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## Note

# Analysis of clonidine in biological tissues and body fluids by gas chromatography with electron-capture detection

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Clonidine, 2-[(2,6-dichlorophenyl)amino]-2-imidazoline hydrochloride, is a highly potent drug which is effective in microgram doses. It has numerous central actions, namely analgesia [1, 2], hypothermia [3, 4], inhibition of conditioned avoidance response [3], sedation [3, 5—7] and hypotension [8]. Clonidine is a clinically useful antihypertensive and its hypotensive action is believed to be mediated by its interaction with a group of central $\alpha$-adrenoceptors [9, 10]. It has also been reported to be a potentially useful therapeutic agent for the treatment of agitated mental patients [11, 12]. We have previously demonstrated that clonidine abolished ischemia-induced seizures in laboratory animals [13]. A number of techniques have been used in the analysis of clonidine, namely gas chromatography—mass spectrometry [7, 14—18], gas chromatography [19—22], radioimmunoassay [23—25] and radiolabelling [20, 26—28]. Since clonidine has numerous pharmacological actions and because of the uncertainty

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regarding its sites of action, we have studied distribution of clonidine in rat brain, liver and blood. This has involved development of a novel gas chromatographic (GC) procedure with electron-capture detection (ECD) for quantitative analysis. The method is rapid, highly sensitive and requires a minimal clean-up procedure.

## EXPERIMENTAL

### *Subjects*

Male Sprague—Dawley rats (180—220 g) were used. The animals were housed in a temperature-controlled room on a 12-h on—12 h off lighting schedule. Food and water were provided ad libitum.

### *Drugs and reagents*

Clonidine hydrochloride (Catapresan®) was a gift from Boehringer Ingelheim and the internal standard, nortriptyline hydrochloride, was obtained from Merck Sharp and Dohme (Canada). Pentafluorobenzoyl chloride was purchased from Aldrich (Milwaukee, WI, U.S.A.). The rats were injected intraperitoneally with clonidine (0.4 mg/kg dose) and killed by decapitation at 15, 30, 60, 120, 180 or 240 min after dosing. Blood was collected immediately in heparinized vials or vials containing ethylenediaminetetraacetic acid (EDTA). The excised brains and livers were frozen solid in isopentane on solid carbon dioxide. Urine samples (24 h) were also collected from separate groups of rats after the same intraperitoneal dose. All samples were stored at −20° C until time of analysis.

### *Brain and liver*

The weighed brain and liver tissues were homogenized in 5 vols. of ice cold 0.4 *M* perchloric acid containing 10 mg% EDTA. The homogenates were centrifuged at 12 000 *g* for 15 min at 0—4° C. The supernatant was retained and to a 2-ml portion internal standard, nortriptyline (5 μg), was added followed by potassium bicarbonate. The resultant potassium perchlorate precipitate was discarded after centrifugation at 1000 *g* for 5 min. The supernatant was basified with sodium carbonate to pH 8—9; this was followed by the addition of the derivatizing reagent pentafluorobenzoyl chloride (4 μl) in 4 ml of toluene. This system was vortexed for 15 min and centrifuged at 1000 *g* for 10 min. The upper toluene layer was retained and dried under a stream of nitrogen. The dried residue was dissolved in 100 μl of toluene and washed with 200 μl of 1.0 *M* ammonium hydroxide. An aliquot of this toluene layer was injected onto a gas chromatograph equipped with an electron-capture detector. Tubes containing known, varying quantities of authentic clonidine and 5 μg of the internal standard were run in parallel with each set of analysis. These standards were used to construct calibration curves from which the amounts of clonidine in the biological samples were determined.

### *Blood*

Weighed blood samples were ultrasonicated in 2 vols. of ice cold 0.4 *M* perchloric acid containing 10 mg% EDTA. These were then centrifuged at

1000 *g* for 5 min. The supernatant (2 ml) was then treated in the same way as brain and liver samples.

*Urine*

Urine samples (2 ml) were basified and extracted in the same manner as brain and liver supernatants.

*Instrumentation*

GC was performed on two column types, capillary or packed, attached to an electron-capture detector with a radioactive source of 15 mCi $^{63}Ni$. The capillary instrument was a Hewlett-Packard (HP) 5880A, equipped with a 25 m × 0.31 mm I.D. 5% phenylmethylsilicone fused-silica capillary column. The GC instrument was attached to an HP 5880A integrator. Helium (flow-rate 2 ml/min) was used as the carrier gas, and methane—argon (10:90) at a flow-rate of 36 ml/min was used as make-up gas at the detector. The injection port temperature was 200°C and the detector temperature was 350°C. The column temperature was programmed to increase from 105 to 330°C at a rate of 25°C/min. Blood samples were analysed on this system.

For packed column analysis, the gas chromatograph employed was an HP 5830A. This instrument was equipped with a 2 m × 4 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh). Methane—argon (10:90) was used as the carrier gas at a flow-rate of 40 ml/min. The temperature at the injection port was 250°C and the detector temperature was 300°C. The column temperature was maintained at 240°C for 13 min (and

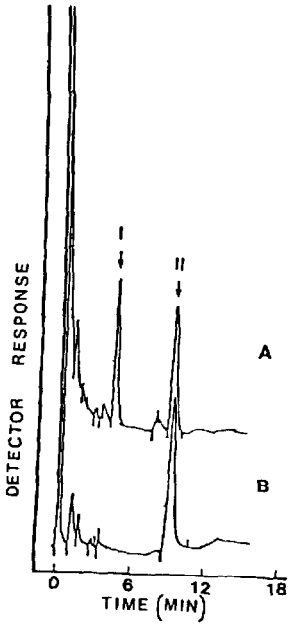


Fig. 1. Gas chromatograms on a 3% OV-17 packed column of extracts of a brain sample from (A) a rat treated with clonidine (0.4 mg/kg intraperitoneally) and sacrificed at 30 min and (B) a control rat brain. Peaks: pentafluorobenzoyl derivatives of clonidine (I) and nortriptyline (II), the internal standard.

programmed to increase subsequently to 275°C at a rate of 15°C/min to bake off impurities). The brain, liver and urine samples were analysed on this system.

Both electron-impact (EI) and chemical-ionization (CI) mass spectrometers were used to confirm the structure of the pentafluorobenzoyl derivative of clonidine. A quadrupole mass spectrometer (HP 5985A) was used in the EI mode and a single-focus magnetic sector instrument (Kratos MS-12) was used in CI mode (using ammonia as the reagent gas).

## RESULTS AND DISCUSSION

The novel GC procedure developed proved to be readily applicable to analysis in biological samples. Although pentafluorobenzoyl chloride has been used in the past for analysis of amines [29—39], this report is the first to show its use in the analysis of clonidine in aqueous biological milieu.

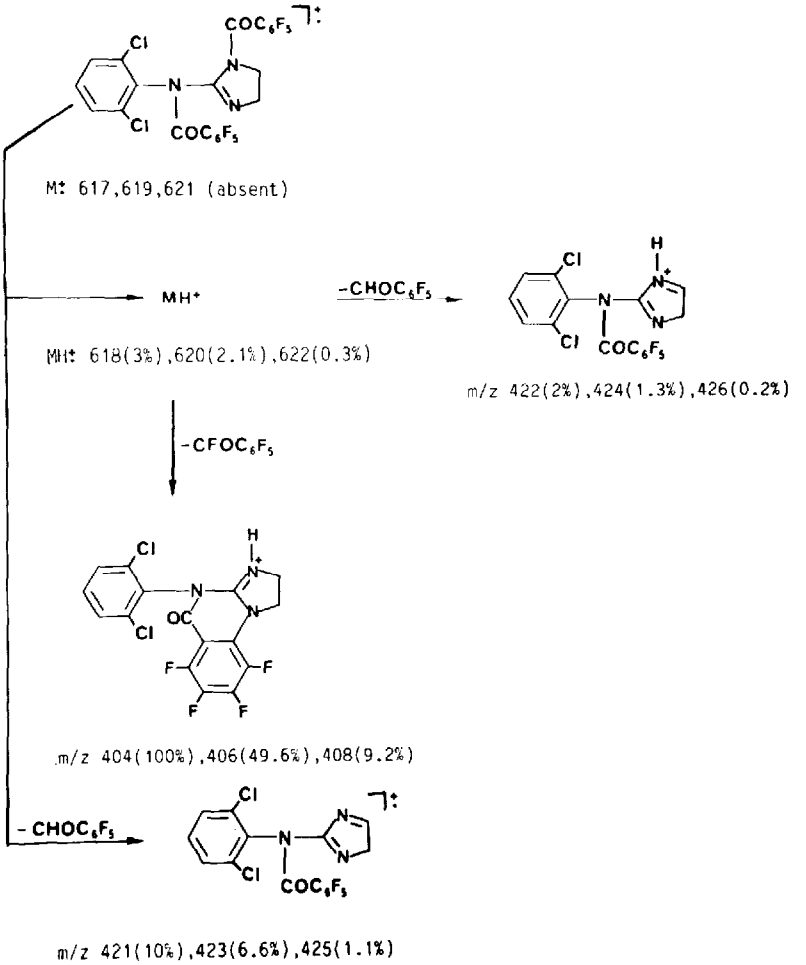


Fig. 2. Proposed chemical-ionization mass fragmentations of the pentafluorobenzoyl derivative of clonidine. Numbers in parentheses are percent relative abundances of the individual fragments. Three *m/z* values are given in each case because of the contribution of chlorine mass isotopes.

Nortriptyline proved to be a suitable internal standard for the assay. It formed a stable derivative with pentafluorobenzoyl chloride, and the resultant derivative separated readily from derivatized clonidine and from other possible substances in the extracts. In addition, the assay was linear over a wide range (100-fold) of clonidine concentrations when nortriptyline was employed as internal standard. The derivative demonstrated good peak shape and high sensitivity (2 pg on-column gave a signal-to-noise ratio of 2:1) and stability with high yields (> 90% recovery). Typical gas chromatograms are shown in Fig. 1. CI mass spectral analysis confirmed that a dipentafluorobenzoyl derivative (Fig. 2) of clonidine was formed during the procedure. The proposed CI and EI

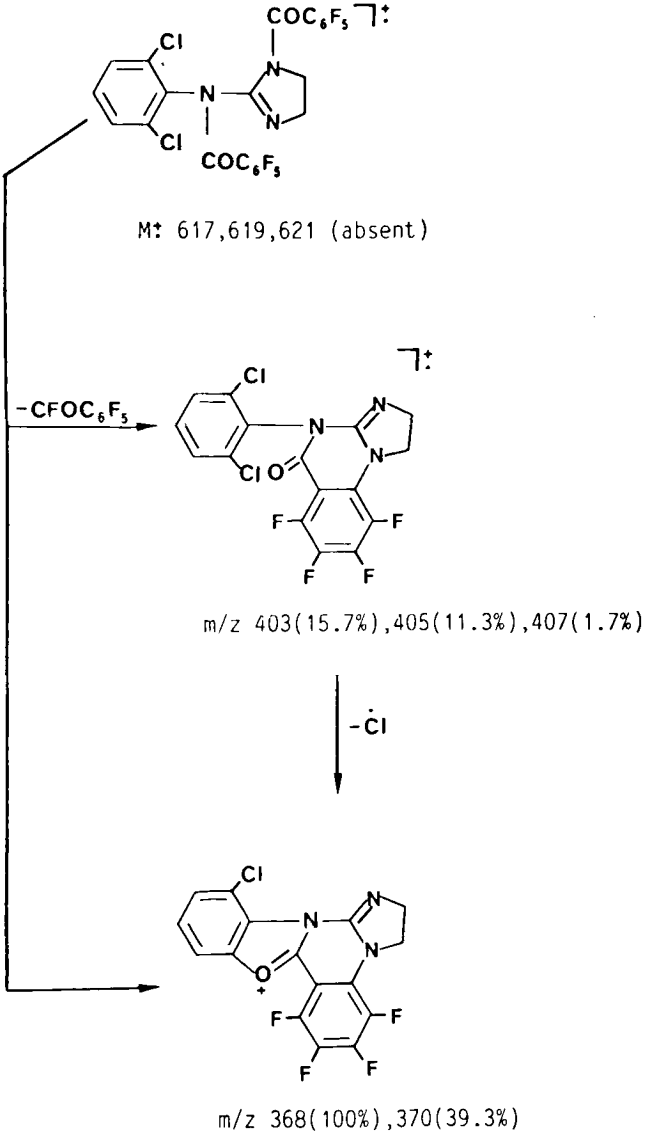


Fig. 3. Proposed electron-impact mass fragmentations of the pentafluorobenzoyl derivative of clonidine. Numbers in parentheses are percent relative abundances of the individual fragments.

mass spectral fragmentations of the derivative are shown in Figs. 2 and 3. The base peaks of the derivatized clonidine were *m/z* 368 (100%) in the EI mode and *m/z* 404 (100%) in the CI mode. The probable structures of these fragments are cyclic structures (Figs. 2 and 3) similar to that proposed by Edlund [19] for the reaction of clonidine with pentafluorobenzyl bromide. Edlund [19] proposed that his reagent formed a monopentafluorobenzyl derivative, but did not indicate if he used the EI or CI mode in his mass spectral analysis.

The procedure has been applied to brain, liver and blood samples from rats, and levels in these samples 3 and 4 h after intraperitoneal injection of clonidine (0.4 mg/kg) are shown in Table I.

Analysis was also carried out in 24-h urines that were collected from rats treated with the same dose; levels in these urine samples were determined to be 2.68 ± 0.26 μg/ml (mean ± standard error of the mean, $n = 5$).

Detailed time—concentration studies were conducted in brain after administration of the 0.4 mg/kg dose. The results are shown in Table II. A log plot of concentration in brain versus time (15—240 min) was linear and from this the half-life ($t_{½}$) was determined to be 150.5 min and the elimination rate constant to be $4.5 \cdot 10^{-3}$ $min^{-1}$. This $t_{½}$ value is similar to the value determined (120 min) by Cho and Curry [20] after administration of an intravenous dose of 0.25 mg/kg. Edlund and Paalzow [21] and Edlund [19] employed GC—ECD for the quantitation of clonidine in plasma. They utilized

TABLE I

CONCENTRATIONS OF CLONIDINE IN RAT BRAIN, LIVER AND BLOOD 3 AND 4 h AFTER ADMINISTRATION OF CLONIDINE (0.4 mg/kg INTRAPERITONEALLY)

| Time (min) | Concentration* (ng/g) | | |
|---|---|---|---|
| | Brain | Liver | Blood |
| 180 | 246.9 ± 32.7 | 423 ± 101 | 75.7 ± 4.8 |
| 240 | 119.3 ± 36.3 | 195 ± 16.3 | 12.7 ± 0.9 |

*Results represent means ± standard error of the mean ($n = 5$—6).

TABLE II

CONCENTRATIONS OF CLONIDINE IN RAT BRAIN AFTER 0.4 mg/kg INTRAPERITONEAL DOSE OF CLONIDINE

| Time (min) | Concentration* (ng/g) | *n* |
|---|---|---|
| 15 | 441.7 ± 86.0 | 5 |
| 30 | 414.5 ± 81.9 | 6 |
| 60 | 353.2 ± 69.6 | 6 |
| 120 | 314.1 ± 60.9 | 6 |
| 180 | 246.9 ± 32.7 | 6 |
| 240 | 119.3 ± 36.3 | 5 |

*Results represent means ± standard error of the mean.

pentafluorobenzyl bromide for the derivatization of clonidine and reported a minimum detectable quantity of 3.3 pg. However their procedure was lengthy and required refluxing for 40 min in a heating block. Chu et al. [22] also used GC—ECD for analyzing clonidine in plasma. Their reported method was also lengthy and derivatization was under anhydrous conditions with heptafluorobutyric anhydride, requiring heat at 45°C for 15 min. Furthermore, a clean-up step involving microcolumns was required following derivatization and before injection onto the GC system to remove the electron-capture sensitive background and achieve the desired sensitivity.

In summary, a sensitive procedure has been developed for quantitation of clonidine in tissues and body fluids. The method is rapid, and extraction and derivatization are performed with pentafluorobenzoyl chloride under relatively mild aqueous conditions. The technique is now being utilized in stroke studies in our laboratories by determining the distribution of clonidine in brains of laboratory animals in which this drug is used to alleviate ischemia-induced seizures.

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