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Determination of Submicrogram Quantities of Clonidine in Biological Fluids

L.-C. CHU ˣ, **W. F. BAYNE, F. T. TAO, L. G. SCHMITT, and J. E. SHAW**

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Abstract □ A sensitive and specific GLC method using electron-capture detection was developed for clonidine in plasma and urine. Di-perfluoroacyl derivatives of both clonidine and the 4-methyl analog of clonidine (used as an internal standard) were formed, and an extraction process was developed for the removal of excess derivatization reagent and endogenous biological compounds; the assay permitted quantification of 25 pg of clonidine/ml in a 4-ml plasma sample. The assay was used to elucidate the time course of plasma concentrations in a normotensive subject following oral administration of 50, 100, and 200 μg of clonidine hydrochloride and also to determine unchanged drug excreted in the urine.

Keyphrases □ Clonidine—GLC analysis in plasma and urine □ GLC—analysis, clonidine in plasma and urine □ Antihypertensives—clonidine, GLC analysis in plasma and urine

Clonidine, 2-[(2,6-dichlorophenyl)imino]imidazolidine, is a potent drug used in the treatment of arterial hypertension. The sympatico-inhibitory activity of clonidine is predominantly due to its influence on α-adrenergic receptors in the brain stem (1). The hypotensive effect of clonidine is absent in tetraplegic subjects with transection of the cervical spinal cord above the sympathetic outflow (2). The absence of the hypotensive effect in these subjects indicates that the action of the drug is centrally mediated in humans.

The therapeutic dose of clonidine is a few tenths of a milligram per day. Clonidine is lipophilic in nature with a large volume of distribution (3). Thus, the resulting plasma concentrations following oral administration of therapeutic doses are in the submicrogram range.

BACKGROUND

The metabolism and disposition of clonidine in various animal species were studied (4) using ^{14}C-labeled clonidine. The time course of total radioactivity in the plasma following oral administration of either 390 or 1440 μg of clonidine was determined. The plasma concentrations of unchanged drug were not ascertained, although approximately 45% of the orally administered dose was excreted in the urine as unchanged drug.

The plasma levels, renal excretion, and urinary metabolic pattern were determined (5) in normotensive subjects. Three hours following oral administration of 300 μg of ^{14}C-labeled clonidine, clonidine contributed less than 50% of the total ^{14}C-radioactivity in the plasma.

A recently developed GLC–mass spectrometric method permits the measurement of clonidine in plasma and urine following administration of unlabeled therapeutic doses to humans (6). The method is based on the selective monitoring of mass fragments produced from clonidine and from a deuterated internal standard under electron-impact ionization. The sensitivity of the method and the chromatographic properties of clonidine and the internal standard were improved by formation of their dimethyl derivative through on-column methylation with trimethylanilinium hydroxide (7). The precision of the method at 0.25 ng/ml was ±11% *SD* with 4 ml of plasma. Davies *et al.* (8) used the improved GLC–mass spectral method to study the pharmacokinetics and pharmacodynamics of clonidine following oral and intravenous administration of 300 μg of clonidine hydrochloride[1].

Cho and Curry (9) developed a GLC method using a ^{90}Sr-ionization detector to determine clonidine levels in the blood and tissues of rats administered 500 μg of drug/kg iv. The minimum detectable quantity of clonidine was 2–3 ng. The drug, which was not derivatized, tended to adsorb onto the chromatographic support, especially with the longer columns, thus decreasing the sensitivity of the assay.

A GLC method with electron-capture detection of the pentafluorobenzyl derivative of clonidine was reported (10). This method was used to determine the temporal pattern of plasma concentrations in rats following administration of 500 μg of clonidine hydrochloride/kg, about 100 times the dose received by humans.

This paper reports an electron-capture GLC method for the determination of clonidine in plasma and urine using a di-heptafluorobutyryl derivative of clonidine and an internal standard. The electron-capture-sensitive background is reduced through the use of silica gel columns. This method was used to elucidate the time course of plasma concentrations in a normotensive subject following oral administration of 50, 100, and 200 μg of clonidine hydrochloride.

EXPERIMENTAL

Materials—Methylene chloride, benzene, and hexane, employed in the extraction procedures, were used as obtained[2]. Ethyl acetate[3], purchased in quart bottles and sealed under nitrogen, was used as the solvent for the derivatization of clonidine and the internal standard.

The carbonate buffers, 1 *M* (pH 9.75) and 0.1 *M* (pH 9.2), were made from potassium bicarbonate and adjusted to the appropriate pH with freshly prepared potassium hydroxide solution. Sulfuric acid (0.1 *N*) was diluted from the concentrated acid.

Heptafluorobutyric anhydride[4] was obtained in 1-ml glass ampuls. After the ampul was opened, the contents were stored in a 3-ml minivial for protection from moisture. A new ampul was used daily.

The internal standard, 2-[(2,6-dichloro-4-methylphenyl)imino]imidazolidine, was used as received[5].

Dimethyldichlorosilane, 5% in toluene, was used to silanize all glassware for at least 2 hr; the glassware was then rinsed with toluene, methanol,

[1] Catapres, Boehringer Ingelheim.
[2] Nanograde solvents, Mallinckrodt, St. Louis, Mo.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Pierce Chemical Co., Rockford, Ill.
[5] C. H. Boehringer Sohn, Ingelheim, Germany.

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and methylene chloride. Microcolumns were used to reduce chromatographic interferences. Each short silanized Pasteur pipet was packed with a plug of silanized glass wool and 0.5 cm of silica gel adsorbent[6].

Apparatus—The derivatives of clonidine and the internal standard were chromatographed on a gas chromatograph[7] equipped with a scandium tritide detector. A 2-mm i.d. × 1.8-m silanized glass column packed with 3% OV-17 on 100–120-mesh Chromosorb W, AW, DMCS[8], was used. The support was inactivated and coated with the liquid phase according to a method described previously (11). Nitrogen was used as the carrier gas.

The retention times for the derivatives of clonidine and the internal standard were 5 and 6.5 min, respectively, at a column temperature of 175° and a gas flow of 25 ml/min. The injector temperature was 220°, and the detector temperature was 250°.

A quadrupole mass spectrometer[9], interfaced with a gas chromatograph[10], was used to establish the structure of the derivatives. The electron energy of the ionization source was 70 ev. A GLC column similar to that already described was employed.

Derivatization of Microgram Quantities of Clonidine—Clonidine base (9.85 mg) was dissolved in 10 ml of ethyl acetate and stored at 0°. A 30-μl aliquot of this solution was placed in a 12-ml conical centrifuge tube with a polytef stopper and taken to dryness with a nitrogen stream. The dried clonidine base was reconstituted in 100 μl of dry ethyl acetate (water content of 50 ppm), followed by the addition of 4 μl of heptafluorobutyric anhydride.

After vortexing, the centrifuge tube was immersed in a water bath at 45° for 15 min. The contents were then taken to dryness under a nitrogen stream. Hexane (400 μl) was added to the dried residue, followed by 1 ml of 0.1 *M* potassium carbonate buffer (pH 9.2). The contents were then vortexed and centrifuged.

The organic layer was transferred to another 12-ml conical centrifuge tube and taken to dryness with a nitrogen stream. The residue was dissolved in 50 μl of methylene chloride, and a flame-ionization detector was employed to check the completeness of derivatization.

Derivatization of Submicrogram Quantities of Clonidine—The working standard of clonidine in ethyl acetate (described in the previous section) was diluted to contain 50–1000 pg/μl, depending on the quantity of clonidine to be derivatized. Aliquots of the diluted solutions were added to 12-ml centrifuge tubes, and the ethyl acetate was removed with a nitrogen stream. Derivatization was conducted as already described; however, the hexane phase following the carbonate wash was not evaporated to dryness.

Reduction of Electron-Capture-Sensitive Background—Microcolumns, prepared as already described and washed with two column heights of benzene followed by two column heights of hexane, were used to reduce the electron-capture-sensitive background. The hexane phase, obtained in the previous procedure, was loaded on a microcolumn. An additional 400 μl of hexane was used to wash the aqueous carbonate phase and also was added to the microcolumn. The microcolumn was then washed with 1.2 ml of hexane.

Elution of the clonidine derivative was effected by washing the microcolumns with 2 ml of 25% (v/v) benzene in hexane. The eluate was taken to dryness with nitrogen at 40°, and the residue was dissolved in 25–50 μl of 20% ethyl acetate in hexane. Aliquots ranging from 2 to 5 μl were injected into the gas chromatograph, and the derivative was detected with the electron-capture detector. The microcolumns could be reused on the same day by washing with two column heights of benzene followed by two column heights of hexane.

Extraction of Clonidine and Internal Standard from Plasma—Extractions of 4-ml plasma samples containing clonidine and the internal standard were performed in 30-ml silanized, separatory centrifuge funnels[11]. A 2-ml aliquot of 1 *M* carbonate buffer (pH 9.75) was added to the plasma. The buffered plasma sample was extracted with 12 ml of 10% ethyl acetate in methylene chloride. The contents were shaken horizontally in a modified shaker[12] for 10 min and centrifuged at 1200 rpm.

The organic layer was then removed through the polytef valve in the bottom of the separator into a second separator. The organic extract was back-extracted with 3 ml of 0.1 *N* H_2SO_4, and the organic phase was discarded. The retained aqueous phase was adjusted to pH 10 with a saturated aqueous solution of sodium carbonate (approximately 5 drops required) and extracted with 3 ml of benzene. The benzene extract was transferred to a 12-ml conical centrifuge tube and taken to dryness at 40° with a nitrogen stream.

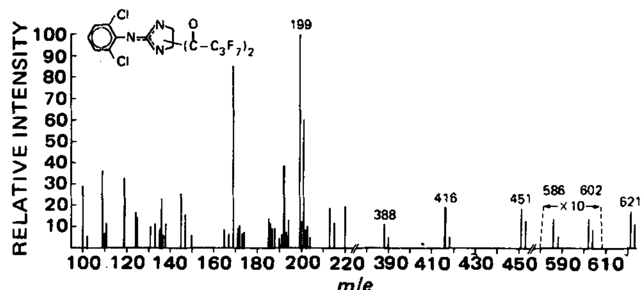


Figure 1—*Mass spectrum of di-heptafluorobutyryl derivative of clonidine obtained with an electron energy of 70 ev and an emission current of 1 mamp.*

Standard Curve for Clonidine in Plasma—Aliquots of human plasma (4 ml) were spiked with varying amounts of clonidine (100 pg–5 ng) and a constant amount of the internal standard (4 ng). A blank plasma sample, containing no clonidine or internal standard, also was carried through the assay. The plasma samples were extracted, derivatized, and chromatographed on the microcolumns as described.

The samples were then injected into the gas chromatograph, and the responses of the derivatives of clonidine and the internal standard were recorded. The areas of the chromatographic peaks were approximated by the product of the height and width at half-height. The standard curve was generated using a linear least-squares analysis of the ratio of the peak areas for the derivatives (clonidine to the internal standard) *versus* the amount of clonidine (slope of 0.392 and correlation coefficient of 0.997).

Assay in Urine—The assay of urine samples was the same as that described for plasma samples, except that 1-ml aliquots of urine and 24 ng of the internal standard were used.

***In Vivo* Studies**—One normotensive subject received 50, 100, and 200 μg of clonidine hydrochloride. Following each dose, 10-ml blood samples were withdrawn into heparin-containing evacuated glass tubes at predetermined times. The blood was centrifuged, and the plasma was separated and frozen until analyzed. Urine was collected over 12-hr intervals for 48 hr.

RESULTS AND DISCUSSION

Ethyl acetate was the solvent of choice for the derivatization of clonidine and the internal standard with heptafluorobutyric anhydride. Complete conversion to a single derivative was achieved with microgram quantities of clonidine, as indicated by the presence of a single peak in the chromatogram obtained with a flame-ionization detector. When acetonitrile was used as a solvent for the derivatization, three peaks were

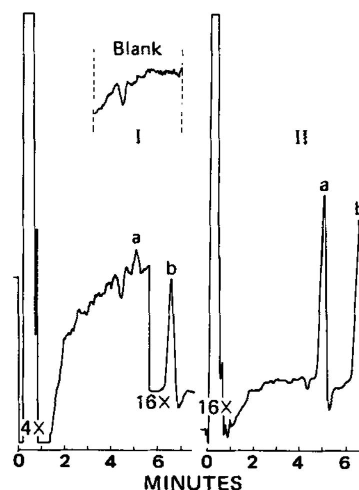
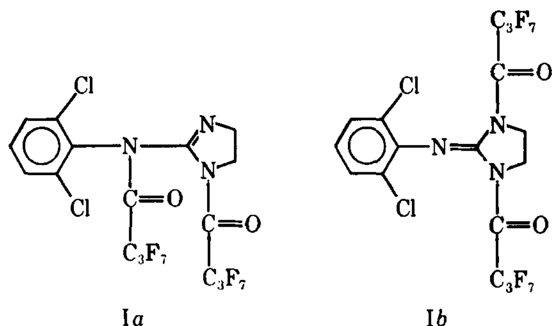


Figure 2—*Representative chromatograms of derivatized clonidine (a) and the internal standard (b) obtained with 100 pg of clonidine (I) and 2.5 ng of clonidine added to 4 ml plasma (II).*

[6] Separations Group, Hesperia, Calif.
[7] Model 2100, Varian, Walnut Creek, Calif.
[8] Johns-Manville, Denver, Colo.
[9] Model 1015 SL, Finnigan, Sunnyvale, Calif.
[10] Model 1700, Varian, Palo Alto, Calif.
[11] Labcrest, Warminster, Pa.
[12] Model 6000, Eberbach, Ann Arbor, Mich.



present in the chromatogram obtained with the flame-ionization detector. The major peak corresponded to that obtained with ethyl acetate as the solvent, and none of the chromatographic peaks had a retention time corresponding to that of underivatized clonidine. With GLC–mass spectrometry, the two other peaks present in the chromatogram subsequently were identified as mono-heptafluorobutyryl derivatives of clonidine.

The mass spectrum of the compound that gave the single chromatographic peak, obtained with ethyl acetate as the solvent for derivatization, is shown in Fig. 1. The spectrum exhibited a molecular ion at m/e 621, consistent with the incorporation of two heptafluorobutyryl groups into the clonidine molecule. The loss of one fluorine atom and the loss of one chlorine atom from the molecular ion produced the m/e 602 and 586 fragments, respectively. The ion at m/e 451 was due to the loss of the C_3F_7 group and a hydrogen atom, and this ion decomposed with expulsion of a chlorine atom to give the m/e 416 ion. This ion further fragmented with the loss of carbon monoxide to give m/e 388. The base peak for the di-heptafluorobutyryl derivative was observed at m/e 199 and can be formulated as arising from the loss of the two acyl groups plus the two methylene groups from the imidazole ring.

There were no prominent ions in the mass spectrum of the di-heptafluorobutyryl derivative to establish unequivocably the exact position of the acyl incorporation. Two possible structural isomers (I*a* and I*b*) can result from the incorporation of two acyl groups into the clonidine molecule.

To establish the structure of the diacyl derivative, macroquantities of the derivative could be made and analyzed using either ^{1}H- or ^{13}C-NMR spectroscopy. Since one isomer possesses a C_2 symmetry element lacking in the other, the proton pattern resulting from the hyperfine interaction of the methylene protons or the ^{13}C-resonances of the methylene carbons would be expected to differ in the two isomers.

The precision of the assay at 60 pg of clonidine/ml in 4 ml of plasma was ±8% *SD*. The recovery of clonidine carried through the entire procedure was 60% relative to an external standard, which was prepared by diluting microgram quantities of derivatized clonidine.

Chromatograms from spiked plasma samples are shown in Fig. 2. The blank plasma sample indicated the absence of interferences. The small positive response in the chromatogram for the 4-ml plasma sample with a clonidine concentration of 25 pg/ml represents approximately 5–6 pg of the clonidine derivative.

To achieve the levels of detection obtained with the method, it was necessary to inactivate the support material in the GLC column. Otherwise, loss in sensitivity resulted due to adsorption of the derivatives on the support. Sufficient inactivation was achieved with the vapor phase silanization technique (11). Furthermore, it was necessary to remove the electron-capture-sensitive background with the microcolumns, following derivatization, to achieve the desired sensitivity. The microcolumns probably removed endogenous biological interferences.

In the electron-capture GLC method developed by Edlund and Paalzow (10), the minimum detectable quantity of the derivative was 3.3 pg. The precision of this method at 0.2 ng in plasma (volume presumably 500 µl) was less than ±20% *SD*. The dose per unit body weight administered to the rats was greater than 100 times that administered to humans for therapeutic benefit, and the volume of the rat plasma sample assayed was generally one-tenth to one-twentieth the volume of the human plasma samples used in this newly developed method. Both factors tend to minimize interference of the chromatographic peaks of interest by endogenous biological materials.

The major known metabolite of clonidine, the *p*-hydroxy analog, was neither extracted nor derivatized under the assay conditions. Chloroform extracts of urine from subjects given ^{14}C-labeled clonidine were shown by TLC to contain only clonidine (5). Metabolites, including the *p*-hydroxy analog, that were more polar than clonidine were extracted with butanol. Chromatograms of patients receiving clonidine therapy and diuretics (either chlorthalidone or hydrochlorothiazide) exhibited no interfering peaks. The peak shapes, in particular peak width, were the same in these chromatograms as those obtained from samples prepared by spiking plasma with clonidine. Moreover, endogenous compounds, at least from individuals not receiving clonidine, did not interfere with the clonidine peaks (insert in Fig. 2). Therefore, except for the remote possibility that a diuretic or one of its metabolites is carried through the assay and has the same chromatographic characteristics as the clonidine derivative, the assay is specific.

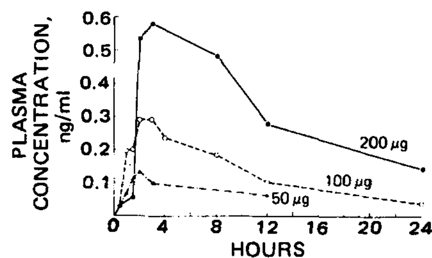


Figure 3—*Plasma concentration–time curves of subject administered three oral doses of clonidine hydrochloride.*

The temporal patterns of plasma concentration in the normotensive subject administered 50, 100, and 200 µg po of clonidine hydrochloride are shown in Fig. 3. The maximum plasma concentrations and the times at which they occurred were 133 (2 hr), 292 (3 hr), and 582 (3 hr) pg/ml, respectively. The peak plasma concentrations were in the ratio 1:2.2:4.4 while the corresponding doses were in the ratio 1:2:4. Since an apparent proportionality existed between peak plasma concentration and dose, the subject apparently behaved as a linear system over the dosing range.

The analysis of unchanged drug excreted in the urine presented no problems since the urinary concentrations were generally two orders of magnitude larger than those in the plasma. Interferences by endogenous compounds were absent. The urinary recoveries were in the range of previously reported values (4, 7, 8).

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