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High-performance liquid chromatographic assay with ultraviolet detection for the determination of etoperidone and two active metabolites, 5-(1-hydroxyethyl)etoperidone and 1-(3-chlorophenyl)piperazine, in plasma

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ABSTRACT

A selective and sensitive high-performance liquid chromatographic assay with ultraviolet detection for the determination of the antidepressant drug etoperidone and two active metabolites in plasma is described. The drug, metabolites and internal standard are isolated from plasma using a two-step liquid–liquid extraction procedure. The resulting sample is chromatographed on a C_{18} column (10 cm × 2.1 mm I.D.) with ultraviolet detection at 254 nm. Standard curves are linear for each compound over the concentration range 2–1000 ng/ml. The accuracy and precision of the assay, expressed as the percentage deviation of measured values from the true value and the relative standard deviation (inter-run), are $\leq 10\%$ at all concentrations except the minimum quantification limit. Using an automated injector and computerized data acquisition, eighty samples can be routinely processed in one day. The assay has been successfully used for the analysis of plasma samples from pharmacokinetic studies in mice, rats, dogs and humans.

INTRODUCTION

Etoperidone hydrochloride, 2-(3-(4-(3-chlorophenyl)-1-piperazinyl)propyl)-4,5-diethyl-2,4-dihydro-3H-1,2,4-triazol-3-one monohydrochloride, is an antidepressant drug marketed in Europe and currently in clinical trials in the U.S.A. Structurally, etoperidone is closely related to trazodone, but unrelated to the tricyclic or tetracyclic antidepressants. Pharmacologically, etoperidone modulates serotonergic function *in vivo* and *in vitro* [1–3].

Following oral administration, etoperidone is extensively metabolized. The 1-(3-chlorophenyl)piperazine (MCPP) cleavage product is known to have pharmacological activity which may contribute to the therapeutic effect of the drug [1,4]. A second metabolite, 5-(1-hydroxyethyl)etoperidone or hydroxyetoperidone, is also pharmacologically active in animal models [5]. Hydroxyetoperidone is a new metabolite which has not been published elsewhere.

Two previous methods for the analysis of etoperidone in plasma have been

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reported. Gilmour and Leary [6] described a gas chromatographic procedure utilizing a nitrogen–phosphorus detector which has a minimum quantification limit of 20 ng/ml for etoperidone in plasma. The second procedure, by Caccia and Fong [7], used high-performance liquid chromatography (HPLC) with UV detection and achieved a quantification limit of 100 and 50 ng/ml for etoperidone and MCPP, respectively, in plasma.

The present study reports the development of a precise and reproducible method for the measurement of etoperidone, hydroxyetoperidone and MCPP in plasma, urine and brain homogenate samples with a linear range from 2 to 5000 ng/ml for each compound. The HPLC analysis, utilizing a narrow-bore column, provides excellent resolution of the three analytes and the internal standard with a total analysis time of less than 13 min. The method has been demonstrated to be reproducible and accurate and has been successfully applied to the analysis of etoperidone and its metabolites in pharmacokinetic studies in animals and man.

EXPERIMENTAL

Instrumentation

A Beckman Model 114 pump, Waters Assoc. WISP® autoinjector and Kratos Spectroflow 783 UV detector (254 nm) comprised the HPLC instrumentation used. Separation of the drug and metabolites was accomplished using a Brownlee C_8 cartridge (10 cm × 2.1 mm I.D.; 5 μm particle size) and guard cartridge (3 cm × 2.1 mm I.D.; 5 μm particle size). The HPLC column was kept at ambient temperature. The aqueous portion of the mobile phase was a pH 3.7 acetate buffer containing 0.01 *M n*-pentylamine and 0.005 *M* heptanesulfonic acid. Methanol and acetonitrile were combined with the aqueous solution in a 3:1:6 volume ratio to form the mobile phase. The mobile phase flow-rate was 0.45 ml/min. A Hewlett-Packard 3354 laboratory automation system with software developed in-house was used for data acquisition and processing.

Reagents and supplies

HPLC-grade methanol, acetonitrile and hexane were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.) and used without further purification. Anhydrous diethyl ether, analytical-reagent-grade concentrated sulfuric acid and glacial acetic acid were purchased from Mallinckrodt (Paris, KY, U.S.A.). Triply purified distilled water was obtained from Ephrata Mountain Water (Manheim, PA, U.S.A.).

N-Pentylamine (99%) and 1-(3-chlorophenyl)piperazine monohydrochloride (98%) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Certified ACS-grade sodium acetate and sodium hydroxide were purchased from Fisher Scientific. Heptanesulfonic acid was obtained from Eastman Kodak (Rochester, NY, U.S.A.).

Etoperidone hydrochloride and hydroxyetoperidone sulfate were obtained in-

house (R. W. Johnson Pharmaceutical Research Institute, Spring House, PA, U.S.A.). The internal standard, trazodone hydrochloride, was obtained from Mead-Johnson Pharmaceuticals (Evansville, IN, U.S.A.). Structures for these compounds and MCPP are given in Fig. 1. All concentrations in this report refer to the free base of each compound.

Extraction procedure

To a 1-ml plasma sample, 1 ml of 0.1 *M* NaOH, 0.1 ml of methanol containing 500 ng internal standard and 4 ml of diethyl ether were added. Samples were shaken on a linear shaker for 10 min and centrifuged (581 *g*) for 5 min. After freezing the aqueous layer by placing the sample containers upright on a bed of dry ice, the organic layer was decanted into a clean centrifuge tube containing 0.2 ml of 0.18 *M* H_2SO_4. The samples were briefly vortex-mixed, capped and shaken on a linear shaker for 10 min. Following centrifugation for 5 min (581 *g*), the

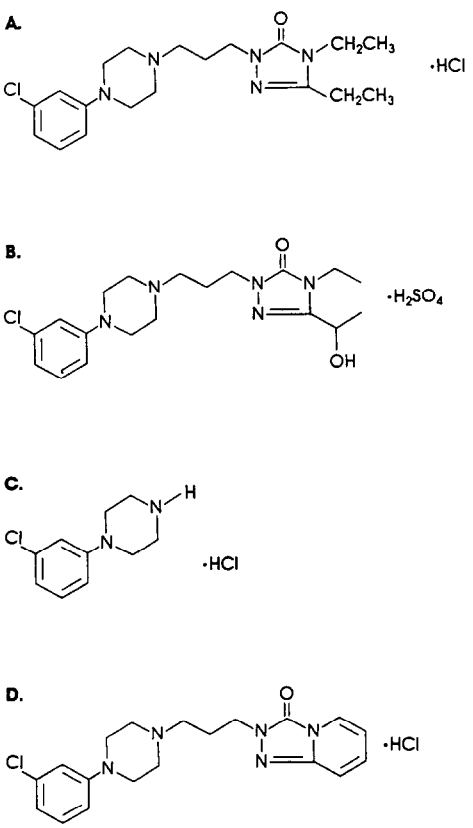

Fig. 1. Structures of (A) etoperidone hydrochloride, (B) hydroxyetoperidone sulfate, (C) 1-(3-chlorophenyl)piperazine hydrochloride (MCPP) and (D) the internal standard.

organic layer was aspirated and discarded. The aqueous layer was transferred to autosampler vials, and 0.15 ml was injected into the HPLC system.

Assay validation

To establish a calibration curve, a series of standards containing etoperidone, hydroxyetoperidone and MCPP (20–10 000 ng/ml of each) and 5000 ng/ml internal standard were prepared in methanol. A 0.1 ml-volume of these solutions (instead of the 0.1-ml solution containing internal standard alone) was added to 1 ml of plasma, and the samples were extracted according to the above procedure. Duplicate standard curves were run on each of three analysis days. The peak-height ratios of each compound and the internal standard were weighted by 1/variance and plotted against concentration. Linear regression analysis gave a calibration line for each compound which was used to calculate the concentrations of each in unknown samples.

As an additional control, spiked plasma pools were prepared at three concentrations (10, 50 and 500 ng/ml of each compound). The pools were separated into 1-ml aliquots and frozen. Two samples from each pool were analyzed with each calibration curve to assess the inter-run precision and accuracy of the assay procedure.

The extraction efficiency of the assay procedure was determined by comparing the peak heights for each compound in extracted standards with those obtained by the injection of unextracted standards.

RESULTS AND DISCUSSION

The chromatographic procedure separated the four compounds within a relatively short run time, despite the large differences in polarity (Fig. 2). Retention times for MCPP, hydroxyetoperidone, the internal standard and etoperidone were 5.5, 6.8, 8.7 and 11.5 min, respectively. No interfering peaks appear in the chromatogram of blank plasma and each compound is well separated. Although the dilute sulfuric acid is injected on the column, no unusual shortening of column life was observed.

For each compound, duplicate calibration curves analyzed on three consecutive days were linear over the concentration range studied (2–1000 ng/ml) (Tables I–III). The correlation coefficients for each of the three day composite curves were 1.00. The inter-run precision of the assay, as indicated by the relative standard deviation of the measured concentrations, was within 10% for each compound except at the minimum quantification limit of the etoperidone and hydroxyetoperidone curves. The accuracy of the assay, as reflected by the percentage deviation of the average measured concentration from the spiked concentration, was within 10% for each compound except at the 2 ng/ml concentration of hydroxyetoperidone and MCPP. The average measured concentrations of the control pools of plasma for each compound were within 10% of the spiked concentrations with relative standard deviations of less than 10%.

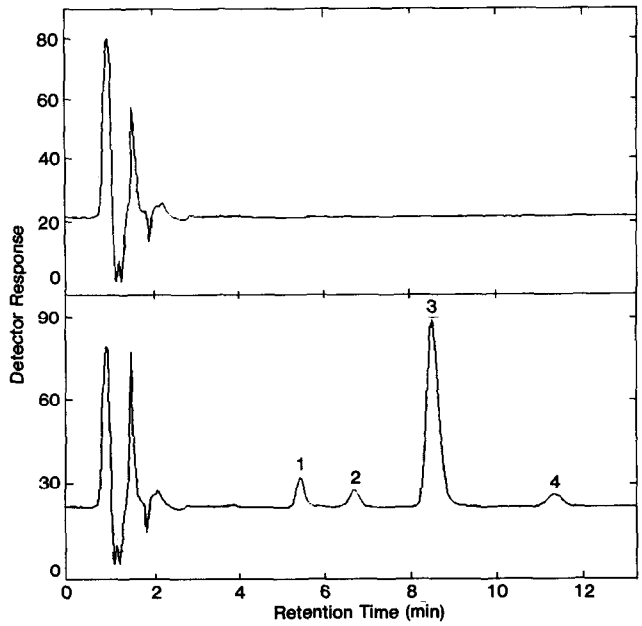

Fig. 2. HPLC patterns of extracted human plasma samples. Top: blank plasma; bottom: plasma containing 50 ng/ml MCPP (peak 1), hydroxyetoperidone (peak 2), etoperidone (peak 4) and 500 ng/ml internal standard (peak 3).

TABLE I

SUMMARY OF CALIBRATION CURVE DATA FOR ETOPERIDONE IN HUMAN PLASMA (n = 6)

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
2	1.85	18.1	7.7
5	4.93	8.6	1.3
10	10.1	2.0	0.9
20	20.0	1.0	0.0
50	50.3	0.5	0.6
100	98.9	0.8	1.1
200	197	0.3	1.5
500	504	0.2	0.8
1000	1005	0.3	0.5

[a] Relative standard deviation.

[b] Relative difference between the mean measured value and the spiked value.

TABLE II

SUMMARY OF CALIBRATION CURVE DATA FOR MCPP IN HUMAN PLASMA (n = 6)

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
2	2.46	8.1	22.8
5	5.35	5.6	7.0
10	9.54	6.1	4.6
20	18.1	5.9	9.5
50	50.6	2.3	1.2
100	95.8	5.6	4.2
200	191	6.3	4.5
500	495	3.5	1.1
1000	1026	1.9	2.6

[a] Relative standard deviation.

[b] Relative difference between the mean measured value and the spiked value.

The extraction efficiency for MCPP, hydroxyetoperidone, the internal standard and etoperidone were 95, 83, 95 and 96%, respectively. The standard deviations of the extraction efficiency results were $<7\%$ (n = 12 for each compound).

The assay has been used to measure etoperidone, hydroxyetoperidone and MCPP concentrations in plasma, urine or aqueous brain homogenate samples from drug metabolism studies in mice, rats and dogs (Fig. 3). Smaller aliquots of

TABLE III

SUMMARY OF CALIBRATION CURVE DATA FOR HYDROXYETOPERIDONE IN HUMAN PLASMA (n = 6)

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
2	2.61	11.9	30.5
5	5.07	7.4	1.4
10	9.85	2.5	1.5
20	19.9	3.2	0.5
50	49.9	0.6	0.1
100	97.5	2.2	2.5
200	195	1.0	2.5
500	502	0.3	0.4
1000	1007	0.7	0.7

[a] Relative standard deviation.

[b] Relative difference between the mean measured value and the spiked value.

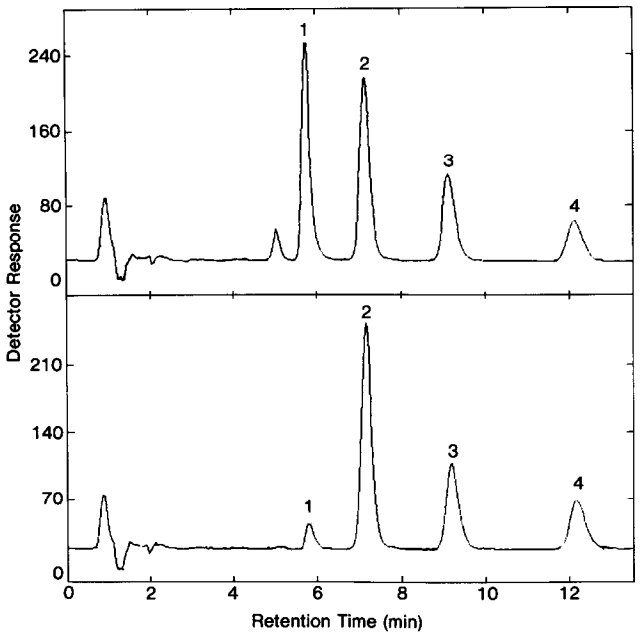

Fig. 3. HPLC patterns of extracted plasma samples. Top: a rat 2 h following the oral administration of an 80 mg/kg dose of etoperidone hydrochloride (MCPP, 805 ng/ml; hydroxyetoperidone, 1200 ng/ml; etoperidone, 390 ng/ml); bottom: a dog 8 h following the oral administration of a 20 mg/kg oral dose of etoperidone hydrochloride (MCPP, 98 ng/ml; hydroxyetoperidone, 1700 ng/ml; etoperidone, 470 ng/ml). Peaks as in Fig. 2.

plasma (as low as 100 μl) were used to measure concentrations above the linear range of the assay. As an example of the utility of the assay, a plasma concentration *versus* time curve for each compound following a single oral dose of etoperidone hydrochloride to a beagle dog is shown in Fig. 4. Due to the sensitivity of the assay, the plasma concentrations of each compound can be detected until 24 h after dose administration.

In summary, an assay has been described which allows the simultaneous quantification of etoperidone and two active metabolites in plasma. The use of a narrow-bore column, coupled with a reproducible and efficient extraction, allows minimum quantification limits for the present assay which represent a four- to twenty-fold improvement over previous methods. Additionally, a previously unreported metabolite, hydroxyetoperidone, was quantified with this procedure. The assay has been successfully applied to pharmacokinetic studies in animals and humans.

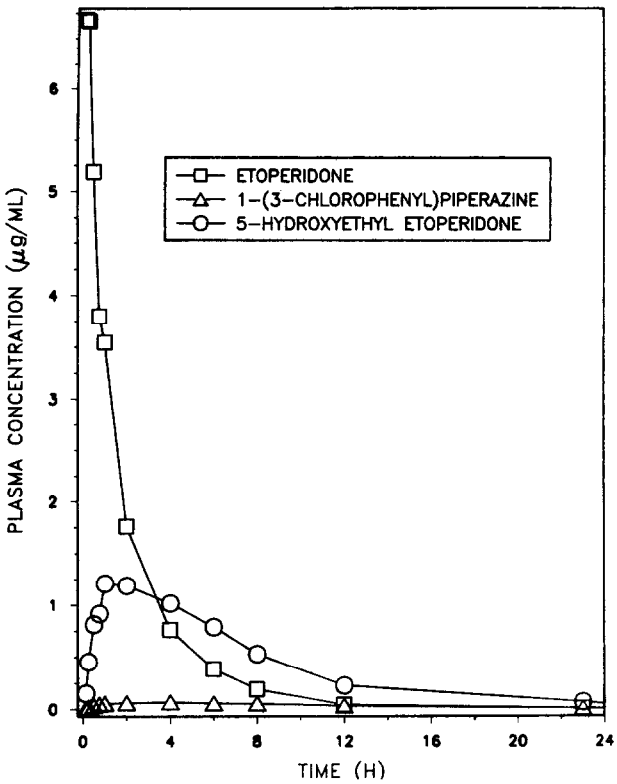

Fig. 4. Mean plasma concentrations of etoperidone, MCPP and hydroxyetoperidone following the intravenous administration of 10 mg/kg etoperidone hydrochloride to four beagle dogs.

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