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Note

Determination of linogliride in biological fluids by high-performance liquid chromatography

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Linogliride fumarate, N-(1-methyl-2-pyrrolidinylidene)-N′-phenyl-4-morpholinecarboximidamide (*E*)-2-butenedioate, is a new oral hypoglycemic agent [1, 2], currently undergoing clinical evaluation.

This paper describes the high-performance liquid chromatographic (HPLC) method which has been employed successfully in analyzing biological samples from clinical and non-clinical studies. The assay presented in this paper is sensitive, specific, and simple to perform. Using an automated injector (WISP) and a Lab Automation Computer System (HP-3354), up to 96 plasma samples are easily analyzed during an 8-h working day.

EXPERIMENTAL

Reagents

Pirogliride (Pg), a pyrrolidine analogue of linogliride (Lg) (Fig. 1) was used as the internal standard. Pirogliride and linogliride were obtained as the sulfate and the fumarate salt, respectively (McNeil Pharmaceutical, Spring House, PA, U.S.A.). Plasma used in the preparation of the standard curve was obtained from the Biological Specialty Corporation (Lansdale, PA, U.S.A.). The chromatography solvents (acetonitrile and methanol) were HPLC grade from Fisher

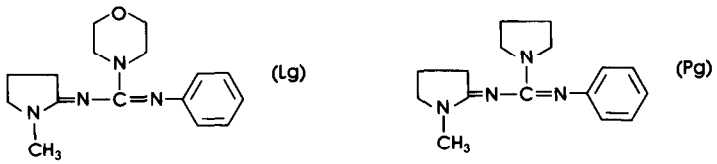

Fig. 1. Chemical structures of pirogliride (Pg) and linogliride (Lg).

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Scientific (Fair Lawn, NJ, U.S.A.). Triply distilled water (Ephrata Mountain Water, Manheim, PA, U.S.A.) was used in the preparation of solutions, buffers and in the mobile phase. The glacial acetic acid, diethyl ether, and potassium hydroxide used were ACS grade (Mallinckrodt, Paris, KY, U.S.A.). The sodium acetate trihydrate was analytical reagent grade from Mallinckrodt.

High-performance liquid chromatography

The HPLC system consisted of a Beckman Model 112 solvent delivery system, and a Beckman Model 160 ultraviolet absorbance detector equipped with a 254-nm wavelength filter. The column was 25 cm × 4.6 mm I.D. packed with 10-μm RP-2 sorbent (Brownlee Labs., Santa Clara, CA, U.S.A.). A 3 cm × 4.6 mm I.D. 10-μm RP-2 guard column from Brownlee Labs. was also used. The mobile phase was composed of acetonitrile—0.1 *M* acetate buffer pH 4.6—methanol (40:40:20, v/v/v). The mobile phase was prepared fresh daily and filtered through a 0.45-μm Millipore® filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approx. 100 ml of mobile phase prior to use. After conditioning the column, the flow-rate was maintained at 2.0 ml/min. Retention times were 4.3 min for linogliride and 6.2 min for the internal standard (Fig. 2). Samples were injected using a Waters Intelligent Sample Processor (WISP 710B, Waters Assoc., Milford, MA, U.S.A.).

Plasma standard solutions

Plasma standards (volume 10.0 ml) containing 2—2000 ng/ml linogliride in plasma were prepared as follows: 0.1 ml of linogliride fumarate (conversion factor to linogliride free base is 1.405) solution in methanol containing the appropriate amount (200—200,000 ng equivalent) of the linogliride free base, was added to 9.9 ml of the drug-free plasma.

Equipment

Disposable screw-top bottles (volume 14.5 ml) with polyethylene-lined caps and 12-ml centrifuge tubes (conical bottom) were employed in the extraction. Prior to use, all glassware was soaked in detergent for 2 h, rinsed thoroughly with distilled water and heat-treated for 3 h at 270°C. Polyethylene-lined screw-caps were soaked in *n*-heptane for 1 h and dried at 60°C prior to use.

Extraction procedure

An aliquot of plasma or urine (0.1—1.0 ml) containing linogliride as a standard or as an unknown was placed in a 14.5-ml disposable screw-top bottle. To this were added 0.1 ml of a methanolic internal standard solution containing 1000 ng/ml internal standard, 0.2 ml of 5 *M* potassium hydroxide solution and 3.0 ml of diethyl ether. The capped bottles were shaken for 10 min on a table-top mechanical shaker (Eberbach) at 240 oscillations per minute. The bottles were then placed upright in a dry ice—methanol bath for approx. 2 min or until the aqueous layer was completely frozen. The diethyl ether (2.7—3.0 ml) was then decanted into a 12-ml conical centrifuge tube to which 0.2 ml of 0.1 *M* acetate buffer at pH 4.6 had been added. Each sample

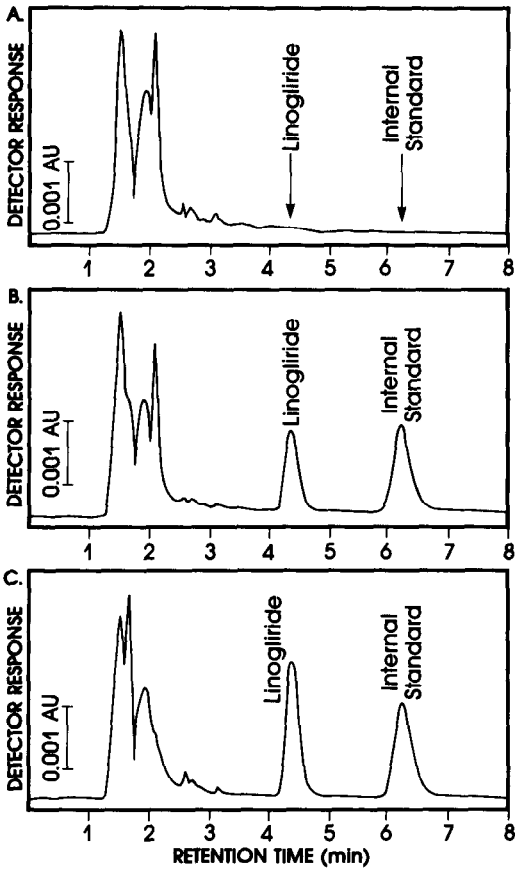

Fig. 2. Typical chromatograms from extracted human plasma samples. (A) Blank. (B) Sample seeded with 50 ng/ml linogliride and 100 ng/ml internal standard. (C) Sample taken 10 h following a 100-mg oral dose; the linogliride concentration was calculated to be 81 ng/ml.

was vortexed for 5 sec using a Vortex-Genie® (Scientific Instruments, Springfield, MA, U.S.A.) at a speed setting of 6. The aqueous and diethyl ether layers were allowed to separate for 1 min, after which the diethyl ether layer was aspirated. All of the acetate buffer layer was transferred to an insert tube which was placed onto the sample carousel of the automatic injector (WISP 710B). A 100-μl aliquot of the acetate buffer layer was injected into the HPLC system.

Quantitation and data handling

Standard curve data were generated by analyzing a series of plasma standards (2—2000 ng/ml). Data were analyzed by linear regression analysis (peak height ratios versus plasma concentrations) using the reciprocal of the variance of the peak height ratios as the weighting factor. Concentrations of linogliride in unknown plasma samples were calculated from the linear regression equation using the measured peak height ratios.

A Hewlett-Packard 3354 Lab Automation System was used for automatic data acquisition, temporary data storage, data analysis and report generation. Calibration curves, calculated linogliride concentrations and final reports were generated using internally developed application software.

RESULTS AND DISCUSSION

Recovery

The recovery of linogliride from plasma/urine was estimated at two concentrations (100 and 1000 ng/ml) with six determinations at each concentration. A mean recovery of 53.9 ± 5.8% was obtained for linogliride in plasma/urine. The recovery of the internal standard from plasma/urine at 100 ng/ml was 54.8 ± 2.0% (twelve determinations).

Stability

Freshly prepared plasma/urine standard solutions were compared to plasma/urine standard solutions frozen at −5°C for one month. The variations in peak height ratios at each drug level between 5 and 1000 ng/ml were insignificant.

Furthermore, linogliride and the internal standard were found to be stable in pH 4.6 acetate buffer at room temperature overnight. Therefore, injection of extracted samples can be performed on the next day without observable changes in peak height ratios.

Sensitivity

Linogliride and the internal standard absorb ultraviolet light strongly at 254 nm in the mobile phase (for linogliride, ϵ_{254} = 21,700). When 1 ng of linogliride was injected into the liquid chromatograph under the stated conditions, a peak with a signal-to-noise ratio of 50 was obtained. It was observed that the ultraviolet absorption of linogliride is twice as strong in a mobile phase composed of acetonitrile—water (1:1, v/v) than in one of methanol—water (1:1, v/v). However, 20% methanol was incorporated into the final mobile phase to reduce the total run time for each injection.

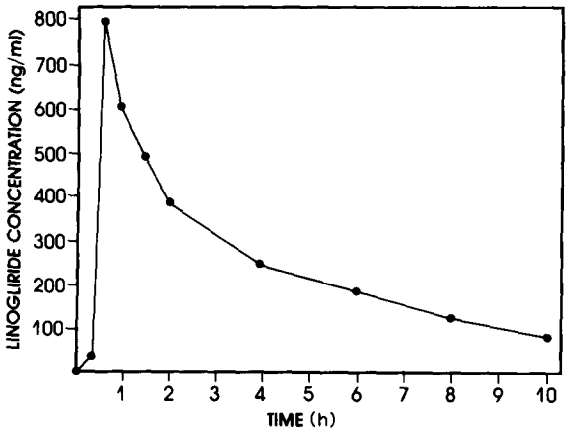

Fig. 3. Plasma concentration—time profile from a subject following oral administration of a 100-mg linogliride dose as a capsule.

The lowest concentration of linogliride that has been determined quantitatively in 1-ml plasma/urine samples is 2 ng/ml. This sensitivity appears more than adequate for clinical studies since the plasma concentration of linogliride in normal volunteers 10 h following oral administration of a capsule dose (100 mg) is approx. 80 ng/ml (see Fig. 3). For smaller sample volumes, assay sensitivity was reduced proportionally. Using 0.1-ml plasma/urine samples, the lower detection limit was 20 ng/ml.

Selectivity

The selectivity of the assay is demonstrated in Fig. 2 which displays chromatograms of processed samples of drug-free plasma, drug-free plasma spiked with known amounts of linogliride and human plasma from a clinical study. Interference from endogenous human plasma/urine components was not observed at the retention times of linogliride and the internal standard.

Other drugs that could potentially be co-administered with linogliride are the antihypertensives, diuretics, and non-steroidal anti-inflammatory agents. Plasma samples spiked with the drugs propranolol, furosemide, hydrochlorothiazide, indomethacin, ibuprofen and tolmetin sodium were analyzed using the present procedure and no interfering peaks were observed except for propranolol. Propranolol was extracted and eluted approx. 0.3 min after linogliride using these assay conditions. In this event, base-line separation of the compounds can be achieved with slight adjustments of the mobile phase composition.

Standard curve

Standard curve data generated by analyzing plasma standard solutions are presented in Table I. Linear regression analysis (peak height ratios versus

TABLE I

SUMMARY OF STANDARD CURVE DATA GENERATED ON FOUR CONSECUTIVE DAYS OF ANALYSIS FOR LINOGLIRIDE IN PLASMA

Linogliride seeded (ng/ml)	*n*	Mean value found* (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
2	7	1.9	0.19	9.8	−2.6
5	7	5.1	0.33	6.5	2.0
10	8	10.1	0.53	5.2	1.1
20	8	19.9	1.3	6.4	−0.5
50	8	49.0	2.1	4.2	−2.0
100	7	99.0	6.9	7.0	−1.0
200	8	200.0	9.6	4.8	−0.1
500	7	486.0	33.0	6.9	−2.8
1000	8	1013.0	55.0	5.4	1.3
2000	8	2076.0	110.0	5.3	3.8

*Calculated from the equation: $[\text{Linogliride}] = \frac{\text{Peak Height Ratio} - 0.001}{0.0194}$ where 0.0194 and 0.001 are the slope and intercept of the regression equation. The regression equation was obtained by method of least squares with data weighted by 1/variance.

linogliride plasma concentrations) gave a slope of $19.36 \cdot 10^{-3} \pm 0.14 \cdot 10^{-3}$ S.D., a *Y*-intercept of 0.0013 ± 0.0013 S.D. and a correlation coefficient equal to 0.998 with a Student's *t* of 135. The composite standard curve is linear between 2 and 2000 ng/ml and passes through the origin, within experimental error. Similar standard curve data have also been generated for spiked urine samples. Excellent accuracy and precision were obtained.

Accuracy and precision

Accuracy and precision of the assay were measured by the relative difference between the mean experimental linogliride concentration and the theoretical value, and the relative standard deviation, respectively (Table I). Frozen seeded control samples at three concentrations were blind-coded and analyzed concomitantly with plasma samples from the clinical study over a period of four weeks. The accuracy and precision (inter-run) at the three concentrations were all within 10%.

Application of the procedure to plasma samples

To date the procedure has been used successfully in the analysis of biological samples from clinical and non-clinical studies. Fig. 3 shows the plasma concentration—time profile obtained from one subject following a single oral 100-mg dose of linogliride. Serial blood samples were drawn at selected time points up to 10 h following dosing. The 81 ng/ml plasma concentration observed at the final time point is well above the detection limit of the assay. In summary, this HPLC assay for linogliride is highly sensitive, selective and precise. The extraction procedure is simple and rapid, and the assay has been automated with excellent results.

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