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Note

High-performance liquid chromatographic method for the determination of methysergide and methylergonovine in human plasma

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Methysergide maleate (Sansert®), 9,10-didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide maleate, is a *d*-lysergic acid derivative and potent serotonin antagonist useful in the prophylactic treatment of migraine and other vascular headaches [1]. Methylergonovine, an active metabolite of methysergide, is used in obstetrics for its uterotonic effect and has been shown to inhibit or block the effects of serotonin in some in vitro systems [2].

In 1981, a procedure for the determination of ergot alkaloids using high-performance liquid chromatography (HPLC) with fluorescence detection was reported by Edlund [3]. This procedure quantitatively measured methylergonovine using methysergide as an internal standard. In 1986, Bredberg et al. [4] reported on the pharmacokinetics of methysergide and methylergonovine in man. The results for this study were obtained using a modification of the method of Edlund [3], but no analytical data was presented.

The HPLC–fluorescence detection procedure reported in this paper involves less sample preparation than previously reported procedures, requires no internal standard and is sufficiently sensitive to accurately measure 20 pg/ml of both methysergide and methylergonovine. The limit of detectability is ca. 5 pg/ml. This procedure has been used for the analysis of methysergide and methylergonovine in more than 1000 human plasma samples demonstrating its utility, reliability and accuracy.

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EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system consisting of a Model 110B pump (Altex Scientific, Berkeley, CA, U.S.A.), a Model LP-21 pulse dampener (Scientific Systems, State College, PA, U.S.A.), an ISS-100 autosampler and LC-100 column oven (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Hitachi F1000 fluorescence detector (EM Science, Cherry Hill, NJ, U.S.A.). Peak-area measurements, baseline integrations and related calculations were performed by an HP-1000 computer system (Hewlett-Packard, Paramus, NJ, U.S.A.) equipped with a computer-automated laboratory system (CALS) software package (Beckman, Waldwick, NJ, U.S.A.).

Reagents and solvents

Chemicals used were methysergide and methylergonovine (Sandoz Pharmaceuticals, East Hanover, NJ, U.S.A.), ethyl acetate and acetonitrile (UV grade, distilled in glass; Burdick & Jackson Labs., Muskegon, MI, U.S.A.), phosphoric acid (85%) and potassium phosphate, monobasic (HPLC grade), sodium hydroxide (50%, w/w) and potassium carbonate (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled water. Human plasma (Sera-Tec Biologicals, North Brunswick, NJ, U.S.A.) was used in the preparation of standards.

Chromatographic conditions

Reversed-phase separations were achieved using a 250 mm × 4.6 mm Supelcosil LC-8 (octyl) 5-μm column (Supelco, Bellefonte, PA, U.S.A.) held at 60 °C and equilibrated with a mobile phase consisting of acetonitrile–0.01 *M* potassium phosphate, pH 7 (3:7, v/v) at a flow-rate of 1.0 ml/min. The mobile phase was filtered/degassed using a vacuum filter system (Millipore, Woburn, MA, U.S.A.) and a 0.45-μm filter (Nylon-66, Rainin Instrument, Woburn, MA, U.S.A.).

Instrumental parameters

The fluorescence detector excitation and emission wavelengths were set at 315 and 440 nm, respectively. The bandpass of each monochromator was fixed at 15 nm. The detector time constant was 0.3 s, and the signal to the data system was 1 V unattenuated.

Standard solutions

Stock solutions of methysergide and methylergonovine were prepared by dissolving 13.29 and 13.42 mg, respectively, of the maleate salts in two separate 100-ml flasks with acetonitrile–0.05 *M* phosphoric acid (1:1, v/v). These solutions (containing 100 μg/ml free base of each compound) were diluted further with 0.05 *M* phosphoric acid prior to preparing plasma standards containing 20–20 000 pg/ml of both methysergide and methylergonovine.

Sample preparation

Into glass-stoppered 40-ml centrifuge tubes pipet 2.0 ml of each blank, standard or subject plasma sample. Add, by use of a Repipet (Labindustries, Berkeley, CA, U.S.A.), 1.0 ml of 0.5 *M* potassium carbonate and mix briefly (5 s) using a Maxi-Mix (Thermolyne, Dubuque, IA, U.S.A.). Using a Repipet, add 10.0 ml of ethyl acetate, stopper and shake horizontally on an oscillating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min. Centrifuge at 750–1000 *g* for 5 min and transfer 8.0 ml of the upper organic phase into a second 40-ml glass-stoppered centrifuge tube. To this tube, add 0.7 ml of 0.05 *M* phosphoric acid, stopper and shake horizontally for 15 min. Centrifuge (750 *g*) for 2 min to separate the phases and aspirate the upper organic phase to waste. Place each centrifuge tube into a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) for 5 min at 50°C to remove any residual organic remaining in the aqueous layer. Inject a 400-μl aliquot of the aqueous layer into the chromatograph.

RESULTS

No interfering peaks have been detected in the plasma used for blank standards or from subjects who have been orally dosed with methysergide. Fig. 1 shows a

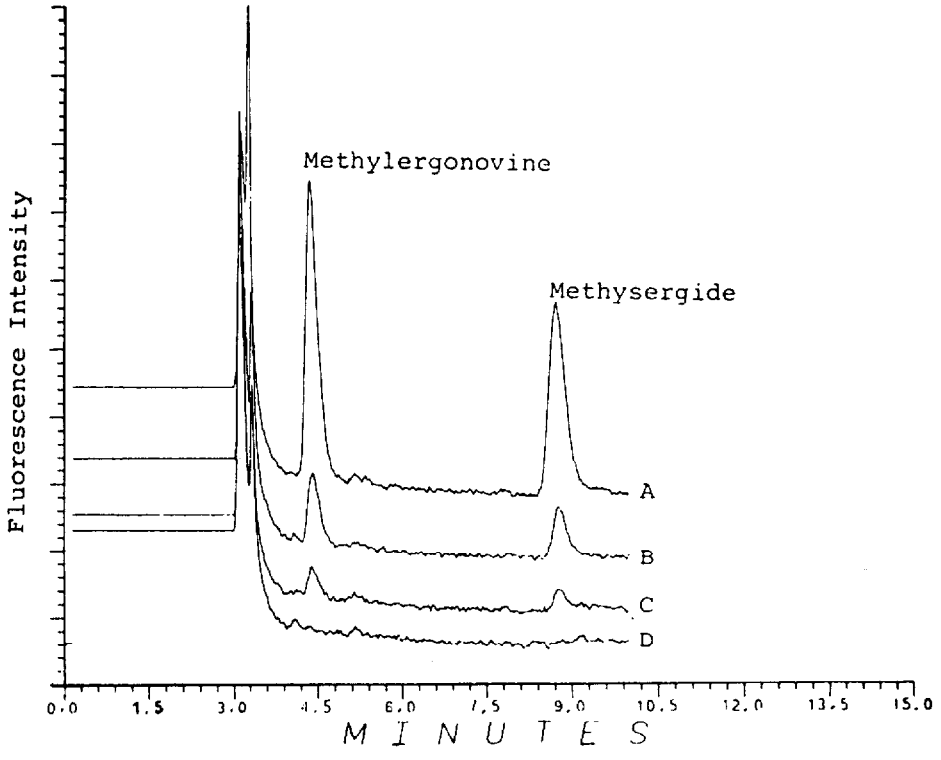

Fig. 1. Representative chromatograms obtained in the analysis of extracted plasma standards containing methysergide and methylergonovine. (A) 50 pg/ml; (B) 20 pg/ml; (C) 10 pg/ml; (D) blank plasma.

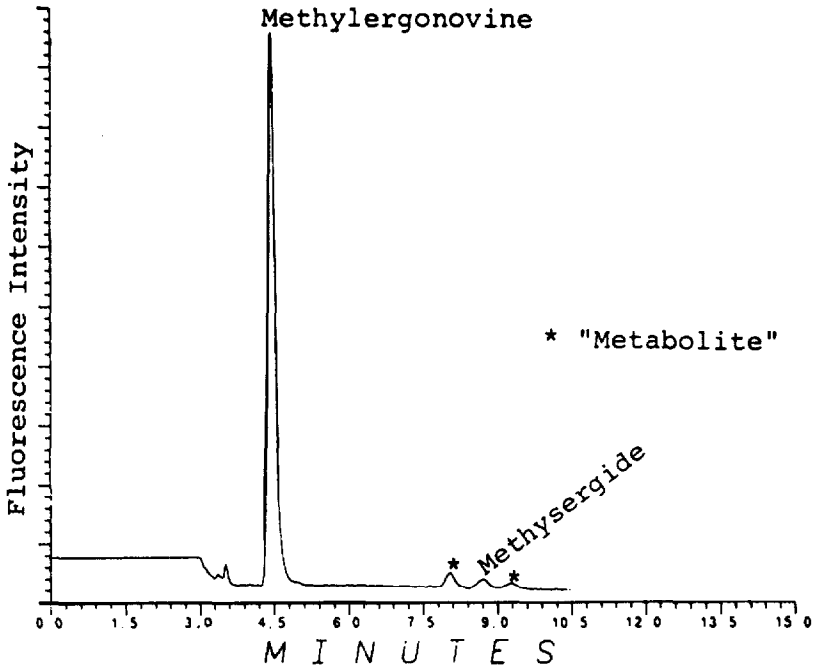

Fig. 2. Chromatogram of a 6-h plasma sample obtained from a subject receiving 1 mg of methysergide maleate. Concentrations of methysergide and methylergonovine are 31 and 970 pg/ml, respectively. Other potential "metabolites" are noted.

chromatogram of a plasma blank, 10, 20 and 50 pg/ml plasma standards. Additionally, there was no interference from other possible "metabolites" of methysergide (methylergonovine is an active metabolite of methysergide) as shown in Fig. 2.

Linearity

Daily standardization curves obtained for methysergide and methylergonovine in plasma over a five-week period resulted in linear concentration–response relationships. Methysergide and methylergonovine plasma concentrations of 0, 20, 50, 200, 1000, 5000 and 20000 pg/ml were extracted in duplicate on each analysis day. A relative response factor (RRF) – defined as drug concentration (pg/ml)/peak area (mV s) – was calculated for each standard and a mean RRF was calculated for the entire set of standards. The daily ($n=11$) mean RRFs obtained over the five-week period ranged from 7.27 to 8.14 (pg/ml)/(mV s) (mean of $7.7 \pm 8.2\%$) for methysergide and from 6.79 to 7.92 (pg/ml)/(mV s) (mean of $7.5 \pm 6.4\%$) for methylergonovine. Typical daily standard curves for methysergide and methylergonovine have RRFs (mean $\pm$ S.D.) of 7.55 ± 0.355 and 7.41 ± 0.273 (pg/ml)/(mV s) with coefficients of variation (C.V.) of 4.7 and 3.7%, respectively.

Accuracy, precision and reproducibility

The accuracy of the method was evaluated by analyzing plasma samples containing known amounts of methysergide and methylergonovine. Using the *t*-value from a one-tailed Student's *t*-distribution table and the variance of absolute differences between theoretical concentrations and the measured concentrations (Table I), the 95% confidence intervals of single determinations of both compounds at all concentrations were calculated. The results indicate that the result of any single analysis at any concentration would fall within ±25.8% and ±28.2% of the true value for methysergide and methylergonovine, respectively.

The precision (within-day variability) and reproducibility (day-to-day variability) of the method are also demonstrated by the data in Table I. The C.V.s for the within-day variation at any concentration of methysergide and methylergonovine in plasma ranged from 1.6 to 19.6% (mean 6.9%) and from 1.4 to 15.7% (mean 5.5%), respectively. The day-to-day C.V.s for the same set of data ranged from 4.5 to 12.3% (mean 9.1%) for methysergide and from 3.9 to 12.2% (mean 8.2%) for methylergonovine.

Sensitivity

The sensitivity of the method was determined using the data in Table I. Based upon the 95% confidence limits and reproducibility obtained at each concentration, a conservative limit of quantitation of 20 pg/ml was chosen for both meth-

TABLE I

VALIDATION OF THE METHOD FOR METHYSERGIDE AND METHYLERGONOVINE IN PLASMA USING SPIKED SAMPLES

Spiked concentration (pg/ml)	Mean found concentration* (pg/ml)				Absolute difference from true value (mean ± S.D.) (pg/ml)	95% Confidence limit (% of true value)
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Days 1–3 ($n=18$)		
Methysergide						
20	20.7 (10.6)	19.6 (7.2)	19.4 (4.6)	19.9 (8.2)	1.29 ± 0.96	±14.8
50	51.2 (3.8)	44.3 (4.5)	42.7 (15.8)	46.1 (11.9)	4.89 ± 4.59	±25.8
400	411 (3.5)	352 (19.6)	387 (6.3)	383 (12.3)	28.3 ± 41.0	±24.9
1000	992 (4.8)	981 (12.4)	1070 (2.2)	1010 (8.2)	66.9 ± 48.5	±15.1
5000	5180 (1.6)	4830 (5.4)	4940 (2.7)	4980 (4.5)	164 ± 145	± 8.3
16000	16700 (2.4)	14300 (13.0)	15900 (3.9)	15600 (9.6)	972 ± 1186	±19.0
Methylergonovine						
20	20.1 (8.3)	20.2 (6.5)	17.3 (5.9)	19.3 (9.7)	1.62 ± 1.11	±17.8
50	49.9 (4.4)	44.7 (4.3)	40.2 (15.0)	44.9 (12.2)	5.63 ± 4.87	±28.2
400	407 (2.7)	360 (15.7)	379 (2.7)	382 (9.8)	24.1 ± 33.7	±20.7
1000	939 (4.2)	994 (10.7)	976 (2.0)	970 (6.9)	52.7 ± 49.9	±14.0
5000	5010 (1.4)	4710 (3.8)	4700 (1.9)	4810 (3.9)	218 ± 159	± 9.9
16000	15800 (1.6)	14400 (10.1)	15100 (2.7)	15100 (6.8)	928 ± 1010	±16.7

*Values in parentheses are coefficients of variation (%).

ysergide and methylergonovine. The limit of detection, based upon a signal-to-noise ratio of 2, approaches 5 pg/ml for both components (Fig. 1).

Selectivity

The selective extraction technique employed combined with fluorescence detection resulted in an assay free from extracted endogenous material and potential metabolites at the retention times of methysergide and methylergonovine (Figs. 1 and 2). No interferences were observed throughout the analysis of more than 1000 human plasma samples and standards.

Stability

The stability of methysergide and methylergonovine solutions in the presence of light was investigated by exposing aqueous solutions (adjusted to pH 2 and 7) containing both compounds to sunlight and to direct fluorescent (laboratory) light for up to 60 min. There was no apparent degradation of either compound when exposed to fluorescent light. However, both methysergide and methylergonovine were found to be very unstable when kept in direct sunlight as demonstrated by half-lives of about 7.5 and 15 min at pH 2 and 15 and 35 min at pH 7, respectively.

The stability of methysergide and methylergonovine in plasma stored at −15 °C was investigated by analyzing, in duplicate, plasma samples containing 20, 200, 1000 and 5000 pg/ml of both compounds on twelve separate days over a 24-day

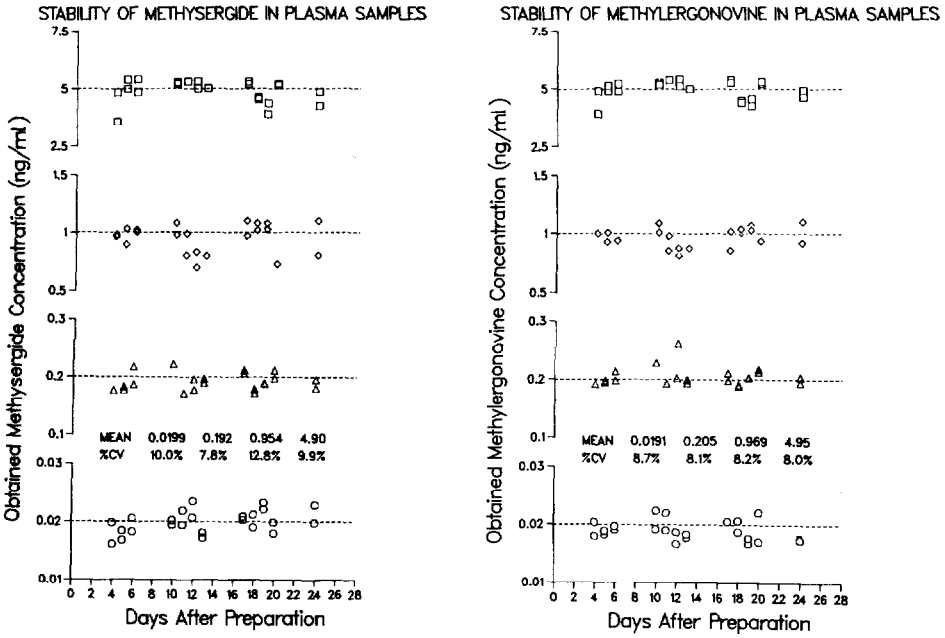

Fig. 3. Stability of methysergide and methylergonovine in plasma stored at −15 °C for 24 days. (○) 0.02 ng/ml; (△) 0.2 ng/ml; (◇) 1.0 ng/ml; (□) 5.0 ng/ml.

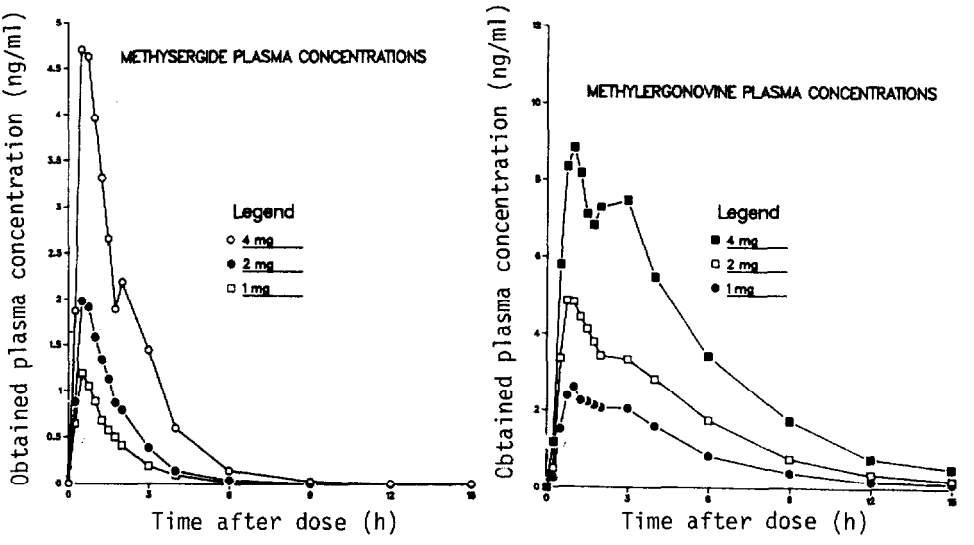

Fig. 4. Mean plasma concentrations obtained for methysergide and methylergonovine in twelve subjects receiving a 1-, 2- and 4-mg single oral dose of methysergide maleate given as a solution.

period. The results are displayed in Fig. 3 and demonstrate that both compounds are quite stable in plasma stored at −15°C for at least 24 days.

Application of the method

Using this procedure, 540 human plasma samples from a methysergide dose-bioavailability study were analyzed. Twelve fasted subjects received 1-, 2- and 4-mg single oral doses of methysergide maleate as a solution on three occasions. Following administration of the drug solutions, serial blood samples were obtained from each subject up to 15 h post-dose. In all cases, plasma concentrations of methysergide and methylergonovine above the limit of quantitation could be followed for more than four half-lives. The mean methysergide and methylergonovine plasma concentrations for the three dose levels are shown in Fig. 4.

DISCUSSION

The only other reported procedure for the analysis of methysergide and methylergonovine [4] used a 2.5-ml sample and a third extraction step in achieving a clean sample extract and a detection limit of 100 pg/ml. The procedure presented here utilizes a smaller sample volume and shorter extraction procedure, requires no internal standard, has greater sensitivity and has demonstrated its utility in the routine analysis of human plasma samples.

The conservative limit of quantitation of 20 pg/ml is significantly better than that previously reported [3,4] and is sufficient to monitor plasma levels of methysergide and methylergonovine for more than four half-lives following a single oral dose of 1–4 mg of methysergide maleate. The use of elevated column temperature increases the efficiency of the column, resulting in sharper peaks and

reduced retention of the compounds of interest. No significant loss in column life has been observed, based upon the use of a single column for the analysis of about 800 plasma samples over a three-week period. In addition, greater column stability would be expected in the present procedure due to a mobile phase pH of 7 compared to a pH of 9 which was used in previous methods [3,4].

In conclusion, the method reported in this paper employs a relatively simple sample preparation and offers the reliability and sensitivity necessary to routinely analyze plasma samples from bioavailability studies. The utility of this method has been demonstrated by the routine analysis of about 1000 human plasma samples.

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