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DETERMINATION OF PROPAFENONE IN BIOLOGICAL FLUIDS BY FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY USING ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas–liquid chromatographic method with electron-capture detection using a capillary column with the inlet in the splitless injection mode is reported for the assay of propafenone. A 25 m×0.31 mm cross-linked, 5% phenylmethylsilicone-coated fused-silica capillary column was employed for all analyses. The present method provides improved selectivity and sensitivity over other existing gas chromatographic and high-performance liquid chromatographic (HPLC) methods. Linearity was observed in the ranges 2.5–50 and 10–100 ng/ml. The coefficient of variation was found to be less than 10% over the concentration ranges studied. Application of the developed method is demon-

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strated by measuring serum propafenone concentrations over 24 h in a normal healthy volunteer after a single oral dose of propafenone and by measuring trough plasma propafenone concentrations at steady state in patients receiving this new antiarrhythmic drug. Validity of the present method is further demonstrated by comparison of analytical results obtained from measurement of patient samples using a modified published HPLC method.

INTRODUCTION

Propafenone, 2′-[2-hydroxy-3-(propylamino)propoxy]-3-phenylpropriophenone, is a potent, well tolerated and orally effective new antiarrhythmic drug with demonstrated effectiveness against a variety of cardiac arrhythmias, including supraventricular and ventricular tachyarrhythmias [1–4]. Its structure and pharmacokinetic characteristics resemble propranolol. Propafenone is almost completely absorbed from the gastrointestinal tract and undergoes extensive first-pass metabolism. The major metabolites of propafenone are 5-hydroxypropafenone and 5-hydroxy-4-methoxypropafenone [5–7]. The metabolite 5-hydroxypropafenone has been found to be pharmacologically active in animal studies [8] and accumulates in the plasma during chronic oral propafenone therapy in patients with frequent ventricular ectopy [9].

Several high-performance liquid chromatographic (HPLC) methods [10–14] and a gas chromatographic (GC) method [15] for the analysis of propafenone have been published. The HPLC techniques [10–13] required a relatively large sample volume (ca. 1–5 ml) and the limit of determination was ca. 5–20 ng/ml. In 1984, Brode et al. [14] published an HPLC method with fluorescence detection with a limit of determination for propafenone as low as 1 ng/ml in 1 ml of human plasma. The GC method [15], using packed-column technology, showed greater sensitivity than the early HPLC methods [10–13] and allowed the determination of 10 ng/ml drug.

With the use of capillary columns for improved separation and peak symmetry, the efficiency and sensitivity of GC techniques increase significantly when combined with the application of the splitless injection technique. The purpose of this study, therefore, was to develop a GC method with electron-capture detection (ECD) for the trace level analysis of propafenone in small plasma volumes.

EXPERIMENTAL

Materials

Propafenone hydrochloride and Li-1115 hydrochloride, internal standard (I.S.), were supplied by Knoll Pharmaceuticals Canada (Markham, Canada). Pesticide-grade toluene (distilled in glass) was purchased from Caledon Labs. (Georgetown, Canada). Triethylamine (TEA) (sequanal grade) and heptafluorobutyric anhydride (HFBA) were purchased from Pierce (Rockford, IL, U.S.A.). Solutions of 1 and 5 *M* sodium hydroxide and 1 *M* hydrochloric acid were prepared from ACS reagent-grade chemicals (American Scientific and Chemical, Seattle, WA, U.S.A.). Distilled water was used in the preparation of stock solutions and during dilutions and extraction.

Instrumentation and chromatographic conditions

A Model 5830A Hewlett-Packard (HP) Reporting gas chromatograph, equipped with a Model 18835B capillary inlet system, an electron-capture detector, a Model 18850A GC terminal for peak integration and a Model 7671A automatic sampler, was used. A bonded-phase fused-silica capillary column, 25 m × 0.31 mm I.D., was used for all analyses (stationary phase, 5% phenylmethylsilicone, cross-linked; film thickness, 0.52 μm; phase ratio, 150; Hewlett-Packard, Palo Alto, CA, U.S.A.). A splitless injection mode was used, employing a fused-silica inlet liner with a small plug of silanized glass-wool 3 cm from the column end. Thermogreen LB-2 septa, low-bleed septa at high inlet temperature, were used and changed routinely to prevent leakage as a result of repeated puncturing of the septum during automatic sampling.

The operating conditions for routine GC analysis were: injection port temperature, 210°C; electron-capture detector temperature, 350°C; initial column temperature, 220°C; temperature programming rate, 4°C/min; final column temperature 270°C; carrier gas (high-purity hydrogen) flow-rate, 1 ml/min; septum purge flow-rate, 2.5 ml/min; make-up gas (argon–methane, 95:5) flow-rate, 60 ml/min.

Stock solutions

Propafenone hydrochloride (0.1 mg/ml, equivalent to base) and I.S. (0.1 mg/ml, equivalent to base) were prepared by dissolving these compounds in water. The solutions were further diluted to the desired concentrations. The stock and diluted solutions were stored at 4°C after preparation for up to two months.

Extraction and derivatization

Blank human plasma (0.3 ml) was spiked with a volume (0.025–1.0 ml) of propafenone solution (0.1 μg/ml) and 0.35 ml of I.S. (0.1 or 0.2 μg/ml). The mixture was adjusted to a total volume of 2.0 ml with water and then alkalinized with 0.5 ml of 1 *M* sodium hydroxide solution (pH 12). Benzene (6 ml) was added and, after mixing for 20 min and centrifugation, the benzene layer was transferred to a clean glass tube, to which 2 ml of 1 *M* hydrochloric acid were added. The solution was mixed for 20 min and, after centrifugation, the benzene layer was aspirated to waste. The aqueous layer was washed twice with 4 ml of benzene. Sodium hydroxide solution (0.5 ml of 5 *M*) was added to the aqueous layer followed by 6 ml of benzene. The solution was mixed for 20 min and, after centrifugation, the benzene layer was transferred to a clean glass tube and was evaporated to dryness under nitrogen at 40°C in a water bath. Samples were then reconstituted with 0.4 ml of toluene containing 0.003 *M* TEA. A 50-μl volume of HFBA was added and the sample was mixed and incubated at 65°C for 15 min. After cooling to room temperature, the excess derivatizing agent was removed by evaporation of the sample at 35°C under nitrogen. The residue was reconstituted with 0.8–1.4 ml of toluene and an aliquot of 2 μl was injected into the gas chromatograph under the conditions described.

Quantitative analysis

A calibration curve for propafenone was obtained by plotting the peak area ratio of propafenone derivative to the I.S. derivative versus the known amount of

propafenone added to the biological sample. The best fit of the calibration curve data points was obtained by linear regression and the coefficient of variation was calculated. Volunteer and patient samples were spiked with the same amount of internal standard and were subjected to the same extraction and derivatization procedure described previously. The peak-area ratios were calculated and the unknown propafenone concentrations of volunteer and patient samples were determined daily from calibration curves. Day-to-day variability was determined by spiking blank plasma with propafenone. Samples with an equal concentration of propafenone were stored in tightly capped vials at −20°C for within-run and between-run analysis of the coefficient of variation of the method.

Gas chromatography–mass spectrometry

Gas chromatographic–mass spectrometric (GC–MS) data analyses were obtained using an HP Model 5987A GC–liquid chromatographic–MS system. The data were processed by a Series 1000E HP computer and displayed on an HP Model 2623A terminal. The column used and the GC operating conditions were the same as mentioned previously. Methane was used as reagent gas. The temperature of the GC–MS interface was 270°C. The MS operating conditions were: electron ionization energy 70, 110 and 130 eV for electron-impact (EI), positive-ion chemical-ionization (PICI) and negative-ion chemical-ionization (NICI) MS, respectively; emission current 0.3 mA; ion source temperature 240°C.

High-performance liquid chromatography

HPLC analyses were performed on an HP Model 1090 liquid chromatograph equipped with a diode array UV detector and a Model 310 HP computer for data analysis. In the present study, a minor modification of the HPLC propafenone measurement method of Harapat and Kates [10] was employed. The mobile phase used was acetonitrile–0.005 *M* phosphate buffer pH 2.9 (38:62) instead of acetonitrile–0.005 *M* phosphate buffer pH 2.4 (25:75) to reduce analysis time and to improve chromatographic peak shape.

Human experiments

Patient samples were obtained during the course of an efficacy study of the effectiveness of propafenone in the treatment of atrial fibrillation and in the treatment of supraventricular tachycardia. Trough plasma samples were drawn on a treatment day immediately prior to the next daily dose. Subjects were screened for multiple drug use and all samples were examined for evidence of multiple drug usage.

A male Caucasian subject, aged 23 years, weight 74 kg, served as the healthy volunteer in the study of the kinetics of propafenone after a single oral dose. The volunteer had no history of cardiac or liver disease and had a normal physical examination and normal electrocardiographic and biochemical/hematological laboratory results at the time of the study. The subject was not taking other medications and alcohol consumption was moderate. Alcohol- and caffeine-containing beverages were not allowed for 48 h prior to and during the study. The subject was fasted overnight and had no breakfast on the day of study. A 300-mg tablet

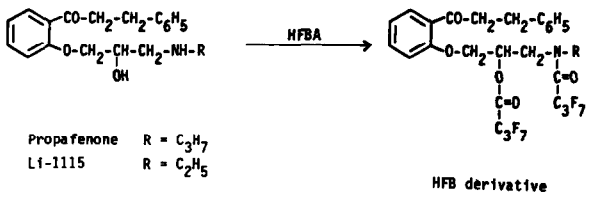


Fig. 1. Derivatization of propafenone and Li-1115 to yield the HFB derivative of propafenone and Li-1115, respectively.

of propafenone was taken with 200 ml of water. The subject sat in a chair 1 h before and 45 min after drug administration to minimize alterations in hepatic blood flow caused by body movement. The contents of the meal were controlled, again to decrease the influence of this factor on propafenone metabolism since these investigators have shown that propafenone kinetics are significantly influenced by a meal [16]. Blood samples were drawn before (0 h) and 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16 and 24 h after drug administration. Samples were stored at $-20\,^{\circ}C$ until analyzed.

RESULTS AND DISCUSSION

Marchesini et al. [15] reported in 1982 a GC–ECD assay method for propafenone using a packed column. A GC–ECD assay method developed in our laboratory demonstrated improved selectivity and sensitivity through the use of fused-silica capillary columns and the splitless injection technique. The sensitivity was further improved by the use of HFBA as an alternative to trifluoroacetic anhydride (TFAA) as the acylating agent (HFBA yielded an increase in sensitivity over TFAA of approximately five-fold in this instance). Fig. 1 shows the structure of propafenone and I.S. and the proposed reaction with HFBA to yield the respective heptafluorobutyryl (HFB) derivatives. Fig. 2A and B shows representative gas chromatograms from the extracts of blank plasma and blank plasma spiked with propafenone and I.S. Fig. 2C shows the chromatogram of a patient's plasma sample spiked with I.S. The HFB derivative of propafenone was completely resolved from the HFB derivative of the I.S. No interfering peaks were observed from endogenous plasma components of the plasma extract (Fig. 2A) and an analysis time of less than 15 min was obtained.

The acylation reaction time was substantially shortened by the use of trimethylamine as a catalyst [17,18]. However, because of the troublesome preparation and instability of trimethylamine, TEA was used. The necessity of using TEA and the optimum reaction time was examined by incubating two sets of samples containing equivalent amounts of propafenone and I.S. for various times (0.5, 1, 2, 3 and 4 h) at $65\,^{\circ}C$, with TEA added to only one set. No significant difference was observed in the peak area of propafenone derivative or the peak-area ratio of propafenone derivative to the internal standard derivative between 0.5 and 4 h when TEA was used, indicating the reaction was maximal in 0.5 h. A further study with incubation times of 0, 5, 15, 30, 45 and 60 min showed that the reaction was, in fact, complete in 15 min, permitting the use of a shorter incu-

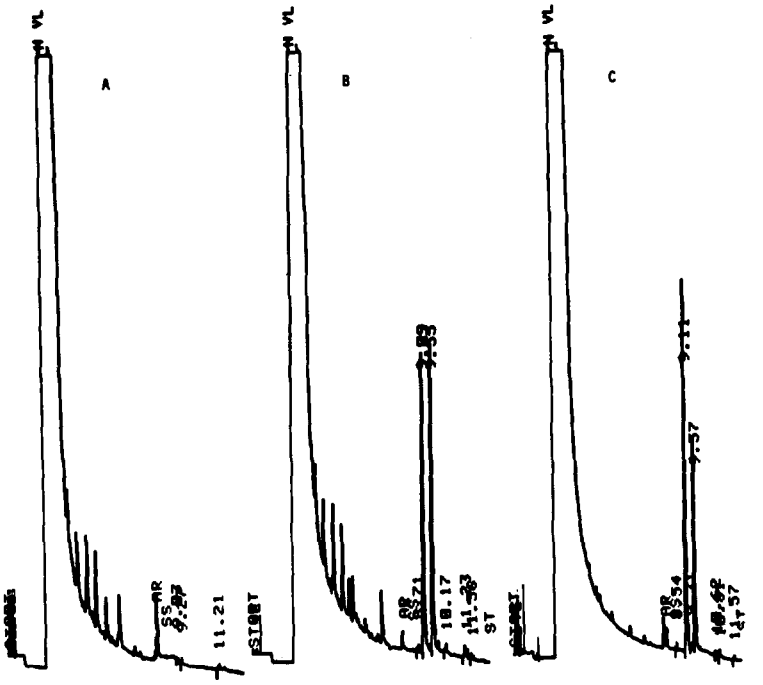


Fig. 2. Chromatograms of extracts from (A) blank plasma, (B) blank plasma spiked with propafenone (0.08 μg, retention time 9.55 min) and Li-1115 (0.07 μg, retention time 9.09 min, internal standard) and (C) sample from patient receiving propafenone spiked with Li-1115 (0.07 μg).

bation time. The amount of TEA required for the reaction was also evaluated. Although 50 μl of 0.05 *M* TEA was recommended [17,18], only half of that amount was used in our analysis to reduce the solvent front band-spreading. This was accomplished without affecting the peak area of propafenone and its internal standard and the area ratio.

The optimal extraction solvent was tested by adding identical concentrations of propafenone to blank plasma and subsequent extraction with four different solvents, viz., toluene, benzene, hexane and a combination of toluene–dichloromethane–propanol-2 (7:3:1). After extraction, an identical concentration of I.S. in methanol was added to the above extracted samples and also to a sample that contained the same concentration of propafenone in methanol (not subjected to extraction). The samples were dried under nitrogen and then derivatized. The peak-area ratio was determined by comparing the peak area of the propafenone derivative to the peak area of the I.S. derivative. The extraction efficiency (E) of these solvents was evaluated by comparing the area ratios of the extracted samples to the unextracted sample with the latter set as 1.0. Benzene was found to provide optimal extraction for propafenone ($E=0.81$), followed by toluene ($E=0.76$). Neither the more non-polar solvent hexane ($E=0.48$) nor the slightly more polar solvent combination of toluene–dichloromethane–propanol-2 (7:3:1) ($E=0.45$) gave satisfactory results. In addition to the slightly higher extraction efficiency, benzene also reduced

TABLE I

EXTRACTABILITY OF PROPAFENONE

Number of samples, $n=3$ (two injections for each sample).

Propafenone added (ng/ml)	Propafenone measured (Mean ± 1S.D.) (ng/ml)	Recovery (mean ± 1S.D.) (%)
10	8.9 ± 1.6	89.0 ± 16.0
20	18.5 ± 1.0	92.5 ± 5.0
40	37.1 ± 1.7	92.8 ± 4.2
60	54.4 ± 0.7	90.7 ± 1.1
80	70.7 ± 3.7	88.4 ± 4.6
100	84.7 ± 3.9	84.7 ± 3.9

extensively the time for sample evaporation during the analytical procedure. This is an important time-saving advantage, due to the large number of samples to be analyzed by this method during pharmacokinetic studies of propafenone.

Propafenone hydrochloride was dissolved in water and methanol, respectively, to make up solutions of equal concentrations (0.1 μg/ml). Various volumes (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml) of these solutions were transferred into two sets of glass tubes, respectively. To the set of propafenone hydrochloride samples in water was added human plasma followed by extraction with benzene. The salt of the I.S. was dissolved in methanol to yield a concentration of 0.2 μg/ml, and 0.35 ml of this solution were added to both sets of samples. The two sets of samples were then subjected to the same derivatization reaction. The amounts of propafenone extracted from the aqueous solutions by benzene were calculated from the standard curve of propafenone hydrochloride in methanol and compared with the actual amount of propafenone added. The recovery of propafenone was approximately 90% over the concentration range 10–100 ng/ml (Table I). Although benzene was used as the extraction solvent, toluene was used as the derivatizing and injecting solvent because of its higher boiling point (lower volatility). This has the advantage of considerably reducing the sample discrimination encountered in the splitless sampling mode [19].

Excess HFBA reagent was removed by evaporation rather than by the hydrolysis technique [18]. The hydrolysis of excess derivatizing agent and subsequent neutralization with ammonium hydroxide solution has been recommended and widely used. However, it is not applicable for the HFB derivative of propafenone as ammonium hydroxide causes a rapid decomposition of the HFB ester (HFB acylation of the hydroxyl group) by basic hydrolysis. Although the use of a phosphate buffer (pH 6.0) is also recommended for the removal of excess HFBA to insure the stability of the HFB ester [20], evaporation of the derivative mixture was used in our procedure and was carried out under a gentle stream of nitrogen in a 35 °C water bath.

Derivatized samples have been found to be stable for at least one week when stored at −4 °C with repeat injections showing no significant decline in absolute peak areas over the time period of storage.

TABLE II

CALIBRATION CURVE DATA FOR PATIENT AND VOLUNTEER PLASMA SAMPLES

Number of samples, $n=2$ (two injections for each sample).

Concentration of propafenone (ng/ml)	Peak-area ratio propafenone/Li-1115 (mean ± 1S.D.)	Coefficient of variation (%)
Patient plasma samples		
10.1	0.191 ± 0.002	1.1
20.3	0.349 ± 0.003	0.9
40.5	0.610 ± 0.002	0.4
60.8	0.866 ± 0.006	0.7
81.0	1.131 ± 0.029	2.5
101.3	1.334 ± 0.016	1.2
Statistics: linear regression, $y=12.593x+0.088$; $r=0.999$		
Volunteer plasma samples		
2.6	0.097 ± 0.002	1.8
5.1	0.171 ± 0.001	0.2
10.2	0.313 ± 0.027	8.7
20.4	0.581 ± 0.005	0.9
30.6	0.827 ± 0.004	0.5
40.8	1.003 ± 0.014	1.4
51.0	1.335 ± 0.042	3.2
Statistics: linear regression, $y=24.747x+0.050$; $r=0.998$		

The data for a representative calibration curve used in the quantitation of volunteer and patient samples are presented in Table II. Linearity was observed over the concentration ranges studied (2.5–50 and 10–100 ng/ml). The best fit through the data points was obtained by linear regression and was described by $y=12.593x+0.088$ with a correlation coefficient (r) of 0.999 and $y=24.747x+0.050$ with a correlation coefficient of 0.998, respectively. The coefficient of variation was less than 10% over the concentration ranges studied.

The day-to-day variability test shows that the coefficient of variation was 0.5–4.5% and 4.5% for within-run (repeatability) and between-run (reproducibility) precision, respectively. Fig. 3 shows the plasma concentration–time curve obtained after administration of a single oral dose (300 mg) of propafenone to a healthy human volunteer. Table III shows the data of trough plasma propafenone concentrations at steady state in patients receiving this new antiarrhythmic drug for control of cardiac dysrhythmia. Table III also permits comparison of the results of propafenone plasma concentrations analyzed by our present GC method and a modification of the HPLC method reported by Harapat and Kates [10]. Statistical analysis (paired t-test, level of significance, 0.05) indicates that there is no significant difference between the results obtained using these two independent methods of propafenone measurement.

Figs. 4–6 provide the GC–MS results and fragmentation ions of the HFB deriv-

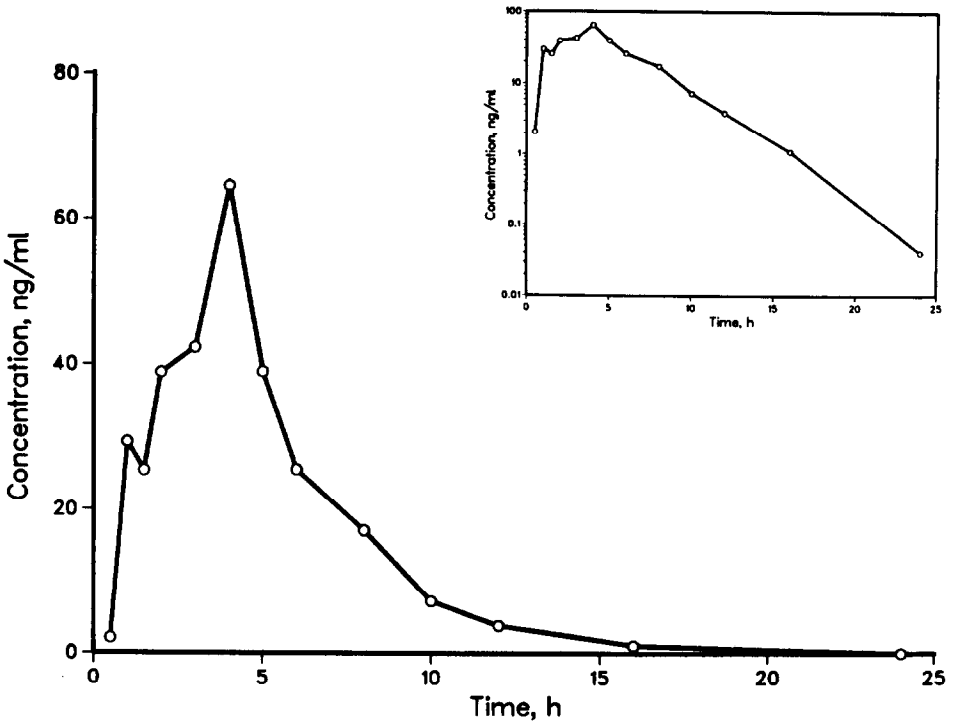


Fig. 3. Representative plasma concentration–time curve from a human volunteer administered a 300-mg oral dose of propafenone. Inset: semi-logarithmic plot of the plasma concentration–time curve in the same subject.

ative of propafenone with EI, PICI and NICI as the ionization modes, respectively. In order of decreasing intensity, the prominent fragment ions for EI were at *m/e* 91, 121, 508, 43, 104, 252, 226, 294, for PICI were at *m/e* 508, 294, 252 and for NICI were at *m/e* 488, 213, 693, 194, 673, 448, 653. The EI-MS fragmentation of the HFB derivative of propafenone in the present study followed a pattern similar to the MS fragmentation of trifluoroacetylated propafenone reported by Hege et al. [21]. Compared to EI-MS, PICI-MS fragmentation of the HFB derivative of propafenone has fewer fragment ions. In addition to the pronounced $(M+1)^+$ ion, characteristic ions of $(M+C_2H_5)^+$ and $(M+C_3H_5)^+$ were also found when methane was used as an ionizing gas. The fragment ions at *m/e* 508, 294 and 252 appeared in both EI-MS and PICI-MS profiles. The NICI-MS fragmentation of the HFB derivative of propafenone in the present study followed a pattern similar to that of HFB derivative of metoprolol and oxprenolol reported by Gaudry and co-workers [22,23]. Structurally, metoprolol and oxprenolol have side-chains that have the same molecular formulae as propafenone (C_3H_7–NH–CH_2–CH(OH)–CH_2–, +2HFB=508), and the fragment ions at *m/e* 488 (508−HF) and 448 (508−3HF) were the common ions in the NICI mass spectra of these three compounds. Other common fragment ions observed were at *m/e* 213 (C_3F_7COO–) and 194 (213−F). The $(M-1)^-$ ion (*m/e* 732) was characteristic of NICI-MS. The molecular ion lost a progressively increasing

TABLE III

TROUGH PLASMA PROPAFENONE CONCENTRATION AT STEADY STATE IN PATIENTS RECEIVING PROPAFENONE

Patient No.	Concentration measured (mean ± 1S.D.) (ng/ml)	
	GC*	HPLC**
1	175.6 ± 7.7	-
2	696.7 ± 6.1	738.2 ± 53.4
3	737.5 ± 7.7	820.2 ± 5.1
4	5.3 ± 0.4	-
5	190.8 ± 10.6	-
6	554.3 ± 84.4	-
7	217.9 ± 6.7	-
8	131.4 ± 6.0	-
9	179.4 ± 6.8	-
10	476.7 ± 23.5	482.1 ± 1.5
11	356.4 ± 1.7	-
12	373.6 ± 8.7	385.0 ± 2.3
13	303.3 ± 2.2	328.7 ± 10.2
14	444.9 ± 2.1	365.6 ± 77.3
15	612.9 ± 7.2	608.8 ± 78.1
16	633.8 ± 17.5	719.9 ± 12.9
17	487.3 ± 35.8	604.5 ± 5.8
18	28.7 ± 0.4	-
19	119.2 ± 6.6	-
20	1740.5 ± 31.9	1873.8 ± 8.5

*Number of samples (n) = 2 (two injections for each sample).
**Number of samples (n) = 1 (two injections for each sample). The method is a slight modification of the HPLC method of Harapat and Kates [10].

number of HF fragments to yield ions at m/e 693 (733 − 2HF), 673 (733 − 3HF) and 653 (733 − 4HF).

CONCLUSIONS

The developed method for the analysis of propafenone is the first GC–ECD assay method for this drug using capillary columns and a splitless injection technique. The improved selectivity and sensitivity of the GC–ECD assay makes it possible for us to measure plasma propafenone concentrations using small plasma volumes (0.01–1.0 ml for patient samples and 0.1–2.0 ml for single-dose pharmacokinetic studies of drug interactions with this drug). The reliability and reproducibility of this new method permit the routine analysis of propafenone during the course of pharmacokinetic experimentation. Furthermore, the sensitivity of the method should be sufficient to make it possible to conduct plasma protein binding experiments using this technique. Such binding and drug–drug interaction experiments are currently underway in our laboratory using the present method.

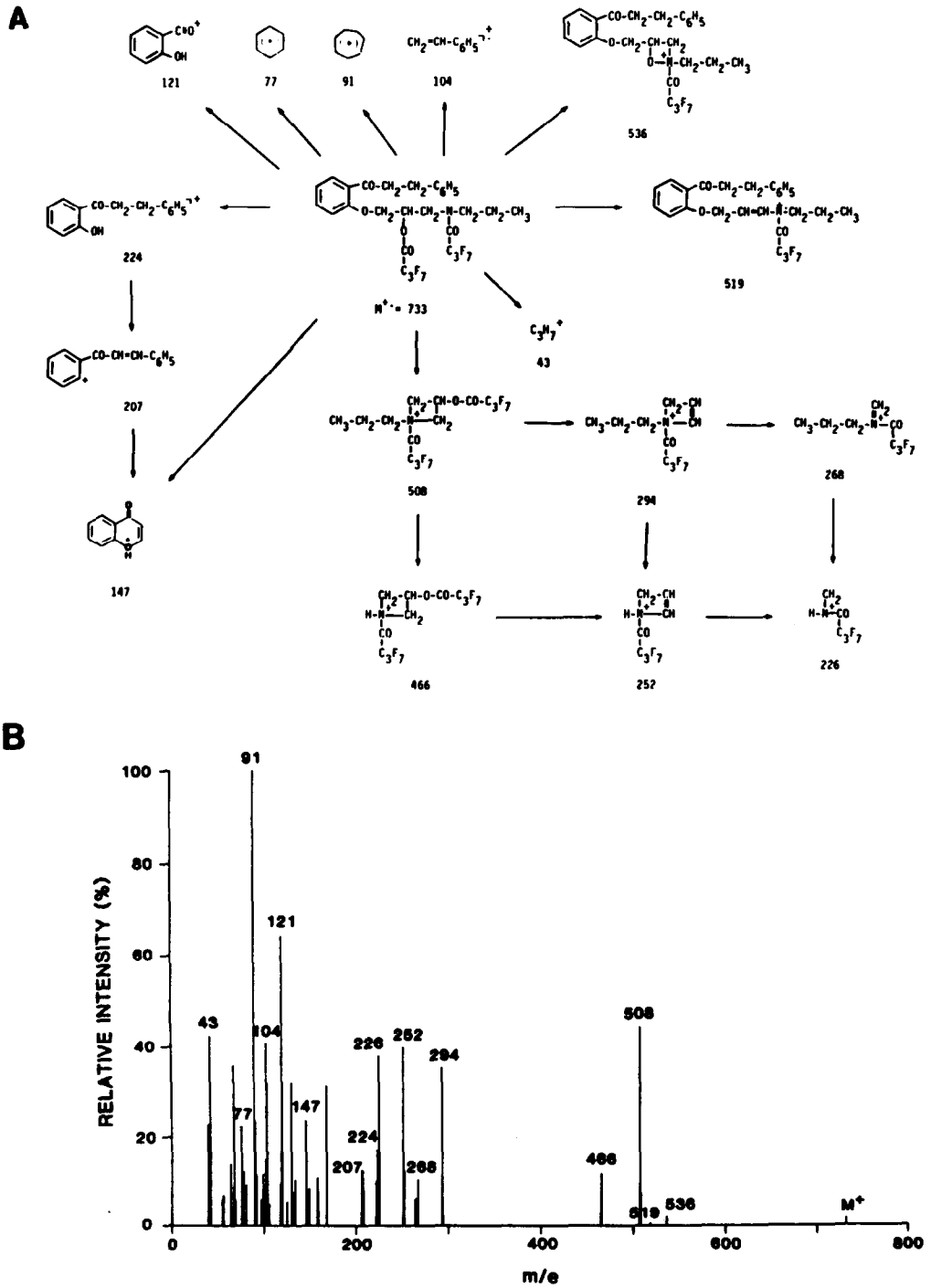


Fig. 4. GC-EI-MS results: (A) a proposed fragmentation pattern of the HFB derivative of propafenone; (B) EI mass spectrum of the HFB derivative of propafenone.

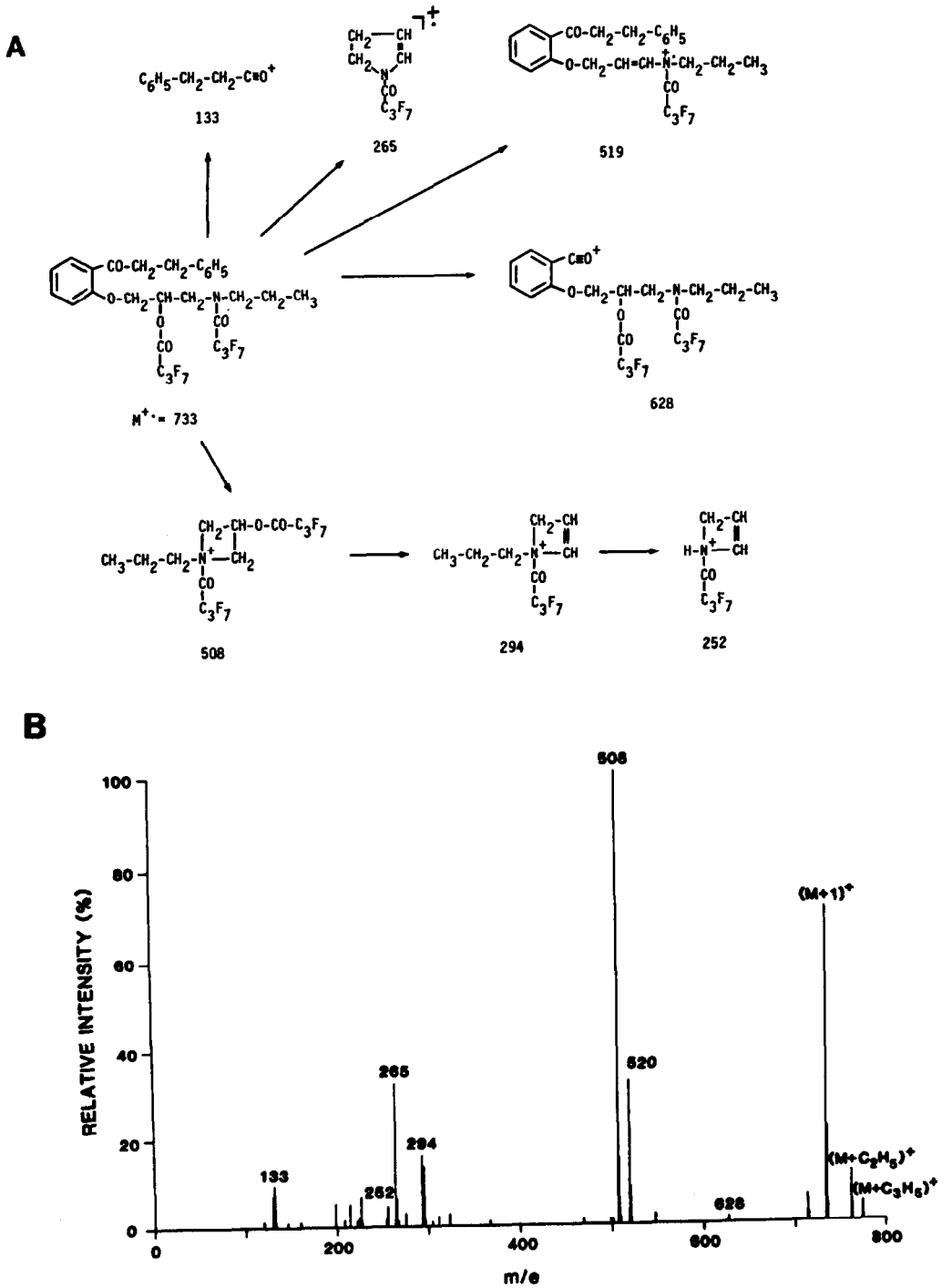
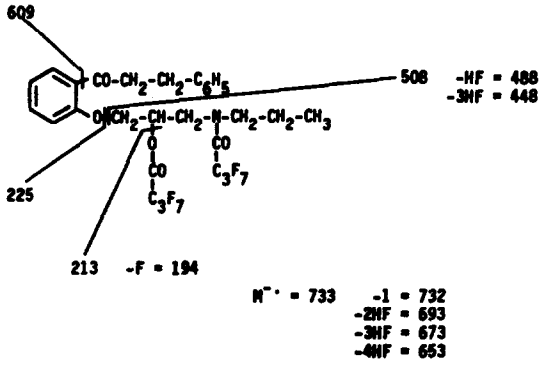


Fig. 5. GC–PICI-MS results: (A) a proposed fragmentation pattern of the HFB derivative of propafenone; (B) PICI mass spectrum of the HFB derivative of propafenone.



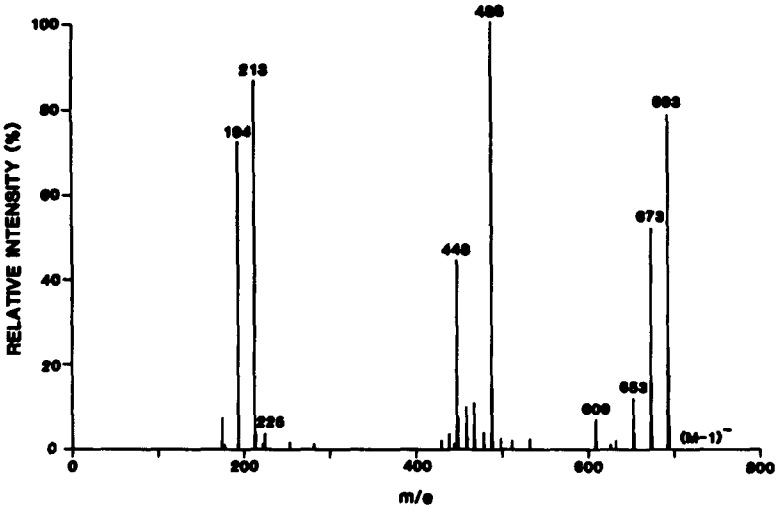


Fig. 6. GC-NICI-MS result of the HFB derivative of propafenone.

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