[12] showed that I is present in the free state, while

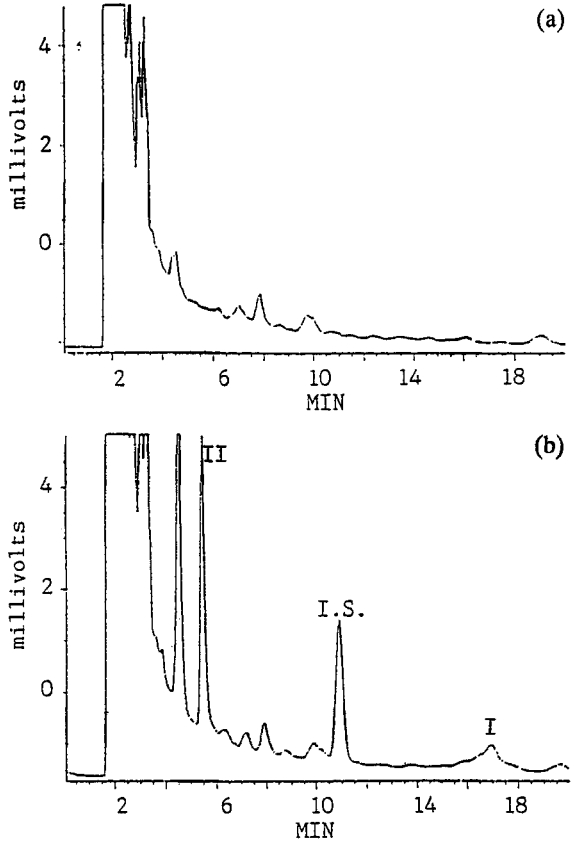



Figure 2. Representative chromatograms from (a) blank plasma and (b) plasma sample 30 min after oral dose of 20 mg/kg of I to a dog. See experimental for chromatographic conditions.
The estimated concentration of I and II was 1.2 μg/ml and 3.5 μg/ml, respectively. I.S. = Internal Standard

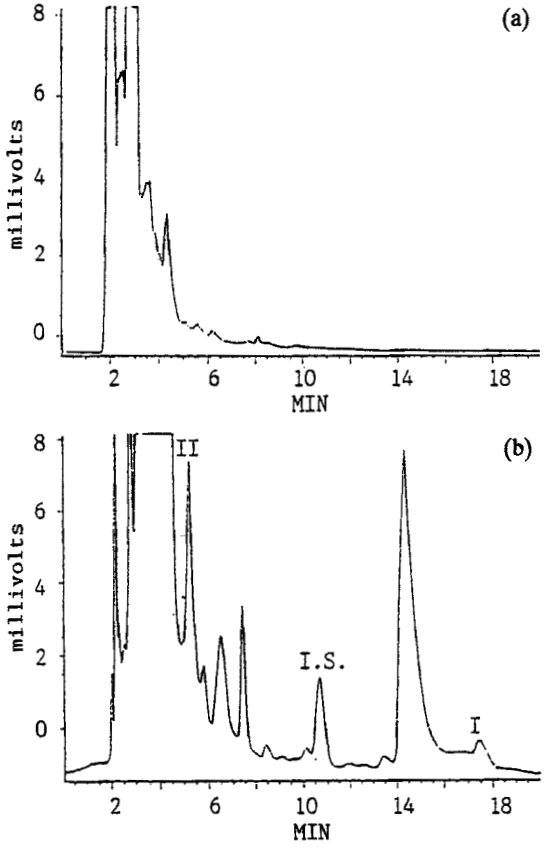



Figure 3. Representative chromatograms from (a) blank urine and (b) urine sample 1 hr after oral dose of 20 mg/kg of I to a dog. See experimental for chromatographic conditions. The estimated concentration (free + conjugated) of I and II was 3.1 μg/ml and 4.2 μg/ml, respectively. I.S. = Internal Standard

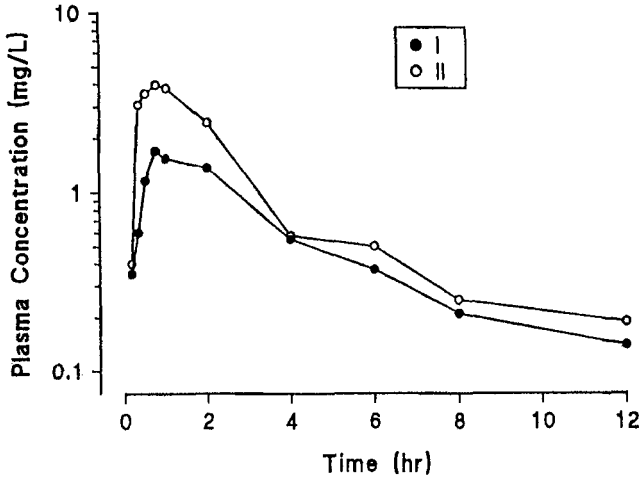



Figure 4. Plasma I and II concentration profile after oral dose of 20 mg/kg in one dog.



II is predominantly present in the form of glucuronide and/or sulfate conjugates in urine.

The results show that the HPLC method described above has a lower quantitation limit of 0.08 μg/ml for I and 0.03 μg/ml for II using a sample volume of 250 μl. As shown in this report, this method is suitable for simultaneous pharmacokinetic studies of this novel antiarrhythmic agent and its S-oxidation metabolite II. In addition, this method can also be used to isolate II and quantitate it from the biological samples containing only II.

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