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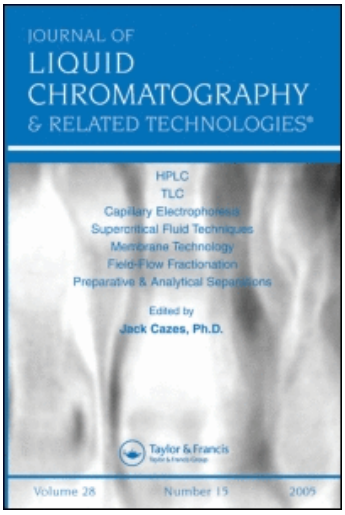

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High-Performance Liquid Chromatographic Assay with Ultraviolet Detection for the Analysis of BW 1370U87 and Its Two Major Metabolites in Human Plasma

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH ULTRAVIOLET DETECTION FOR THE ANALYSIS OF BW 1370U87 AND ITS TWO MAJOR METABOLITES IN HUMAN PLASMA

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ABSTRACT

A sensitive and selective high performance liquid chromatographic assay with UV detection was developed for the analysis of BW 1370U87, a novel MAO-A inhibitor, and its major metabolites, BW 183U88 and BW 1003U88 in human plasma. The compounds were isolated from plasma by liquid extraction with methyl-t-butyl ether. The extracts were chromatographed on a LiChrospher 100 RP-8 column (4.6 mm x 25 cm) using a gradient elution with a mobile phase consisting of acetonitrile-water containing 0.025% phosphoric acid (25:75, v/v, initial conditions increasing to 40:60, v/v, over 25 min). The compounds were detected at 226 nm with a lower limit of quantitation of 10 ng/ml. The accuracy and precision of the assay, expressed as the percent deviation of measured values from true values and the percent coefficient of variation,

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respectively, were ≤8%, at all concentrations. The assay has been successfully applied to the analysis of samples from a clinical pharmacokinetic study in man.

INTRODUCTION

BW 1370U87, 1-ethylphenoxathiin-10,10-dioxide (Fig. 1), is a structurally novel reversible, selective inhibitor of brain monoamine oxidase-A (MAO-A) (1) currently in Phase I clinical trials in the U.S.A. Preclinical pharmacology showed that BW 1370U87 elevated brain amine levels in rats without significantly potentiating the blood pressure effects of orally-administered tyramine (2, 3). Consequently, BW 1370U87 is being developed as a potential anti-depressant which can be expected to be free of the tyramine-potentiating blood pressure effects that may lead to a hypertensive crisis with traditional monoamine oxidase inhibitors.

BW 1370U87 is extensively metabolized in animals and man. Two major metabolites have been identified in plasma by gas chromatography-mass spectrometry as the 1-(1-hydroxyethyl), BW 183U88, and 1-(1,2-dihydroxyethyl), BW 1003U88, which are active MAO-A inhibitors (4). To support clinical studies, a high performance liquid chromatographic (HPLC) method with UV detection was developed and validated for the determination of BW 1370U87 and its major metabolites in human plasma. The assay has been successfully applied to the analysis of BW 1370U87 and its major metabolites in pharmacokinetic studies in man.

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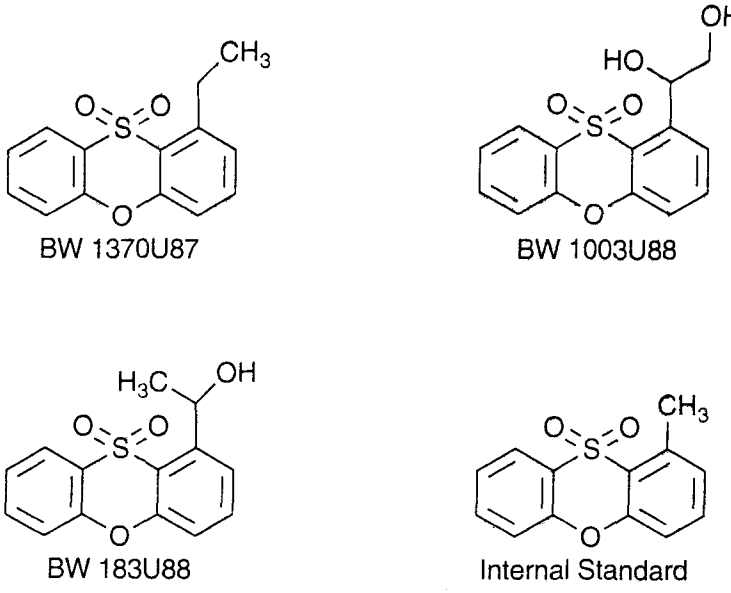

Figure 1. Structures of BW 1370U87, BW 183U88, BW 1003U88 and the internal standard.

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MATERIALS AND METHODS

Materials

BW 1370U87 and its metabolites, BW 183U88, 1-(1-hydroxyethyl)phenoxathiin-10,10-dioxide and BW 1003U88, 1-(1-phenoxathiinyl)-1,2-ethanediol-10,10-dioxide, were synthesized at Burroughs Wellcome Co., Research Triangle Park, NC. The internal standard, 1-methylphenoxathiin 10,10-dioxide, was also synthesized at Burroughs Wellcome Co. Phosphoric acid (85%) was A.R. grade (Mallinckrodt, Paris, KY.). Methanol, acetonitrile, methyl-t-butyl ether and water were HPLC grade (Omnisolv, EM Science, Cherry Hill,

NJ.). Control human plasma was obtained from in-house donors.

Instrumentation

Waters 510 HPLC pumps (Waters Assoc., Milford, MA.) were used with a 4.6 mm x 25 cm LiChrospher 100 RP-8 column (EM Science, Gibbstown, NJ) for the separation of BW 1370U87 and its metabolites. Samples were injected onto the column with a Waters 712 autosampler and the analytes quantitated at 226 nm with a Waters 490E fixed wavelength ultraviolet detector. A gradient elution was used with a mobile phase consisting of acetonitrile and water containing 0.025% phosphoric acid as follows: 0-16 min, initial conditions, acetonitrile-water 25:75, v/v; 16-25 min, acetonitrile increasing linearly to 40:60, v/v acetonitrile-water; 25-27 min flushing period using 100% acetonitrile; 27-35 min equilibration period using initial conditions. With a flow-rate of 1 ml/min, the retention times of BW 1003U88, BW 183U88, internal standard and BW 1370U87 were approximately 10.9, 16.0, 20.6 and 22.8 min, respectively. Chromatographic data acquisition, peak-area analysis and data reduction were performed by an IBM AT personal computer and Waters Maxima software (Waters Assoc., Milford, MA).

Extraction Procedure

Experimental plasma samples (1.0 ml) and calibration standards (10-2000 ng/ml) were spiked with

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internal standard (50 µl of 10 mg/ml) and extracted with 6 ml of methyl-t-butyl ether. Samples were shaken for 15 min and then centrifuged. Aliquots (5 ml) of the organic phase were removed and evaporated to dryness under nitrogen. The residues were reconstituted in 50 µl of methanol and aliquots (30 µl) were injected onto the HPLC.

Assay Validation

To determine the intra-day accuracy and precision of the assay, replicate (n=6) plasma samples at seven different concentrations (10-2000 ng/ml) were analyzed. The peak height ratios of each compound and the internal standard were calculated and a least squares regression analysis was performed on the $1/concentration^2$ weighted data. Precision was estimated from the standard deviation expressed as a percentage of the mean (% coefficient of variation, CV). Accuracy was calculated as the percentage difference between the mean calculated concentration and the amount added (% bias).

To determine the inter-day accuracy and precision of the assay, spiked plasma pools were prepared at three concentrations (20, 500 and 1500 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 over a period of six weeks to determine the inter-assay accuracy and precision of the assay. To differentiate between the intra- and inter-day variability in these data, an analysis of variance analysis (ANOVA) was performed using SAS statistical software (SAS Institute, Cary, NC).

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

The stability of BW 1370U87 and its metabolites in human plasma stored at -20°C was determined. Plasma was spiked with 20, 500 or 1500 ng/ml of BW 1370U87, BW 183U88 and BW 1003U88. Samples were analyzed immediately and at various times after storage at -20°C. Stability was also determined in plasma samples (20 and 500 ng/ml) after five freeze-thaw cycles.

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Biomedical Application

A clinical study was designed to investigate the pharmacokinetics of BW 1370U87 and its metabolites in human volunteers. Informed written consent was obtained from healthy male volunteers prior to receiving oral doses of BW 1370U87. The compound was formulated in Labrafil (Gattefosse Corp., Hawthorne, NY) and administered in capsules. Venous blood samples were taken at various time points. Plasma was separated by centrifugation and samples frozen at -20°C until analyzed.

RESULTS AND DISCUSSION

A sensitive and selective assay has been developed for the quantitation of BW 1370U87 and its major metabolites in human plasma. The assay is specific

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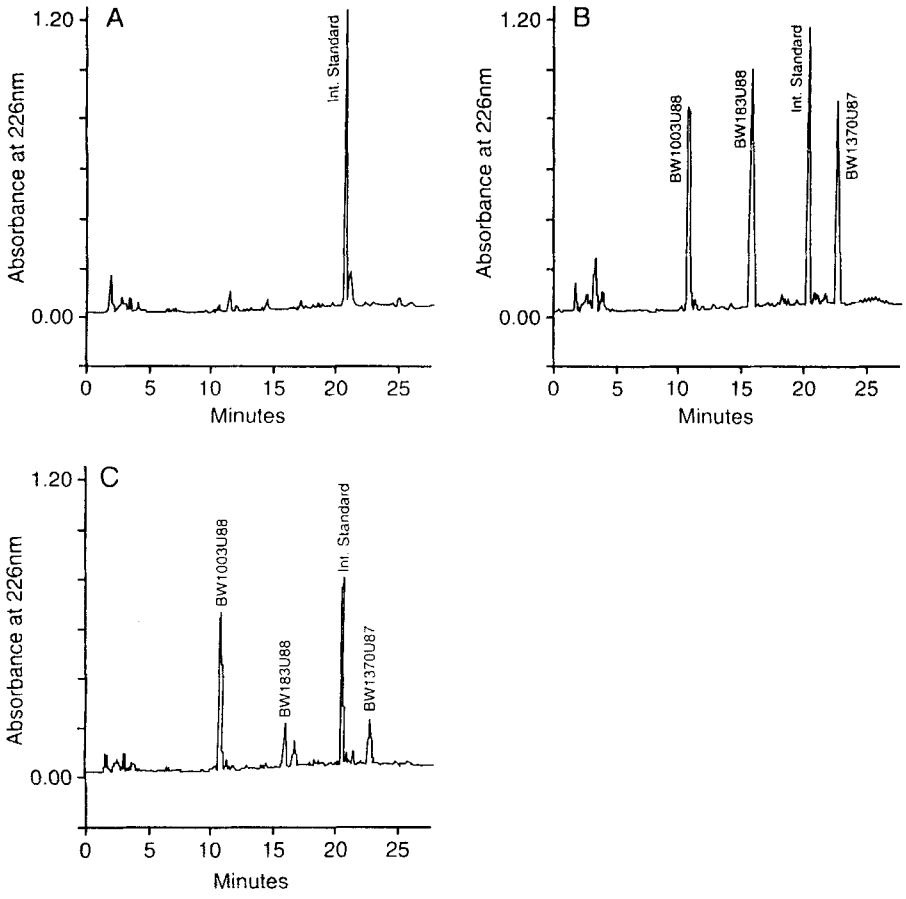

Figure 2. HPLC chromatograms of (A) extracted control human plasma,(B) a 500 ng/ml calibration standard, and (C) an extract of a plasma sample taken from a human volunteer at 0.5 hr after an oral dose of BW 1370U87.

TABLE 1 Intra-day Precision and Accuracy of BW 1370U87, BW 183U88 and BW 1003U88 in Human Plasma

Conc. Added (ng/ml)	Mean Conc. Found (ng/ml)	CV %	Bias %
BW 1370U87			
10	10.1	0.5	0.9
25	24.6	1.6	-1.5
50	49.4	1.5	-1.1
100	98.8	4.1	-1.2
500	493.9	4.7	-1.2
1000	1019.9	2.0	2.0
2000	2007.1	3.9	0.4
BW 183U88			
10	10.2	1.1	1.7
25	24.2	3.4	-3.1
50	49.3	1.9	-1.5
100	96.7	4.6	-3.3
500	495.1	4.8	-1.0
1000	1027.0	1.7	2.7
2000	2040.3	4.4	2.0
BW 1003U88			
10	10.3	1.9	2.9
25	24.6	5.3	-5.6
50	48.3	1.7	-3.3
100	98.3	3.5	-1.7
500	496.0	4.7	-0.8
1000	1026.9	2.4	2.7
2000	2064.7	3.6	3.2

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for BW 1370U87 and its metabolites and no interfering peaks appear in chromatograms of control plasma (Fig. 2). Fig. 2 also shows a calibration standard (500 ng/ml) and a chromatogram of a sample extract from a human volunteer taken at 30 min after an oral dose of BW 1370U87. The lower limit of quantitation for each of the compounds was 10 ng/ml. Three other

TABLE 2 Inter-Day Accuracy and Precision for the Analysis of BW 1370U87, BW 183U88 and BW 1003U88 in Human Plasma

Compound	Target Conc. (ng/ml)	Mean Conc. Found (ng/ml)	CV[a] %	Bias %
BW 1370U87	20	20.9	2.6	4.5
	500	525.4	0.8	5.1
	1500	1523.5	2.8	1.6
BW 183U88	20	21.6	3.6	8.0
	500	527.5	2.3	5.5
	1500	1548.8	0.6	3.3
BW 1003U88	20	21.0	3.0	4.8
	500	523.2	1.6	4.6
	1500	1527.0	2.2	1.8

n = 28 for low concentration.
n = 40 for medium and high concentrations.
[a]ANOVA was performed to differentiate between intra- and inter-day variability.

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minor metabolites of BW 1370U87 have been identified in plasma with this assay. These metabolites are completely resolved from BW 1370U87, BW 183U88 and BW 1003U88 so that they do not interfere in the assay. These minor metabolites are present in very low concentrations and they were not quantitated in clinical studies.

The intra-day accuracy and precision data for the assay of BW 1370U87, BW 183U88 and BW 1003U88 in human plasma are shown in Table 1. The intra-day % CV was

TABLE 3 Extraction Efficiencies of BW 1370U87, BW 183U88 and BW 1003U88 in Human Plasma

Compound	Conc. (ng/ml)	Recovery (mean ± S.D.) (%)
BW 1370U87	20	80.4 ± 3.2
	500	96.2 ± 3.8
	1500	110.5 ± 1.9
BW 183U88	20	83.8 ± 4.2
	500	93.3 ± 4.1
	1500	95.3 ± 1.7
BW 1003U88	20	101.7 ± 3.4
	500	103.2 ± 3.9
	1500	102.5 ± 1.4

n = 6 at each concentration.

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5% or less for BW 1370U87 and its metabolites over the concentration range 10-2000 ng/ml. The % bias was less than 6% for all compounds. The inter-day accuracy and precision of the assay, measured over a 6-week period, is shown in Table 2. Analysis of variance analysis of these data showed that the inter-day %CV was 4% or less for all three compounds. The % bias was 8% or less for all three compounds.

The extraction efficiencies for BW 1370U87, BW 183U88 and BW 1003U88 are shown in Table 3. The mean extraction efficiencies ranged from 80-111%, 84-95%, and 102-103% for BW 1370U87, BW 183U88 and BW 1003U88, respectively, over the concentration range of 20-1500 ng/ml. The extraction efficiency of

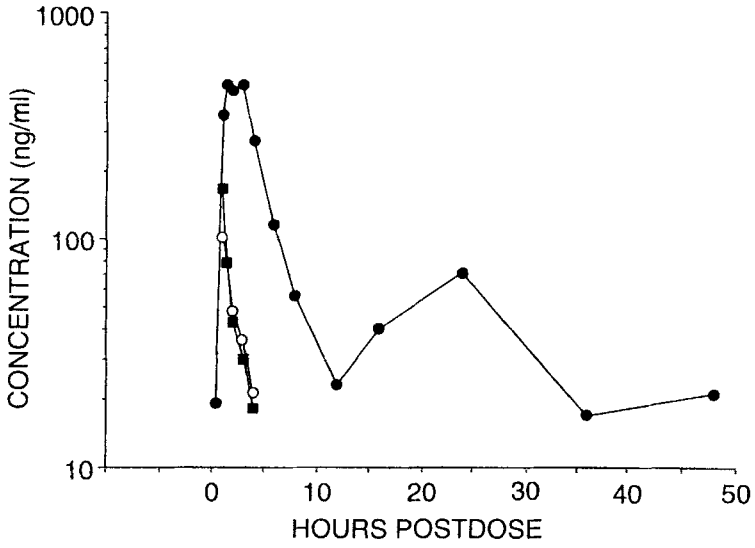

Figure 3. Plasma concentrations of BW 1370U87 (■), BW 183U88 (○) and BW 1003U88 (●) in a human volunteer after a 400 mg oral dose of BW 1370U87.

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BW 1370U87 and BW 183U88 was lower at 20 ng/ml than at higher concentrations. However, the accuracy data shown in Tables 1 and 2 indicate that use of an internal standard compensates for the lower recovery at low concentrations.

The stability of BW 1370U87 and its metabolites in plasma stored at -20°C was studied. No degradation of either BW 1370U87, BW 183U88 or BW 1003U88 was detected in plasma samples stored for at least 7 weeks at this temperature. In addition, no degradation of either compound was detected in plasma samples after five freeze-thaw cycles.

The assay has been used to quantitate plasma concentrations of BW 1370U87 and its metabolites in

volunteers after oral administration of BW 1370U87. As an example of the utility of the assay, a plasma concentration versus time curve for each compound following an oral dose of 400 mg of BW 1370U87 in one human volunteer is shown in Fig. 3. BW 1370U87 is extensively metabolized in man after oral dosing. The major compound detected in human plasma was BW 1003U88, with lower concentrations of the parent drug and BW 183U88.

In summary, a sensitive and selective HPLC assay has been developed and validated for the simultaneous quantitation of BW 1370U87 and its two active metabolites in plasma. The method has been used to study the pharmacokinetics of BW 1370U87 and its metabolites in man.

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