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DETERMINATION OF (—)-*threo*-CHLOROCITRIC ACID IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—POSITIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring (—)-*threo*-chlorocitric acid in human plasma. Plasma is acidified to pH 1 to minimize lactonization and a ^{13}C analogue of (—)-*threo*-chlorocitric acid is added as internal standard. The acidified plasma is then extracted with ethyl acetate containing 10% methanol. The ethyl acetate—methanol extract is back-extracted with acetate buffer (pH 5). This extract, following adjustment to pH 1, is reextracted with ethyl acetate. The residue after removal of the ethyl acetate is treated with ethereal diazomethane. The wet residue is reconstituted in ethyl acetate and a portion of this solution is analyzed by gas chromatography—chemical ionization mass spectrometry. The mass spectrometer is set to monitor *m/z* 269 [MH^+ of trimethylated (—)-*threo*-chlorocitric acid] and *m/z* 270 [MH^+ of trimethylated (—)-*threo*-[^{13}C]chlorocitric acid] in the gas chromatographic effluent. The *m/z* 269 to *m/z* 270 ion ratio in a sample containing an unknown amount of (—)-*threo*-chlorocitric acid is converted to an amount of compound using a calibration curve. The calibration curve is generated by analyzing control plasma spiked with various known amounts of (—)-*threo*-chlorocitric acid and a fixed amount of (—)-*threo*-[^{13}C]chlorocitric acid. The limit of quantitation is 0.1—0.6 $\mu g\ ml^{-1}$, depending on the characteristics of the calibration curve generated with each set of samples. The precision (relative standard deviation) at a concentration of 2 $\mu g\ ml^{-1}$ is 3.3%.

INTRODUCTION

(—)-*threo*-Chlorocitric acid is currently undergoing testing as an anorectic agent [1—3]. This paper reports a specific and relatively simple gas chromatographic—mass spectrometric (GC—MS) procedure for (—)-*threo*-chlorocitric acid. The assay features the use of a ^{13}C-labeled analogue of (—)-*threo*-chlorocitric acid as internal standard and the GC- chemical ionization MS (CIMS)

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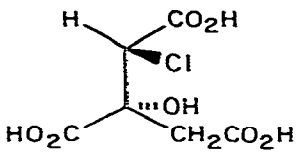
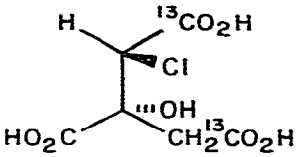
analysis of both (−)-*threo*-chlorocitric acid and its internal standard as their respective methyl tri-ester.



(−)-*threo*-chlorocitric acid



(−)-*threo*-[^{13}C]chlorocitric acid (50% labelled at each position)

EXPERIMENTAL

Equipment and operating conditions

Gas chromatography. A Finnigan Model 9500 gas chromatograph was equipped with a glass U-shaped column (152 cm × 2 mm I.D.) packed by Whatman (Clifton, NJ, U.S.A.) with OV-17 on μPartisorb®. The column was conditioned overnight at 295°C with a nitrogen flow-rate of 10 ml min^{-1}. The temperatures of the injector, column oven, interface oven and transfer line were 300, 170, 250 and 250°C, respectively. Methane (1.5 kg cm^{-2}) was used as carrier gas. The retention time of derivatized (−)-*threo*-chlorocitric acid under these conditions was approximately 95 sec.

Mass spectrometry. A Finnigan Model 3200 quadrupole mass spectrometer was tuned to give the maximum response consistent with reasonable ion peak shape and unit resolution. Methane (68 Pa) was used as CI reagent gas. The voltage across the continuous dynode electron multiplier was −2.0 kV and the voltage on the conversion dynode was +2.5 kV.

Peak monitor. A Finnigan Promim® was used to set the mass spectrometer to monitor *m/z* 269 and *m/z* 270. Each Promim channel was operated at 100 msec dwell-time, a 0.5-Hz frequency response, and a sensitivity of 10^{-8} A/V. Ion chromatograms were recorded on a four-channel Rikadenki pen recorder.

Glassware. Culture tubes (16 ml, Pyrex 9825), with Teflon®-lined screw caps, were used for plasma extraction and the ethyl acetate back-extraction. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the evaporation of the final solvent extract and subsequent reaction with diazomethane. Prior to use, all tubes were cleaned with detergent, rinsed with water, treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.), and finally rinsed with methanol and dichloromethane (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Solvent evaporator. Solvents were removed at 35°C using a nitrogen evaporator (N-Evap, Organomation Assoc.).

Shaker. Extractions were carried out by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach).

Centrifuge. Centrifugations were done at 10°C using a Damon/IEC Model CRU-500 refrigerated centrifuge operated at 1600 *g*.

Chemicals

(−)-*threo*-Chlorocitric acid and (−)-*threo*-[^{13}C]chlorocitrate were obtained

from Dr. W. Scott and Dr. C.W. Perry, respectively, Hoffmann-La Roche, Nutley, NJ, U.S.A. Nanograde ethyl acetate and methanol were obtained from Burdick and Jackson Labs., Muskegon, MI, U.S.A. Glacial acetic acid, 12 *N* hydrochloric acid and sodium acetate were obtained from J.T. Baker, Phillipsburg, NJ, U.S.A. Diazald® and ethyl ether were obtained from Aldrich, Milwaukee, WI, U.S.A. and Mallinckrodt, St. Louis, MO, U.S.A., respectively.

Solutions

(−)-threo-Chlorocitric acid, stock solution. Dissolve 1.00 mg of titled compound in 1.00 ml of methanol.

(−)-threo-Chlorocitric acid, spiking solutions. Using the stock solution, prepare four solutions containing either 0.5, 2.5, 5 or 10 μg of the titled compound per 50 μl of methanol (solutions A, B, C, D, respectively).

(−)-threo-[^{13}C] Chlorocitric acid, stock solution. Dissolve 1.00 mg of the titled compound in 1.00 ml of methanol.

(−)-threo-[^{13}C] Chlorocitric acid, spiking solution. Using the stock solution, prepare one solution containing 5 μg of the titled compound per 50 μl of methanol (solution E).

10% methanol in ethyl acetate. 100 ml of methanol is diluted to 1 l with ethyl acetate.

0.2 M pH 5 acetate buffer. 16.41 g of sodium acetate and 5.2 ml of glacial acetic acid are dissolved in 1000 ml of distilled water.

6 N HCl. Dilute 1 volume of 12 *N* HCl with 1 volume of distilled water.

2 N HCl. Dilute 1 volume of 12 *N* HCl with 5 volumes of distilled water.

Ethereal diazomethane. Ethanol-free ethereal diazomethane is prepared from Diazald® using the manufacturer's suggested procedure.

Procedure

The calibration curve samples are prepared as follows. Transfer a 50-μl aliquot of methanol containing either 0, 0.5 μg (solution A), 2.5 μg (solution B), 5 μg (solution C) or 10 μg (solution D) of (−)-*threo*-chlorocitric acid to 16-ml culture tubes, followed by another 50-μl aliquot of methanol containing 5 μg of (−)-*threo*-[^{13}C] chlorocitric acid (solution E). Add 0.5 ml of 2 *N* hydrochloric acid and 1.0 ml of control human plasma and vortex each mixture thoroughly.

Spike the plasma samples (1 ml) containing unknown amounts of (−)-*threo*-chlorocitric acid with 5 μg of (−)-*threo*-[^{13}C] chlorocitric acid and then acidify and vortex them as described for the calibration curve samples.

Extract samples by shaking for 10 min with 5 ml of ethyl acetate containing 10% methanol. Centrifuge the tubes; transfer 4 ml of the top layer to another culture tube and back-extract by shaking the tubes for 5 min with 0.5 ml of 0.2 *M* acetate buffer pH 5. Centrifuge the tubes and discard as much of the organic layer as possible. Add 2 ml of ethyl acetate containing 10% methanol, vortex the tubes briefly, centrifuge the tubes and discard the organic solvent wash. Acidify the aqueous phase with 0.2 ml of 6 *N* hydrochloric acid and extract with 3 ml of ethyl acetate (without methanol). Shake the tubes for 5 min, centrifuge, and transfer a 2.5-ml aliquot of the ethyl acetate extract to a 5-ml conical centrifuge tube. Evaporate the ethyl acetate extract almost to dryness.

Add 0.6 ml of ethereal diazomethane solution and vortex the tubes. Allow the stoppered tubes to stand at room temperature for 10 min. To minimize losses of the derivative, evaporate the methylating solvent without heating by keeping the tubes positioned out of the water bath. Occasionally immerse the tubes in the water bath to remove condensation. Remove the tubes from the N-Evap just before the evaporation is completed. Add 100 μl of ethyl acetate to the wet residue and inject 2—5 μl of this solution into the GC—MS system. Turn off the GC valve 30 sec after injection. Turn on the ionizer 45 sec after injection.

Calculations. The peak heights are measured with a ruler and the *m/z* 269 to *m/z* 270 ion ratio is calculated. The data obtained from the calibration curve samples are fitted by a nonlinear least-squares program to the isotope dilution equation $R = (x + A)/(Bx + C)$ [4, 5]. In this equation x is the amount of (−)-*threo*-chlorocitric acid added to plasma, R is the ion ratio obtained from the spiked samples, and A, B, C are constants determined by the nonlinear least-squares fit. To find an unknown x given an experimentally determined R, the above equation is rearranged to solve for x: $x = (RC - A)/(1 - RB)$.

Clinical samples

A 90-kg healthy male volunteer did not eat anything for 7.5 h prior to receiving a 200-mg oral dose of (−)-*threo*-chlorocitric acid. Aliquots (10 ml) of whole blood were drawn at −0.25, 0.5, 0.67, 1, 1.25, 1.5, 2, 3 and 4 h post-dosing into heparinized Vacutainer® 6527 from Becton-Dickinson. Plasma is obtained following centrifugation of the blood at 2000 *g* at 5°C for 30 min. The plasma is then immediately acidified with 0.025 ml of 12 *N* hydrochloric acid per ml of plasma and stored at −10°C until analyzed.

RESULTS AND DISCUSSION

Complicating the analysis of (−)-*threo*-chlorocitric acid is the tendency of the drug, especially in an alkaline or neutral environment, to lactonize with generation of HCl. To minimize this possibility, plasma samples are acidified to pH 1 immediately after they are obtained from freshly drawn blood, and a mild derivatizing reagent, diazomethane, is used to convert the drug into a compound which can be gas chromatographed.

The methane positive CI mass spectra of derivatized (−)-*threo*-chlorocitric acid and derivatized (−)-*threo*-[^{13}C]chlorocitric acid are shown in Fig. 1. The mass spectra consist of MH^+ ions and base peak ions reflecting the loss of the elements of acetic acid from the MH^+ ions. The fact that the fragment ion in the mass spectrum of (−)-*threo*-[^{13}C]chlorocitric acid shows no loss of label suggests that the loss is of the carboxyl group attached to the carbon bearing the hydroxyl group. For the assay, the MH^+ ions at *m/z* 269 and *m/z* 270 are monitored in the GC effluent for (−)-*threo*-chlorocitric acid and its ^{13}C-labeled internal standard, respectively.

Typical selected ion current profiles from the analyses of 1 ml of either control plasma spiked with 0.5 μg of (−)-*threo*-chlorocitric acid (A), or plasma from a subject either 15 min before (B) or 2 h after (C) receiving a 200-mg dose of (−)-*threo*-chlorocitric acid are shown in Fig. 2. The small response in select-

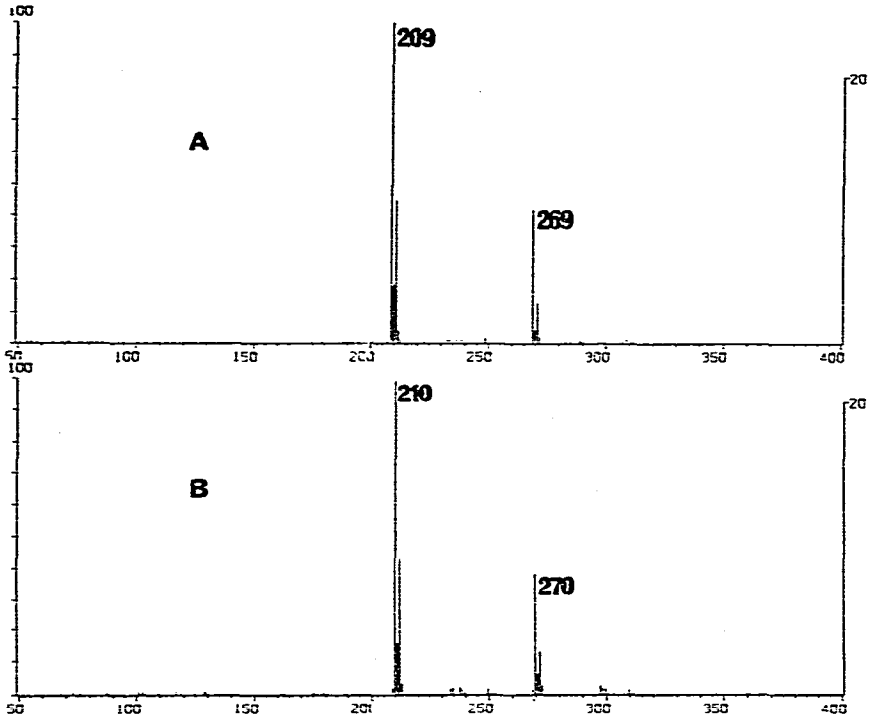


Fig. 1. Methane positive CI mass spectra of trimethylated derivatives of (—)-*threo*-chlorocitric acid (A) and (—)-*threo*-[^{13}C]chlorocitric acid (B).

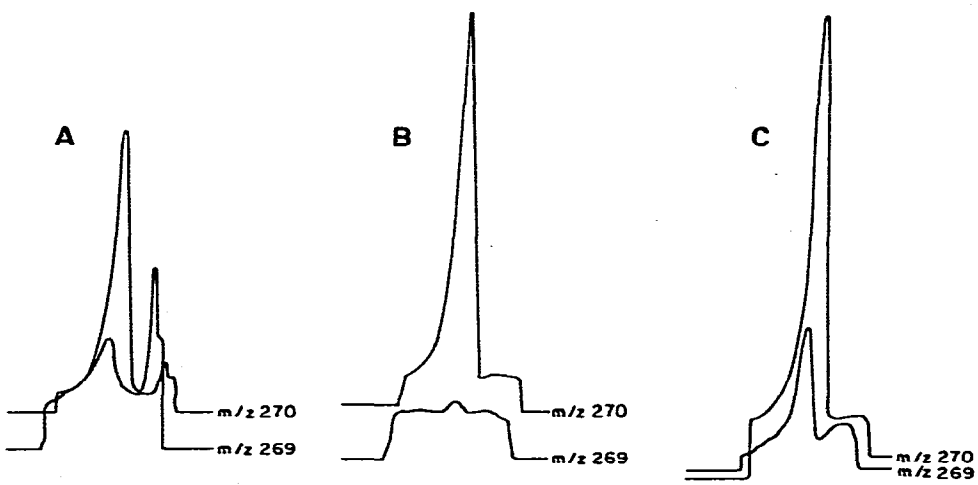


Fig. 2. Selected ion current profiles from the analyses of 1 ml of either control plasma spiked with 0.5 μg of (—)-*threo*-chlorocitric acid (A) or plasma from a subject either 15 min before (B) or 2 h after (C) receiving a 200-mg oral dose of (—)-*threo*-chlorocitric acid. All samples were spiked with 5 μg of (—)-*threo*-[^{13}C]chlorocitric acid. The measured concentration of (—)-*threo*-chlorocitric acid in the 2-h post-dose sample was 1.0 μg ml^{-1}.

ed ion current profile B in Fig. 2 at *m/z* 269 is from charge exchange ionization of (−)-*threo*-[^{13}C]chlorocitric acid and currently defines the limit of quantitation. When using 5 μg of the internal standard, this response typically represents between 0.05 and 0.2 μg of (−)-*threo*-chlorocitric acid. For any given set of samples, the limit of quantitation is considered to be three times the response at *m/z* 269 in the selected ion current profiles from the calibration curve plasma samples spiked only with (−)-*threo*-[^{13}C]chlorocitric acid. Typically this response is equivalent to a concentration of (−)-*threo*-chlorocitric acid of between 0.15 and 0.6 μg ml^{-1}.

Assay precision and recovery of (−)-*threo*-chlorocitric acid were determined by spiking six separate 1-ml plasma samples with 2.0 μg of authentic compound and analyzing the samples using the procedure described. The mean (± S.D.) concentration found was 2.10 ± 0.07 μg ml^{-1} and indicates a precision (relative standard deviation) of 3.3% at this concentration. The mean recovery (± S.D.) of the compound from these samples, based on a comparison of the responses of derivatized (−)-*threo*-chlorocitric acid from the calibration samples with the responses of external standard solutions containing known amounts of derivatized (−)-*threo*-chlorocitric acid, was 25 ± 6%.

The stability of (−)-*threo*-chlorocitric acid in acidified plasma upon prolonged storage was determined. To 20 ml of control plasma were added 100 μg of (−)-*threo*-chlorocitric acid. The plasma was acidified with 0.5 ml of 12 *N* hydrochloric acid. Duplicate 1-ml aliquots of the spiked plasma were analyzed six months later. The measured concentrations of (−)-*threo*-chlorocitric acid were within 6% of the initial plasma concentration measured on day 1.

The plasma concentration–time curve for (−)-*threo*-chlorocitric acid in a male volunteer following a 200-mg oral dose of the drug is shown in Fig. 3. The data can be fitted, using the program NONLIN [6], to a one-compartment open model [7] with complete absorption (correlation coefficient = 0.99). A peak

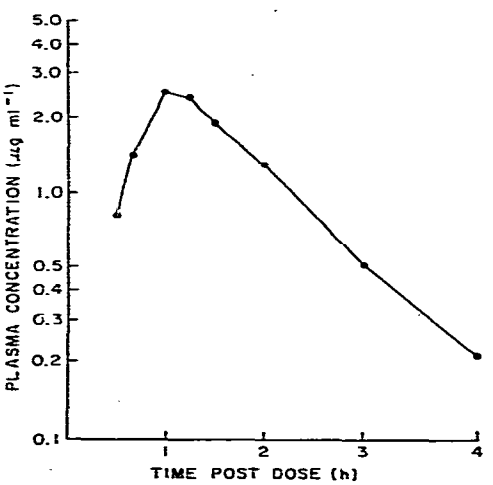


Fig. 3. Plasma concentration–time curve for a male volunteer who had received a 200-mg oral dose of (−)-*threo*-chlorocitric acid. The drug concentrations at −15, 10 and 20 min post-dose were all nonmeasurable.

plasma concentration of 2.5 μg/ml occurred at 1 h and the drug disappeared from the plasma with a half-life of 35 min. The volume of distribution, 39 liters, was 43% of body weight.

CONCLUSION

A specific and relatively simple GC—MS procedure has been described which can measure (−)-*threo*-chlorocitric acid in plasma for up to 4 h following a 200-mg dose of the drug.

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