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Gas Chromatographic Analysis of Cetiedil, a Candidate Antisickling Agent, in Human Plasma With Nitrogen-Sensitive Detection

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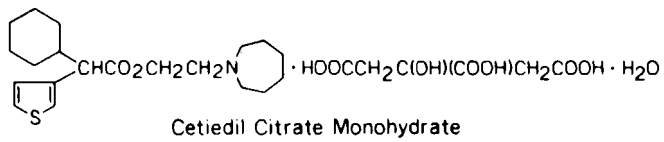
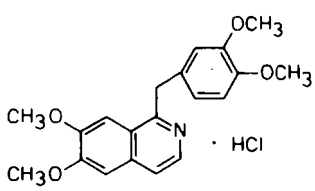
Abstract □ In this report a sensitive gas chromatographic assay for cetiedil, a candidate antisickling agent, in human plasma is described. After a triple extraction procedure, cetiedil was analyzed without derivatization with a nitrogen-phosphorus detector (with papaverine used as the internal standard.) Cetiedil was measured in plasma samples taken from human volunteers administered the drug intravenously.

Keyphrases □ Gas chromatography—cetiedil, human plasma, nitrogen-phosphorus detector □ Antisickling agents—cetiedil, GC analysis in human plasma, nitrogen-phosphorus detector □ Cetiedil—antisickling agent, GC analysis in human plasma, nitrogen-phosphorus detector

At present there is no pharmacological agent available to prevent or attenuate the sequelae of sickle cell crisis. For acute crisis, analgesics, hydration, and symptomatic care remain the standard therapy. In 1977, Cabannes reported on the clinical use of cetiedil citrate monohydrate for management of the painful thromboembolic crises characteristic of sickle cell disease (1).



Cetiedil Citrate Monohydrate



Papaverine Hydrochloride

Cetiedil[1] was first introduced for the treatment of peripheral and cerebral vascular disease (2). Subsequent studies have demonstrated that this vasoactive agent was also active in erythrocytes, in that it could prevent or reverse sickling by altering the permeability characteristics of the erythrocyte membrane (3–5). Several of its pharmacological properties (vasodilation, anticoagulation, analgesia, and inhibition of platelet aggregation) would be favorable for management of the thromboembolic episodes associated with sickle cell disease (6–8).

In this report, a reliable and sensitive gas chromatographic (GC) assay for cetiedil in human plasma, employing a triple extraction procedure and a nitrogen-phosphorus detector is described. The assay is similar to a previously reported method for analysis of papaverine in blood samples (9).

EXPERIMENTAL SECTION

Chemicals—Cetiedil citrate monohydrate[2] (white powder) and papaverine hydrochloride[3] were used without further purification. All solvents[4] were either nanograde or organic residue-analysis grade. All other reagents were certified ACS reagent grade.

Chromatographic Conditions—The gas chromatograph[5] was equipped with a nitrogen-phosphorus detector. A glass column (1.5 m × 2 mm i.d.) packed with 2% OV-101 on Chromosorb W HP 100–120-mesh support[6] was conditioned overnight (300°C). High-temperature septa[5] and graphite ferrules[6] were used. Air, helium, and hydrogen of the highest purity available[7]

[1] Stratene; Laboratoire Innothera, Arcueil, France.
[2] Johnson and Johnson Baby Products Co., Skillman, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] American Scientific Products, Hanrahan, La.
[5] Model Sigma 3B; Perkin-Elmer, Norwalk, Conn.
[6] Supelco, Bellefonte, Pa.
[7] Air Products, Mobile, Ala.

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were used. Operating conditions were as follows: isothermal column temperature, 252°C; injection port temperature, 300°C; detector temperature, 300°C; carrier gas (helium) flow, 20 mL/min; air flow, 54 mL/min; hydrogen flow, 1.5 mL/min; range 1; and attenuation, 2–8. Injections (0.5–2 μL[8]) were made directly on column, the chart[9] speed was 1.3 cm/min.

Glassware Preparation—All glassware was washed in phosphate-free detergent[10] and rinsed with glass-distilled acetone. Silanization was accomplished by treatment with a 5% solution of dimethyldichlorosilane[11] in toluene, followed by rinsing in methanol. (Due to the toxicity of the silanizing agent, it is best handled with gloves and safety glasses in a well-ventilated hood. Addition of methanol produces fumes of hydrogen chloride gas if excess silanizing agent is present.) The glassware was then sonicated[12] in dichloromethane.

Internal Standard—Papaverine hydrochloride (55 ± 1 mg) was accurately weighed and transferred to a 125-mL separatory funnel. Glass-distilled water (25 mL) was added to dissolve the powder; the free base was formed by the addition of 10 M KOH (2 mL) and extracted with dichloromethane (3 × 30 mL). The combined extracts were filtered through anhydrous sodium sulfate (1 kg washed with 6 × 200 mL of methylene chloride) and brought to 100.0 mL (stock solution I). A total of 1 mL of stock solution I was diluted to 100.0 mL with dichloromethane (stock solution II). A total of 5 mL of stock solution II was placed in a 1000-mL volumetric flask and evaporated under a nitrogen[7] stream. 2-Propanol (50 mL) was added to dissolve the residue, and the volume was brought to the mark with *n*-heptane (working internal standard solution).

Cetiedil Standard Solutions—Cetiedil citrate monohydrate (16.0 mg, equivalent to 10.0 mg of free base) was dissolved in water and diluted to 200.0 mL (50 μg/mL). Serial dilutions with water gave working standard solutions of 10, 20, 40, 80, and 160 ng/mL.

Buffer Solution—Boric acid (12.4 g) and potassium chloride (14.9 g) were dissolved in water and diluted to 1 L. Of this solution, 50 mL was adjusted to pH 7.0 with 0.2 M KOH and diluted to 200 mL with water.

Extraction Procedure[13]—*Standards for Calibration Curve*—Buffer solution (pH 7, 4 mL) and blank plasma[14] (2.0 mL) were added to each of six 35-mL centrifuge tubes[15]. To one tube was added 1.0 mL of water; to each of the remaining tubes was added a 1.0-mL aliquot of each of the cetiedil working standard solutions, respectively. After addition of working internal standard solution (20.0 mL) to each tube, the mixtures were shaken at highest speed[16] (30 min) and centrifuged (30 min at 2500 rpm)[17]. The top layer was transferred to another tube containing 1 M HCl (3 mL)[18]. The mixtures were vortexed at highest speed (1 min) and centrifuged, and the top layer was discarded. The acidic extract was washed with *n*-heptane (5 mL) by vortexing for 30 s, centrifuging, and discarding the top layer. To each tube was added 10 M KOH (0.5 mL); after cooling to room temperature, dichloromethane (4 mL) was added. The mixtures were vortexed (1 min) and centrifuged, and the top layer was discarded. The organic layer was transferred directly[19] to a 5-mL vial[6], and the solutions were evaporated[20] to dryness under a nitrogen stream at 40°C. After rinsing the vial walls with dichloromethane (100 μL) and evaporating, the residue was dissolved in 2-propanol (10 μL), and an aliquot was injected.

Subject Samples—The procedure described above was followed for subject samples, except that 2.0 mL of the plasma was used in place of the blank plasma, and 1.0 mL of water was used in place of the cetiedil standard solution.

Calculations—The ratio of peak height of cetiedil to peak height of papaverine was determined for each of the calibration standards; linear regression analysis of these ratios *versus* the known cetiedil concentrations gave a slope and intercept, from which the equation for a straight line was obtained. Cetiedil concentrations in subject samples were obtained by substitution of the experimentally determined peak height ratio into the equation for the standard curve prepared for analysis of every set of samples.

Plasma Level Studies—Three adult male volunteers were admitted[21] to the study after signing informed-consent releases[22]. The subjects received a 0.15-mg/kg dose of cetiedil citrate monohydrate injectable[2] as a 30-mm intravenous infusion[23], followed by a 72-h observation period. Blood samples were obtained through a catheter placed in the arm contralateral to the infusion drip at 0 (prior to infusion), 5, 10, 15, 30, and 45 min and 1, 1.5, 2, 4, and 6 h postinfusion; the catheter was kept patent with heparinized saline. Before each blood collection, dead-space volume heparin solution was removed. After each collection, the catheter was refilled with heparinized saline (33 U of heparin/mL). Samples were collected into heparinized tubes[24] and centrifuged; the plasma was separated and stored at −15°C until assayed.

RESULTS

Standard Curve—In Fig. 1 is shown a typical chromatogram obtained for the analysis of unchanged cetiedil, with papaverine as internal standard. There

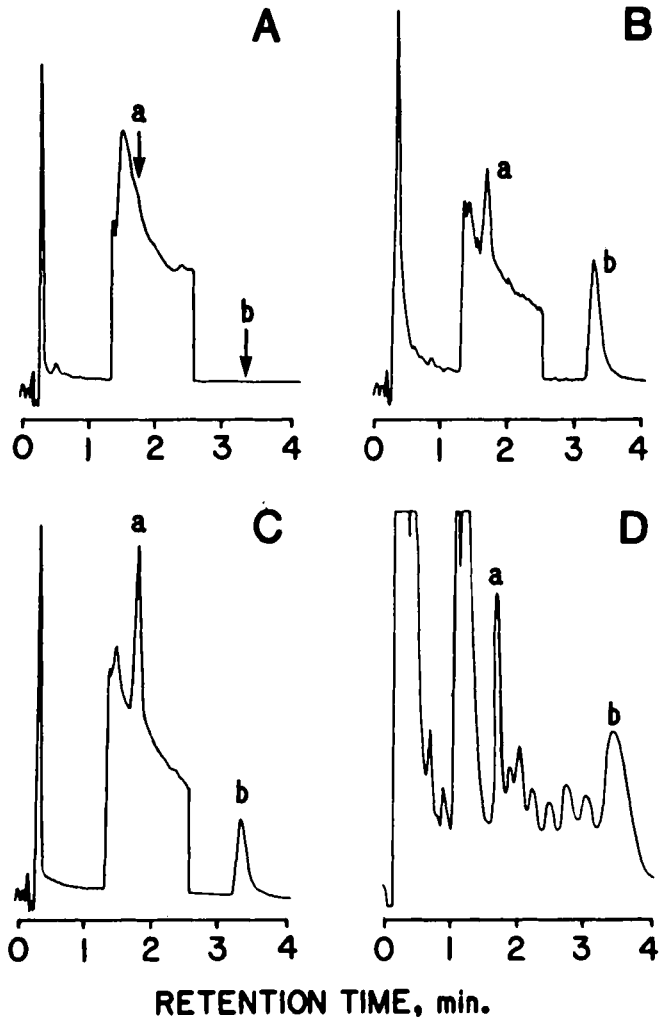

Figure 1—*Typical chromatograms of drug-free plasma carried through the procedure: (A) drug-free plasma to which 5 ng/mL of cetiedil (a) and papaverine (b) were added; (B) plasma from a subject receiving cetiedil; (C) with papaverine as the internal standard; (D) effects of filtration through sodium sulfate with possible contributions from contaminated solvents or glassware. The sudden jumps in the base line are due to programmed changes in recorder attenuation.*

[8] Model 701-RNWG, 10 μL; Hamilton, Decatur, Ga.
[9] Speedomax XL Flatbed; Leeds and Northrup, North Wales, Pa.; or 3390A Reporting Integrator; Hewlett-Packard, Avondale, Pa.
[10] Liquinox; Alconox Products, New York, N.Y.
[11] Kodak Laboratory Chemicals, Rochester, N.Y.
[12] Branson Ultrasonic Cleaner; Fisher Scientific Co., Atlanta, Ga.
[13] Preliminary unpublished work on this procedure was conducted at Cooper Laboratories, Inc.; Analytical Testing Procedure No. 78-15.
[14] Both heparinized and citrated plasma from volunteers gave identical results.
[15] Bellco Glass Co., Vineland, N.J.
[16] Wrist-Action Shaker (setting 10); Burrell, Pittsburgh, Pa.
[17] The long centrifugation time was needed for separation of an interface layer present after shaking. Small amounts of this layer may still be present after separation; great care must be taken to avoid any transfer since particulates may form on addition of HCl.
[18] Washed with 3 × 100 mL of 5% isopropanyl alcohol-toluene.
[19] Filtration through anhydrous sodium sulfate (even after extensive washing with dichloromethane) is not recommended since this procedure consistently produced spurious peaks.
[20] N-Evap; Organomation Associates, Northborough, Mass.
[21] Clinical Pharmacology Research Unit, University of South Alabama College of Medicine, Mobile, Ala.
[22] Both the consent form and the study protocol were approved by the Committee for Protection of Human Subjects and the Scientific Review Committee, University of South Alabama College of Medicine.
[23] Intravenous Infusion Delivery Set; Travenol Laboratories, Deerfield, Ill., and Dialaflo; Sorensen, Salt Lake City, Utah.
[24] Vacutainer; Becton, Dickinson and Co., Rutherford, N.J.

Table I—Cetiedil Determination in Human Plasma [a]

Cetiedil Added, ng/mL	Cetiedil Determined, ng/mL	*SD*	*n*
5	4.99	0.43	4
10	4.87	0.84	8
	11.45	1.63	5
20	19.54	2.66	5
	22.60[b]	1.43	5
40	49.76	4.05	5
200	181.35	16.14	8

[a] Different concentrations of cetiedil were added to drug-free plasma, and samples were then analyzed by the procedure described in the text. [b] Using 4 mL of blank plasma.

were no interfering peaks at the retention times for cetiedil or papaverine. The retention times for cetiedil and papaverine were 1.7 and 3.3 min, respectively. There was a linear relationship between the ratios of the peak height of cetiedil to the peak height of papaverine and the corresponding concentrations of cetiedil. The resulting general equation for the standard curve as described above ($y = 0.119x - 0.0259$) was linear ($r = 0.984$; $n = 37$) over the concentration range of 5–80 ng/mL, where y was the peak height ratio, and x was cetiedil concentration.

Precision—The precision of the method was determined by adding different concentrations of cetiedil and analyzing samples of each concentration by the described procedure (Table I). The average coefficient of variation over the concentration range of 5–200 ng/mL was 11%.

Reproducibility—To determine the day-to-day reproducibility, five different cetiedil concentrations were added to drug-free plasma, and the samples were analyzed over a period of 3 months (Table II). The average day-to-day coefficient of variation over the concentration range of 5–80 ng/mL was 16.3%. These results also indicate that cetiedil in plasma samples was stable for at least 3 months when kept frozen.

Recovery—2-Propanol solutions of cetiedil citrate were prepared so that injection of 1 μL would give a peak representing complete recovery of cetiedil from extracted plasma samples. The ratio of cetiedil peak height from the extracted samples to the standard solutions gave an average cetiedil recovery of 67.5 ± 15.5% over a concentration range of 10–80 ng/mL. Papaverine recovery was determined similarly (57.4 ± 5.2%).

Sensitivity—At an added cetiedil concentration of 0.5 ng/mL, the cetiedil peak was not routinely detected under optimal conditions (chromatograph attenuation, 2; injection volume, 2.0 μL). At 1 ng/mL, cetiedil peaks were detectable; however, the determined value was 2.56 ± 0.09 ng/mL ($n = 5$). This unacceptably large value may reflect nonlinearity of the standard curve at <5 ng/mL. When 2.0 mL of plasma was used, 5.0 ng/mL (average *CV*, 12.9%; Table I) was the sensitivity limit. From the general standard curve equation ($y = 0.0119x - 0.0259$), the theoretical sensitivity limit of the procedure would be 2.18 ng/mL. This large value may indicate that the plasma background was producing a significant amount of signal. Alternatively, the tailing of the solvent peak under maximal detection conditions may contribute to the large amount of background signal.

Plasma Level Studies—In Fig. 2 is shown the decline of unchanged cetiedil in human plasma after a 30-min intravenous infusion at a dose of 0.15 mg/kg in three subjects. The disposition of cetiedil is described by a two-compartment model (10).

DISCUSSION

The described procedure is sensitive and reproducible over a 5–80 nanogram per milliliter range. Detector response was linear over a wide concentration range. When kept in the dark at −15°C, the samples remained stable for at least 3 months without any added preservative. Standard solutions were kept in the dark at room temperature and remained stable for at least 2 weeks. All glassware were silanized to minimize drug losses.

In summary, this report, along with many other recent studies, illustrates the utility of the nitrogen-phosphorus detector in measuring drug levels over a 5–80 nanogram per milliliter range. Although combined GC-MS may provide greater sensitivity and require less time for sample analysis, such instrumentation is not universally available. Application of the nitrogen-sensitive detection technique to measurement of the candidate antisickling agent cetiedil has provided preliminary information regarding its plasma levels in humans.

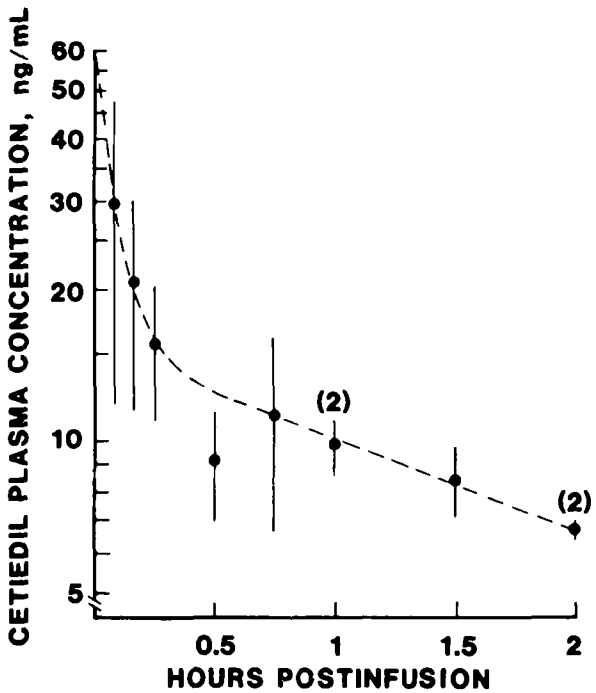

Figure 2—*Plasma level decline of unchanged cetiedil after an intravenous infusion of 0.15 mg/kg;* n = *3 unless otherwise indicated by numbers in parentheses.*

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Table II—Reproducibility of Cetiedil Determination over Time [a]

Amount of Cetiedil Added, ng/mL	Concentration of Cetiedil Found, ng/mL						
	1 d	3 d	5 d	7 d	30 d	90 d	Mean ± *SD*[b]
5	7.1	5.7	5.4	5.4	5.6	5.2	5.64 ± 0.82
10	12.2	11.2	6.0	9.2	11.2	8.6	9.75 ± 2.25
20	22.3	20.6	18.4	28.4	24.8	22.8	22.86 ± 3.46
40	49.2	42.3	36.1	46.0	57.4	42.4	45.58 ± 7.26
80	96.6	84.9	80.7	111.4	100.0	84.1	92.93 ± 11.82

[a] Different concentrations of cetiedil were added to drug-free plasma and kept frozen in aliquots. Each aliquot was thawed on the day of analysis. [b] Reproducibility at 10 months could be demonstrated only at cetiedil concentrations of 5 ($n = 4$), 20 ($n = 3$), and 40 ($n = 3$) ng/mL; the respective determined mean ± *SD* values were 4.05 ± 0.46, 19.74 ± 2.15, and 32.76 ± 6.25 ng/mL. Determined values for 10 and 80 ng/mL were 71–75% of the theoretical added values.

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Radiopaque Liposomes: Effect of Formulation Conditions on Encapsulation Efficiency

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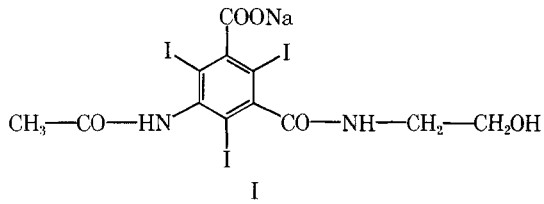
Abstract □ Liposomes containing sodium ioxitalamate were prepared by sonication. Suitable amounts of purified soybean phosphatidylcholine and cholesterol were used at various molar ratios. Stearylamine or dicetylphosphate were added to this lipid composition when charged liposomes were required. After sonication and removal of unencapsulated solute, this manufacturing process yielded small multilamellar vesicles as confirmed by electron microscopy. These liposomes did not exhibit a narrow range of size distribution; the mean particle size varied from 135 to 145 nm. With respect to the efficiency of encapsulation, two parameters were distinguishable: the volume of encapsulated aqueous space per unit of lipid weight and the percentage of the contrast agent added that became encapsulated in the liposomes. Investigation of the preparative parameters revealed that increased molar ratios of cholesterol yielded higher aqueous volume and iodine contents in the liposomes, which were attributed to a reduction of the liposome permeability to the contrast agent. However, the inclusion of cholesterol into the bilayer liposomal membrane was limited, probably by solubility restrictions. Negatively and positively charged liposomes had higher rates of encapsulation than did neutral liposomes. This result was expected since efficient encapsulation of polar compounds requires formation of large aqueous spaces within the vesicles per mole of lipids. Increase of the lipid fractions at a constant ratio of phosphatidylcholine–cholesterol, with all the other factors kept constant, reduced the aqueous volume entrapped per millimole of lipid and, consequently, the iodine content in the liposomes. However, an increase in the initial sodium ioxitalamate concentration diminished the aqueous volume entrapped in the liposomes but increased the iodine content.

Keyphrases □ Liposomes—radiopaque, effect of formulation conditions on encapsulation efficiency □ Formulations—radiopaque liposomes, effect on encapsulation efficiency □ Encapsulation—radiopaque liposomes, effect of formulation conditions

The possibility of utilizing liposomes as vehicles for therapeutic agents had been widely considered over the last 16 years. The potential applications have been extensively reported and clearly summarized (1–3). The ultimate goal has been the targeting of drugs and other biologically active ingredients to specific cells within the body. However, this cannot be easily accomplished since it has been shown in numerous reports that intravenously injected liposomes localize predominantly in the liver, spleen, and other organs rich in cells of the reticuloendothelial system (4).

The specific hepatic uptake can be exploited to design a selective delivery system based on liposomes containing sodium ioxitalamate, which is a water-soluble iodinated contrast agent characterized by its high iodine content (57%). The radiopaque agent, sodium ioxitalamate (I), is primarily used for urography because of its pharmacokinetic properties, (5, 6); however, side



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effects, allergic reactions, and intolerance have been reported. The preparation of liposomes containing the radiopaque agent is, therefore, also aimed at reducing or avoiding these adverse reactions since selective liver uptake enables administration of smaller amounts of radiopaque agent for a similar or even improved diagnostic result. The liposomal preparation will probably be able to produce a visible contrast enhancement of the liver, which will help improve the diagnostic accuracy of computerized tomography of this organ. Some attempts, including the preparation of brominated radiopaque liposomes (7) and the encapsulation of diatrizoic acid salts within liposomes (8), have already been performed, yielding preliminary results that are promising. However, this interesting approach is limited by the amount of contrast agent that can be entrapped in the aqueous phase of liposomes and the potential release of the opaque ingredient from the liposomes. The objective of this study, therefore, is to incorporate the contrast agent within liposomes and to investigate some of the physical and chemical factors which may control the efficiency of the encapsulation rate. The results of such an investigation offer the possibility of optimizing liposome formulation, enabling a high encapsulation rate and, consequently, an increase in the opacification of the liposomes caused by their high iodine content.

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