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DETERMINATION OF CLOFILIUM, A NEW ANTIFIBRILLATORY AGENT, IN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A sensitive and selective method for the assay of the new quaternary amine antifibrillatory agent clofilium is described. Plasma samples were extracted with dichloromethane (98.5 ± 0.2% recovery) and analyzed by gas chromatography—mass spectrometry operating in the electron-impact mode. The method involves a Hofmann elimination of an N-alkyl radical from clofilium and the internal standard in the presence of a strong nucleophile in the injector of the gas chromatograph. The resulting tertiary amines are chromatographed and detected by selective ion monitoring. The ratio of the clofilium base peak (*m/z* 224) to the internal standard peak (*m/z* 210) was linear relative to the plasma clofilium concentration over the range of 25—1000 ng/ml plasma.

INTRODUCTION

Clofilium (4-chloro-N,N-diethyl-N-heptylbenzene butanaminium phosphate) is a new quaternary amine antifibrillatory agent which has been shown to elevate the ventricular fibrillation threshold, reduce defibrillation threshold and allow electrically induced ventricular fibrillation to convert to normal sinus rhythm in pentobarbital anesthetized dogs [1, 2]. [^{14}C]Clofilium has been used to study the plasma and tissue kinetics of clofilium since a suitable non-radioactive assay for clofilium had not been available. However, myocardial levels of radioactivity after intravenous (i.v.) administration of [^{14}C]clofilium have been shown to closely parallel the kinetics of the biological response in dogs [3].

Various investigators have shown that pyrolytic degradation of quaternary amines may reproducibly lead to products which can be resolved by gas chromatography. The quaternary compound may be subjected to chemical degradation prior to injection into the gas chromatograph [4, 5] or they may be thermally decomposed in a gas chromatograph equipped with a pyrolyzer fitted to

the injection port [6]. In this paper, we report on the development of a gas chromatography—mass spectrometry (GC—MS) assay for clofilium which utilizes an on-column Hofmann elimination reaction and selective ion monitoring.

METHODS

Plasma extraction

To 200-μl samples of plasma were added 100 ng of internal standard (4-chloro-N,N-dimethyl-N-heptylbenzene butanaminium bromide) in aqueous solution, 1.5 ml of 0.1 *M* sodium bromide and 5 ml of dichloromethane. After centrifugation, the organic phase was transferred to a glass stoppered centrifuge tube previously treated with 1% dichlorodimethylsilane. The aqueous phase was re-extracted with 5 ml of dichloromethane and the organic phase was combined with the first organic extract. The extract was evaporated to dryness at room temperature under vacuum. The walls of the centrifuge tube were washed with 150 μl of methanol and again evaporated to dryness. The residue was then taken up in 10 μl of methanol containing 0.01 *M* potassium hydroxide and 3 μl were injected into the gas chromatograph—mass spectrometer. Extraction efficiency studies were performed in triplicate using dog plasma to which [^{14}C]clofilium (3.7 μCi/mg) was added to a final concentration of 2 μg/200 μl plasma. Samples were extracted as outlined above and radioactivity in the organic fraction was quantitated. The efficiency of extraction was 98.5 ± 0.2.

Gas chromatography—mass spectrometry

An LKB-9000 gas chromatograph—mass spectrometer (electron-impact mode) was used throughout. Samples were injected into a 0.61-m silanized glass column containing 1% (w/w) SP-2100 on Supelcoport (100—120 mesh) maintained at 195°C. The injector and separator were maintained at 295 and 230°C, respectively. The helium carrier gas flow-rate was 30 ml/min. The retention times of the internal standard and clofilium were 2.0 and 2.4 min, respectively. The mass spectrometer was operated in the selective ion monitoring mode at mass settings of *m/e* 210 (I.S.) and *m/e* 224 (clofilium). Ionizing voltage was 22 eV.

Alternatively, a Hewlett Packard 5840A gas chromatograph equipped with a flame ionization detector (FID) was used to investigate the parameters for the dequaternization of clofilium. Samples of clofilium or tertiary amine standard (4-chloro-N-ethyl-N-heptylbenzene butanamine) were chromatographed on a 0.61-m silanized glass column containing 3% (w/w) SP-2100 on Supelcoport (100—120 mesh) maintained at 200°C. The detector temperature was held at 300°C and the injector temperature was varied between 250 and 375°C. Helium carrier gas flow-rate was 38 ml/min. The area under the peak after injection of clofilium was compared to the area under the peak after injection of tertiary amine standard and expressed as percent N-deethylation of clofilium.

Standard curve

Standard solutions of 10 μg/ml clofilium and internal standard were prepared in water. Appropriate volumes of clofilium standard were added to

plasma samples to give final concentrations of 25—1000 ng/ml plasma. Internal standard was added to a final concentration of 500 ng/ml plasma. Plasma samples were then extracted and assayed for clofilium as outlined above.

Dosing

Clofilium was administered i.v. to 3 female mongrel dogs weighing approximately 12 kg at a dose of 5 mg/kg in saline. Blood samples were collected at various times in heparinized Vacutainer tubes. Samples were centrifuged and plasma was separated and stored at −20°C until assayed.

RESULTS AND DISCUSSION

The goal of the present investigation was to develop a selective and sensitive assay for clofilium in plasma. Although clofilium can be chromatographed using ion-exchange high-performance liquid chromatography (HPLC), the ultraviolet absorbance is too small to detect the low plasma concentrations of drug. Although flame ionization or electron-capture detection might be sensitive enough, ordinary GC was not successful because of the quaternary amine structure of the drug. However, the possibility of performing a Hofmann elimination in the injector of the gas chromatograph prompted the following investigation. When clofilium was injected into the gas chromatograph—mass spectrometer with injector temperatures between 290 and 375°C, a peak was detected which had a mass spectrum indicating the formation of 4-chloro-N-ethyl-N-heptylbenzene butanamine (Fig. 1). This deethylation is analogous to the pyrolytic dealkylation of choline esters observed by Szilagyi et al. [7]. The effect of injector temperature on the formation of the tertiary amine is shown in Fig. 2. Comparison of the peak area after injection of clofilium to the peak area after injection of an equimolar quantity of standard 4-chloro-N-ethyl-N-heptylbenzene butanamine showed that only 30% of the clofilium was deethylated at

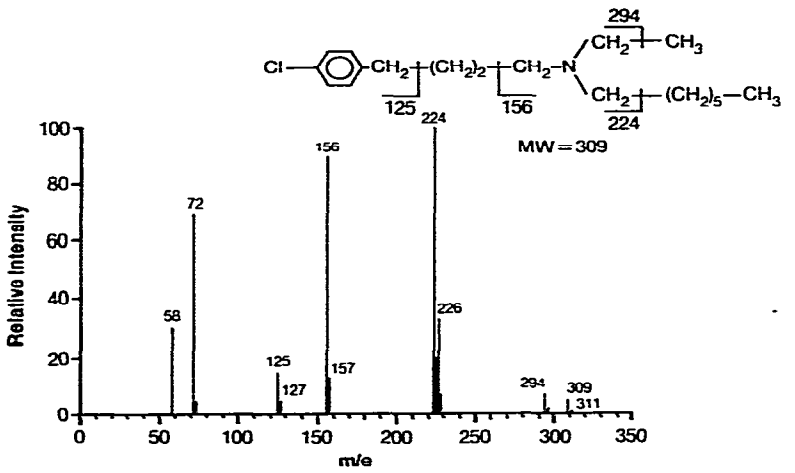


Fig. 1. Mass spectrum obtained after GC of clofilium with an injector temperature of 300°C. No molecular ion was observed at *m/e* 338 corresponding to the molecular weight of clofilium. A parent peak at *m/e* 309 was observed corresponding to N-deethylated clofilium.

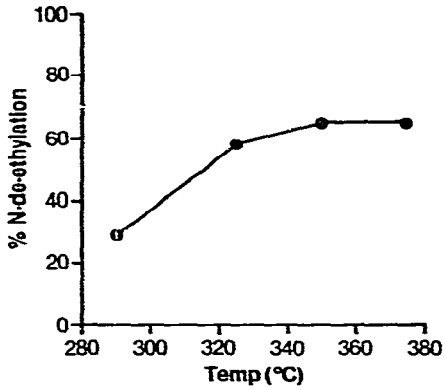
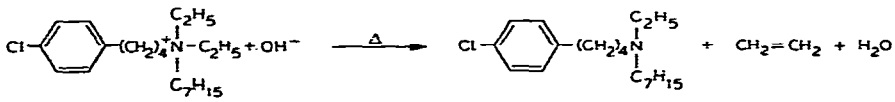


Fig. 2. The effect of injector temperature upon the N-deethylation of clofilium.

290°C which increased to a maximum of 65% at 350°C. Hofmann elimination reactions involve nucleophilic attack on quaternary amine alkyl groups eliminating ethylene which carries the smallest number of alkyl substituents. Therefore, the addition of a nucleophilic hydroxide ion in methanol to the extract before injection onto the gas chromatograph should improve the efficiency of the elimination reaction consistent with the following scheme:



At a constant injector temperature, an increase in hydroxide ion concentration lead to increased deethylation of clofilium as shown in Fig. 3. Using a hydroxide ion concentration of 0.01 *M*, the deethylation reaction was quantitative and independent of injector temperature between 250 and 325°C (data not shown). Thirty repetitive injections of 650 ng of clofilium in 3 μl of 0.01 *M* potassium

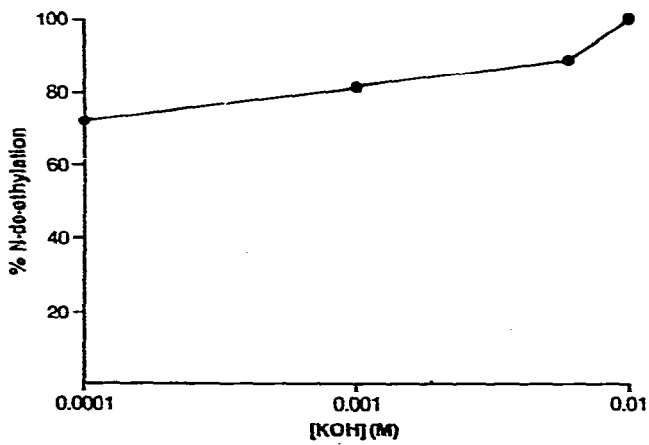


Fig. 3. The effect of hydroxide ion concentration upon the N-deethylation of clofilium at a constant injector temperature of 325°C.

hydroxide—methanol did not result in any loss of column or detector efficiency (standard error of the mean peak areas is 0.6%).

Although the GC of clofilium was possible, the sensitivity of flame ionization or electron-capture detection was not sufficient for the low plasma levels of clofilium anticipated. Therefore, the base peak (*m/e* 224) in the mass spectrum of clofilium originating from the Hofmann elimination reaction was used for a sensitive selective ion monitoring method. The clofilium analogue, 4-chloro-N,N-dimethyl-N-heptylbenzene butanaminium bromide, also undergoes N-dealkylation in the gas chromatograph to yield the 4-chloro-N-methyl-N-heptyl tertiary amine (Fig. 4) and has chromatographic properties similar to

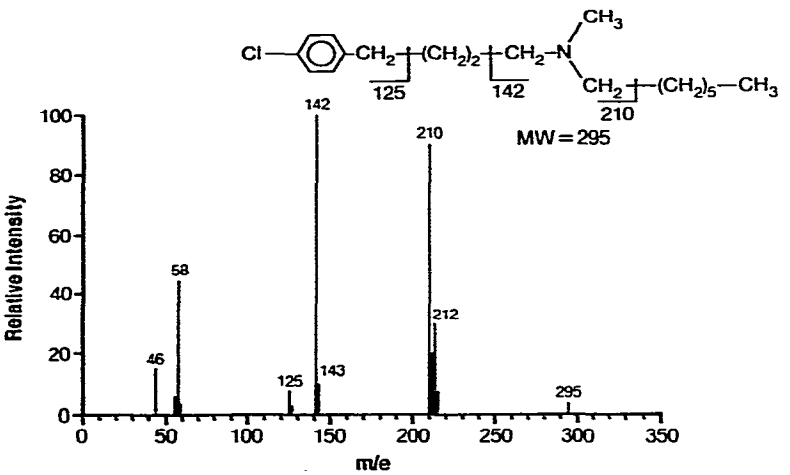


Fig. 4. Mass spectrum obtained after GC of the internal standard with an injector temperature of 300°C. No molecular ion was observed at *m/e* 310 corresponding to the molecular weight of the internal standard. A parent peak at *m/e* 295 was observed corresponding to N-demethylated internal standard.

clofilium. In addition, the compound has a prominent ion at *m/e* 210 in the mass spectrum. These properties allowed its use as an internal standard. A standard curve was then constructed with control dog plasma to which was added clofilium and internal standard. By monitoring the ratio of the peak height of the *m/e* 224 ion (clofilium) to the peak height of the *m/e* 210 ion (internal standard), a linear relationship was observed relative to clofilium plasma concentration. Linearity was observed between 25 and 1000 ng clofilium per ml plasma. The accuracy and precision of the assay are shown in Table I. The limit of detection was 10 ng clofilium per ml plasma.

TABLE I

ACCURACY AND PRECISION OF THE GC—MS ASSAY OF CLOFILIUM

Clofilium added to plasma (ng/ml plasma)	Clofilium assayed in plasma (ng/ml plasma)	
	Mean ± S.D. (n=4)	C.V. (%)
100	99.5 ± 11.0	11.0
500	478.0 ± 20.0	4.2

Dogs were administered 5 mg/kg of clofilium by i.v. injection and plasma samples were assayed for clofilium at various times. The results are shown in Fig. 5. Clofilium plasma levels rapidly decreased within the first hour and continued to decline throughout the 24-h observation period. Within 24 h, plasma levels had decreased to 36 ng/ml plasma. The selectivity and sensitivity of this assay will allow its use in further characterizing the plasma kinetics of clofilium in the dog and other laboratory animals.

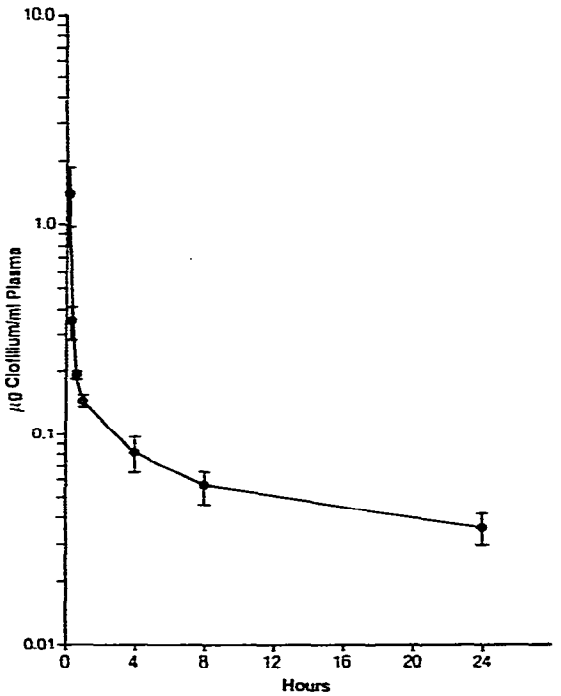


Fig. 5. Plasma levels of clofilium in dogs following a single intravenous injection of 5 mg/kg of clofilium. The values are the mean ± standard error for 3 dogs.

REFERENCES

1 M.I. Steinberg and B.B. Molloy, Life Sci., 25 (1979) 1307.
2 W.A. Tacker, M.J. Niebauer, C.F. Bobbs, W.J. Combs, B.M. Hahn, M.A. Barker, J.F. Seipel, J.D. Bourland and L.A. Geddes, Crit. Care Med., 8 (1980) 177.
3 T.D. Lindstrom, P.J. Murphy, J.K. Smallwood, S.A. Wiest and M.I. Steinberg, J. Pharmacol. Expt. Ther., 221 (1982) 584.
4 E.C. Jennings, Jr. and H. Mitchner, J. Pharm. Sci., 56 (1967) 1590.
5 H. Mitchner and E.C. Jennings, Jr., J. Pharm. Sci., 56 (1967) 1595.
6 W. Bianchi, L. Boniforti and A. di Domenico, in A. Frigerio and N. Castagnoli, Jr. (Editors), Mass Spectrometry in Biochemistry and Medicine, Raven Press, New York, 1974, p. 193.
7 P.I.A. Szilagyi, D.E. Schmidt and J.P. Green, Anal. Chem., 40 (1968) 2009.