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Report

A High-Performance Liquid Chromatographic Microassay Employing a Liquid–Solid Extraction Technique for Etintidine in Plasma

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This paper describes a new, rapid solid extraction method for the determination of etintidine in plasma. The method employs a semiautomatic sample preparation system. Plasma samples and the internal standard (cimetidine) were applied onto octyl-bonded silica extraction columns. The extraction columns were then subjected to Tris buffer and water wash and were subsequently loaded onto an automatic sample injection system. The contents of the extraction columns were eluted on-line with a mobile phase of acetonitrile:methanol:0.1% ammonium hydroxide (85:10:5, by volume) onto a silica analytical column and detected by UV absorption at 229 nm. The chromatographic condition separates etintidine from some of its metabolites and other endogenous components in plasma. The detection limit for etintidine was 0.02–0.05 μg/ml when 0.2 ml of plasma was used. This method has been used for the determination of plasma etintidine levels in humans and mice after oral administration of etintidine and was found to be suitable for pharmacokinetic/bioavailability studies of etintidine in humans and animals. The method can also be used for the quantitative determination of cimetidine and certain metabolites of etintidine.

KEY WORDS: etintidine; high-performance liquid chromatography (HPLC); solid extraction; determination of etintidine in plasma.

INTRODUCTION

Etintidine (E; *N*-cyano-*N'*-[2-[(5-methyl-1H-imidazol-4-yl)methylthio]ethyl]-*N''*-(2-propynyl)guanidine) is an H_2-receptor antagonist which has been studied for the treatment of ulcer diseases (1) (Fig. 1). A high-performance liquid chromatographic (HPLC) method employing liquid–liquid extraction for determining plasma levels of etintidine has been developed (2) and used for pharmacokinetic/bioavailability studies of etintidine in humans (3,4) and animals (5). This report describes an HPLC method using liquid–solid extraction for plasma samples. The method incorporates semiautomatic sample preparation and on-line elution techniques and, hence, is rapid and more sensitive than the previously developed method (2) for the quantitative determination of etintidine in plasma. Cimetidine (C) was used as an internal standard in this method. The method can also be used for the measurement of some metabolites of etintidine: etintidine sulfoxide (ES), 5-hydroxymethyletintidine (HE), and 5-hydroxymethyletintidine sulfoxide (HES) in plasma.

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MATERIALS AND METHODS

Chemicals and Reagents. Etintidine HCl, cimetidine, etintidine sulfoxide (ES), 5-hydroxymethyletintidine (HE), and 5-hydroxymethyletintidine sulfoxide (HES) were supplied by the Chemical Development Division at Ortho. Glass-distilled acetonitrile and methanol (HPLC grade, Burdick & Jackson Laboratories, Muskegon, Mich.), tris(hydroxymethyl)aminomethane (Fisher Scientific, Fairlawn, N.J.), and ammonium hydroxide (Mallinckrodt, Paris, Ky.) were used as received.

Apparatus and Chromatographic Conditions. The chromatographic system consisted of a Waters M-6000 pump (Waters Associates, Milford, Mass.), a Varian Ad-

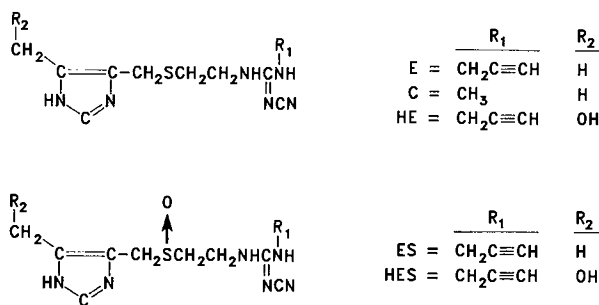

Fig. 1. Chemical structures of etintidine (E), cimetidine (C), 5-hydroxymethyletintidine (HE), etintidine sulfoxide (ES), and 5-hydroxymethyletintidine sulfoxide (HES).

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vanced Automatic Sample Preparation (AASP) system (Varian Instruments Group, Sugar Land, Tex.), an LDC Model 1203 UV detector (Milton Roy, Riviera Beach, Fla.) operated at 229 nm, and a Linear recorder (Linear Instruments, Irvine, La.) set at 30 cm/hr and 10 MV. The AASP system initiated chromatographic peak height measurement by an HP3357 Laboratory Automation System (Hewlett Packard, Paramus, N.J.) through an HP18652 A/D converter. A dry-packed saturation column (Whatman precolumn) filled with 10μ silica was placed between the pump and the injector (AASP). A silica column (Dupont Zorbax, 4.6 mm × 25 cm) was used for separation and was operated at room temperature. The mobile phase consisted of an 85/10/5 (by volume) mixture of acetonitrile/methanol/0.1% ammonium hydroxide (final apparent pH 9.4). The mobile phase was prepared daily and filtered through a 0.45-μm HA filter (Millipore, Bedford, Mass.) before use. At a flow rate of 1 ml/min, etintidine, cimetidine, ES, HE, and HES eluted at 6.8, 10.1, 15.5, 7.9, and 14.4 min, respectively. The valve reset time on the AASP was 0.3 min.

Standards. Stock solutions of etintidine HCl, cimetidine, HE, ES, and HES were prepared in methanol. A methanolic solution containing 0.02 mg/ml of cimetidine was used as an internal standard.

Extraction Procedure. The extraction of plasma samples was performed on AASP C-8 cassettes (Analytichem International, Harbor City, Calif.) using a Vac-Elut sample preparation system (Analytichem International). The cassettes (each containing 10 extraction cartridges) were conditioned by passing through ~1.5 ml each of methanol, mobile phase, methanol, and water, followed by Tris buffer (0.1 *M*, pH adjusted to 9.3 with HCl). After conditioning, 0.2-ml aliquots of plasma samples along with 5 μl of the internal standard solution and 1.5 ml of Tris buffer were applied to these cassettes. After the eluant was discarded, the cassettes were further washed with 1.5 ml each of Tris buffer and water, followed by another water wash. The cassettes were then loaded onto the AASP system for injection.

Quantification. Blank plasma fortified with etintidine was simultaneously analyzed with study samples. Peak height ratios were used for quantification. All plasma level values reported in this study are base equivalent values.

Extraction Recovery. Plasma samples fortified with 0, 0.05, 0.5, 1, 2, and 5 μg/ml of etintidine were extracted according to the extraction procedure described above. The extraction recovery was calculated by comparing the peak height of extracted etintidine or cimetidine with that of a methanolic standard injected directly onto the column.

RESULTS AND DISCUSSION

Figure 2 depicts typical chromatograms. The extraction of etintidine or cimetidine from plasma using C-8 extraction columns was good. The mean (±SD; $N = 3$) recoveries of etintidine were 112 (±8.1), 95.4 (±4.9), 84.3 (±2.7), 83.5 (±7.6), and 92.7% (±7.5%) for etintidine concentrations of 0.05, 0.5, 1, 2, and 5 μg/ml, respectively. The recovery of cimetidine (0.1 μg on column) from the above plasma samples ranged from 88.2 ± 5.5 to 103 ± 5.0%.

Tables I and II present the intra- and interday assay precision data, respectively. The limit of detection of this assay for etintidine in plasma was determined to be 0.02–0.05

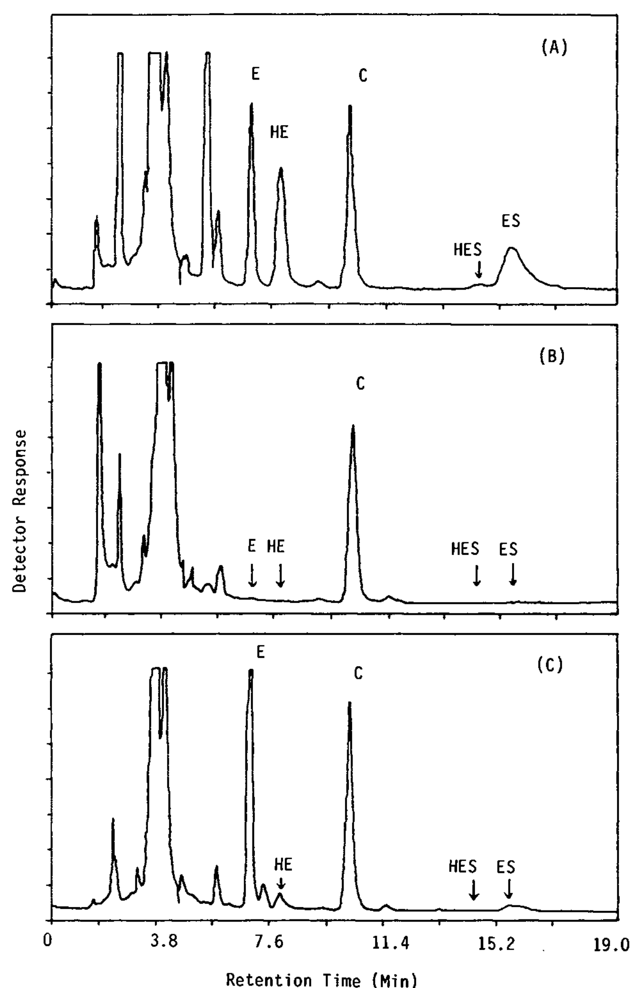

Fig. 2. Typical chromatograms obtained from on-line elution of (A) human plasma fortified with 0.5 μg/ml of E, HE, ES, and HES, (B) predose plasma, and (3) plasma sample obtained 2 hr following oral administration of 400 mg of E in one human subject.

Table I. Intraday Assay Precision

Spiked concentration of etintidine (μg/ml)	Measured concentration[a] (CV)[b] (μg/ml)		
	Day 1	Day 2	Day 3
0	0.006 (NA)[c]	0.002 (200%)	0.002 (30%)
0.01	0.014 (7.1%)	0.014 (7.1%)	0.014 (NA)[c]
0.02[d]	0.023 (4.4%)	0.024 (4.2%)	0.022 (9.1%)
0.05	0.055 (5.4%)	0.052 (3.8%)	0.055 (NA)[c]
0.5	0.49 (2.8%)	0.48 (NA)[c]	0.51 (NA)[c]
1	1.00 (4.8%)	1.00 (NA)[c]	1.04 (1.9%)
2	1.89 (4.8%)	1.86 (1.6%)	1.95 (1.5%)
5	4.91 (1.4%)	4.80 (2.5%)	4.96 (1.6%)

[a] Determined by single point calibration using one plasma standard containing 0.5 μg/ml of etintidine as a calibration standard; blank plasma from one volunteer (Subject A) was used for preparation of these standards.
[b] Coefficient of variation; $N = 3$.
[c] $N = 2$; coefficient of variation not available.
[d] Determined as the limit of detection.

Table II. Interday Assay Precision[a]

Spiked concentration of etintidine (μg/ml)	Number of days	Measured concentration of etintidine (μg/ml)[b]	% precision[c]	% deviation[d]
0	20	0.012	71	—
0.01	19	0.014	36	40
0.02	21	0.021	17	5
0.05[e]	24	0.052	15	4
0.5	28	0.48	9.2	−4
2	28	1.92	8.7	−4
5	29	4.83	6.6	−3.4

[a] Samples analyzed on 19–29 different days over a period of 6 to 7 months: blank plasma from one volunteer (Subject B) was used for preparation of these standards.
[b] Determined by single point calibration using one plasma standard containing 0.5 μg/ml of etintidine as a calibration standard during each day's analysis.
[c] Coefficient of variation.
[d] Percentage deviation = 100% × (measured concentration − spiked concentration) ÷ spiked concentration.
[e] Determined as the limit of detection.

μg/ml, depending on the background readings of the plasma samples. For example, when the background plasma reading was 0.002–0.006 μg/ml (Subject A in Table I), the limit of detection was determined to be 0.02 μg/ml (≥3 times the background noise level). When the background plasma reading was 0.012 μg/ml (Subject B in Table II), the limit of detection was determined to be ~0.05 μg/ml. In a human three-way crossover bioequivalence study of etintidine conducted in 29 subjects (15), the detection limits were determined to be ≤0.02 μg/ml in 22 subjects and were 0.03–0.05 μg/ml in 7 subjects.

This assay is linear for plasma samples containing 0.02 to 5 μg/ml of etintidine. The linearity was demonstrated in the accuracy of the mean calculated concentrations for a series of plasma standards when a single point calibration was used (Table II). The assay is also linear for plasma samples containing 0.05 to 0.5 μg/ml of ES, HES, or HE. The reproducibility data for ES, HES, and HE are shown in Table III.

No endogenous components were found to elute at the retention times of etintidine, HES, HE, or ES. Theophylline (4.9 min), caffeine (5.4 min), and theobromine (5.6 min) did not interfere with the present assay for etintidine, HES, HE, or ES.

A mobile phase of acetonitrile, methanol, and 0.1% ammonium hydroxide (85/10/5, v/v) was found to give optimal separation of E, C, HE, HES, and ES. The high percentage of organic solvent in the mobile phase was necessary to elute quantitatively E, C, ES, HES, and HE on-line from the C-8 extraction columns to the analytical column. The small amount of base was needed to elute effectively E, C, HE, HES, and ES from the analytical column. After a column had been used extensively (e.g., after approximately a thousand injections or after the column had been used with the alkaline mobile phase continuously for months), the mobile-phase composition may have to be modified between volume ratios of 85/10/5 and 87/10/3 in order to achieve optimal separation of all components. The increase in acetonitrile content, with a corresponding decrease in ammonium hydroxide content, results in longer retention times for E, C, HE, HES, and ES. However, the precision, accuracy, linearity, and specificity of the assay for etintidine were not affected by this modification of the mobile phase.

Table III. Reproducibility Data of HE, HES, and ES

Spiked concentration (μg/ml)	Measured concentration[a] (CV)[b] (μg/ml)		
	HE	HES	ES
0.05	0.056 (3.6%)	0.049 (24.5%)	0.052 (3.8%)
0.1	0.101 (3.0%)	0.083 (0%)	0.094 (2.1%)
0.5	0.528 (11.0%)	0.507 (14.4%)	0.510 (5.1%)

[a] Determined by single point calibration using one plasma standard (0.5 μg/ml) as a calibration standard.
[b] Coefficient of variation; $N = 3$.

This HPLC assay for etintidine has several advantages over the former method (2). The modified method is rapid and requires no organic solvent extraction. Injecting the entire eluate from the solid extraction column improves the assay's sensitivity (0.02 to 0.05 μg/ml with 0.2 ml of plasma).

This HPLC method has been used for the quantification of etintidine in human plasma obtained following oral administration of 400 mg base equivalent of etintidine HCl in tablets, capsules, or solution dosage form to 29 normal subjects (Fig. 3). The method has also been used for the quantification of some metabolites of etintidine: ES and HE in human plasma. Figure 4 shows typical plasma level profiles of E, ES, and HE obtained following oral administration of 400 mg of etintidine in solution to normal subjects. HES was not detectable (<0.05 μg/ml) in these plasma samples.

This method has also been used for determination of etintidine levels in small volumes (0.1–0.2 ml) of mouse plasma. In Fig. 5 (after 2 years of continuous daily oral administration of 100, 400, and 1000 mg/kg of base equivalent of etintidine HCL in the diet to three groups of male BALB/C mice) the mean etintidine levels in mouse plasma obtained in the mornings (9 AM–12 noon) are shown. Since the diet intake in the mouse is erratic and occurs mainly at night, considerable variation in the plasma E levels (in the

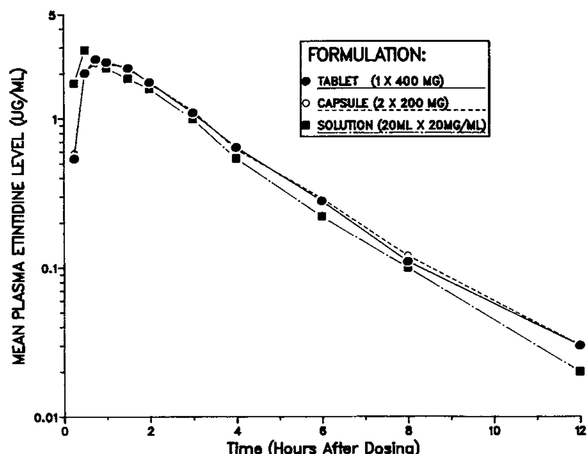

Fig. 3. Mean plasma etintidine concentration versus time data obtained following oral administration of 400 mg base equivalent of etintidine HCl in 29 subjects.

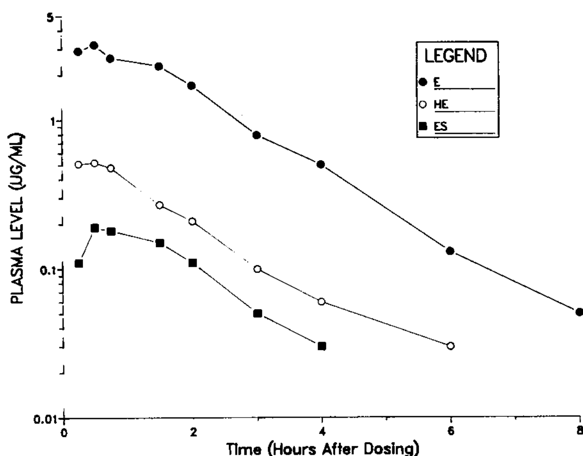

Fig. 4. Typical plasma concentration of E, HE, and ES versus time data obtained following oral administration of 400 mg of E in solution to human subjects.

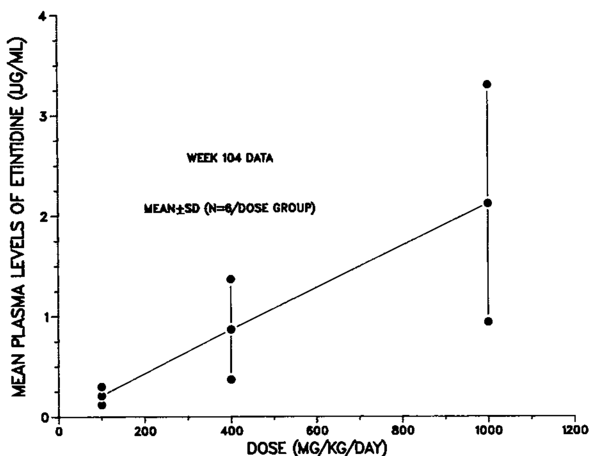

Fig. 5. Mean (± SD) morning plasma etintidine levels obtained following 2 years of daily oral administration of 100, 400, and 1000 mg/kg of base equivalent of etintidine HCl in the diet of male BALB/C mice.

morning) was expected and observed. Nonetheless, these plasma levels clearly demonstrated a significant dose-related increase in the amount of E absorbed as indicated by the increase in plasma E levels when the E dose (in the diet) was increased (Fig. 5). Thus, our present assay method was found to be suitable for pharmacokinetic/bioavailability studies of etintidine in humans and animals.

The present method may also be used for determination of cimetidine in plasma. Most of the published assays for cimetidine in plasma with an assay sensitivity of 0.05 to 0.1 μg/ml used liquid–liquid extraction procedure (6–9,11–14) and required a larger sample volume: 0.5–5 ml (7,9,11,12,14). In addition, most of these methods measure only cimetidine. One method (14) also measures some metabolites of cimetidine in plasma. However, this method (14), like other cimetidine assays (7,10,12) entails elaborate sample preparation procedures. Thus, our present micro, liquid–solid extraction procedure for etintidine and its metabolites presents a new, simple analytical procedure for this class of compounds.

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