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# Determination of an antipsychotic agent (ICI 204,636) and its 7-hydroxy metabolite in human plasma by high-performance liquid chromatography and gas chromatography–mass spectrometry

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ABSTRACT

ICI 204,636 (I) is an orally active antipsychotic agent under development for the treatment of schizophrenia in humans. It is partially converted in animals to an active 7-hydroxy metabolite (II). Methods were developed for the simultaneous determination of both analytes in human plasma using high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). The analytes were extracted from plasma using phenyl solid-phase extraction columns. Quantification by isocratic HPLC was performed in the reversed-phase mode with detection at 250 nm. Extracts were derivatized to trimethylsilyl ethers for quantification by GC MS using selected-ion monitoring. Both assays were evaluated for consistency of response, precision, accuracy and specificity. Limits of quantification for I and II by HPLC were 15 and 20 ng/ml, respectively; limits of quantification for I and II by GC–MS were 2 and 5 ng/ml, respectively. Both methods were applied to the analysis of clinical samples from oral dosing studies with I.

## INTRODUCTION

ICI 204,636 (I) is an antipsychotic compound intended for the treatment of schizophrenia and other psychotic syndromes in humans [1]. Based on its pharmacological profile in animals, it should be less likely to produce extrapyramidal side effects and tardive dyskinesia than other neuroleptics [2]. Work was initiated in our laboratory to develop and validate a plasma assay to provide definitive clinical pharmacokinetic information of I and its active 7-hydroxy metabolite (II) found in animals.

Physicochemical methods for the determination of antipsychotic compounds in biological fluids have been reviewed by McKay *et al.* [3] and Curry [4]. Initial efforts were directed at developing a reversed-phase high-performance liquid chromatographic (RP-HPLC) method using UV detection and a solid-phase extraction (SPE) approach. The assay was validated and applied to the analysis of clinical samples. However, it was apparent that a lower limit of quantification would be required to define analyte pharmacokinetics at the low doses administered to normal volunteers.

Methods for antipsychotic compounds with quantification limits in the low ng/ml range, or better, include HPLC with electrochemical detection, and gas chromatography with nitrogen–phosphorus detection (GC–NPD), electron-capture detection (ECD) or mass spectrometric (MS) detection [3,4]. GC–MS has not been applied extensively to the analysis of routine clinical samples because of high instrumental costs, limited

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sample througput and the need for specialized personnel to operate the instrument [3].

Jemal *et al.* [5] reported a technique for the analysis of fluphenazine in human plasma with a 50 pg/ml limit of quantification using capillary GC–MS with a commercially available benchtop quadrupole mass spectrometer. Sample injection, data acquisition and data reduction were accomplished in an automated fashion which permitted the routine analysis of over 100 samples in a 24-h period. The benchtop GC–MS system was sufficiently rugged to permit the analysis of 300–400 samples between ion source cleanings.

The aim of this study was to develop a rugged GC–MS method for I and II with a lower quantification limit than the existing HPLC method. The performance of the two procedures was subsequently compared.

## EXPERIMENTAL

### *Materials*

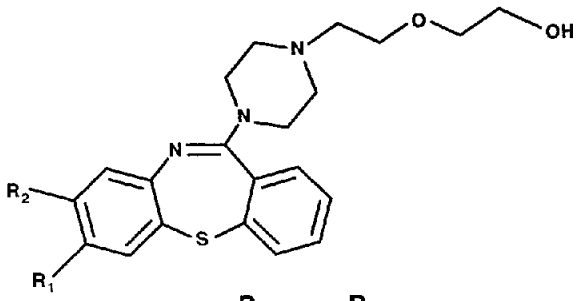
Compound I, 2-[2-(4-dibenzo[*b*,*f*]-1,4-thiazepin-11-yl-1-piperazinyl)ethoxy]ethanol, and its 7-hydroxy metabolite (II), 8-hydroxy (III, the internal standard, I.S., for II) and 8-fluoro (IV, the I.S. for I) derivatives were provided by the ICI Pharmaceuticals Group (ICI Americas, Wilmington, DE, USA) (Fig. 1). Acetonitrile, methanol, toluene and water were HPLC grade purchased from J. T. Baker (Phillipsburg, NJ, USA). Potassium phosphate, dibasic (99.4%), phosphoric acid (85.4%), triethylamine (100%) and hydrochloric acid (37.2%) were Baker-analyzed-grade reagents (J. T. Baker). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine were silylation grade (Pierce, Rockford, IL, USA). Blood for blank plasma was obtained from apparently healthy human volunteers using Vacutainer collection tubes containing sodium heparin anticoagulant (Becton Dickinson, Rutherford, NJ, USA). The blood samples were centrifuged at 1000 *g* for 15 min and the plasma was separated and stored at approximatley −20°C.

### *Sample preparation*

*Standard preparation.* Plasma calibration standards were prepared fresh daily by spiking 1 ml of blank human plasma with 10 μl of an appropriate I or II standard spiking solution prepared in methanol. Plasma calibration standards were prepared in duplicate at seven levels during four separate validation runs. The HPLC assay calibration ranges for I and II were 16–1000 and 21–1300 ng/ml, respectively. The GC–MS calibration ranges for I and II were 2–100 and 5–200 ng/ml, respectively.

Pooled plasma quality control (QC) samples for I were prepared at 260 and 30 ng/ml and for II at 330 and 40 ng/ml for the HPLC assay and at 50 and 5 ng/ml for both analytes for GC–MS.

*Plasma extraction.* The same plasma extraction procedure was used for both methods. Bond Elut 100 mg per 1 ml phenyl SPE columns (Analytichem International, Harbor City, CA, USA) were placed in a thirty-column vacuum manifold (Applied Separations, Bethlehem, PA, USA) and prepared for the plasma extraction by washing with 2 ml of methanol followed by 2 ml of water. Plasma samples were spiked with 10 μl of a methanolic I.S. solution of IV (100 μg/ml) for the HPLC assay and III and IV (10 μg/ml each) for the GC–MS assay. The samples were acidified to pH 3.5 with 85 μl of 1 *M* hydrochloric acid, vortexed for 30 s and centrifuged at 1000 *g* for 10 min. The plasma was applied to the extraction columns and drawn through under vacuum (approximately 3400 Pa). The columns were washed with two 0.5-ml volumes of acetonitrile–water (4:6, v/v), then dried under vacuum for 10 min. The columns were washed twice with 0.5 ml of toluene before the analytes were eluted into poly-




| | $R_1$ | $R_2$ |
|---|---|---|
| I | H | H |
| II | OH | H |
| III | H | OH |
| IV | H | F |

Fig. 1. Analyte structures.

propylene tubes with two 0.5-ml volumes of 0.2% triethylamine in methanol.

*Preparation for HPLC analysis.* The samples were evaporated under a stream of dried nitrogen in a 40°C water bath. Samples were reconstituted by adding 0.25 ml of mobile phase and vortex-mixing for 1 min. The samples were transferred to polypropylene microvials, capped and placed on the HPLC autosampler. Absolute recovery for the plasma extraction procedure was evaluated by HPLC peak-height comparison with non-extracted reference standards prepared in mobile phase.

*Derivatization for GC–MS analysis.* The eluted samples were transferred to silanized [6] glass reaction vials and evaporated under dried nitrogen in a 40°C water bath. Pyridine and BSTFA (20 μl each) were added to the dried extracts and the vials were capped and vortex-mixed for 15 s. The samples were derivatized by incubation at 65°C for 40 min. After incubation, the samples were vortex-mixed for 15 s, transferred to glass microvials, capped and placed on the GC–MS autosampler.

*High-performance liquid chromatography*

The HPLC apparatus consisted of an ACS Model 351 pump (Peris Industries, State College, PA, USA), an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, USA), a NewGuard RP-8, 7 μm particle size, 15 mm × 3.2 mm I.D. cartridge guard column (Brownlee Labs., Santa Clara, CA, USA), a 250 mm × 4.6 mm I.D. analytical column packed with ODS Hypersil, 5 μm particle size (Keystone Scientific, State College, PA, USA) and a Spectroflow 783 UV detector equipped with a deuterium light source to monitor absorbance at 250 nm (ABI Analytical, Ramsey, NJ, USA). The mobile phase was acetonitrile–methanol–5 m*M* potassium phosphate (pH 7.0) (2:1:2, v/v). The mobile phase was filtered through a 0.45-μm Nylon 66 membrane filter and degassed by helium sparging before use. The flow-rate was 1 ml/min with an injection volume of 25 μl and run time of 16 min. All separations were performed at ambient temperature (22–26°C).

*Capillary GC–MS*

The chromatographic system consisted of a Model 7673A autosampler, a 5890A gas chromatograph, a 5971A quadrupole mass-selective detector (electron impact ionization, 70 eV) and a 59970C MS ChemStation (HP 9000 Series 300 computer) with associated software (MS-MSD operating system and sequencing software) and peripheral components (35731B monitor, 9144A tape drive, 7957B disc drive and 2225A printer) (all from Hewlett-Packard, Palo Alto, CA, USA). The analytical column was a J & W DB-1 (cross-linked methylsilicone bonded phase), 30 m × 0.25 mm I.D., 0.1 μm film thickness. Ultrapure carrier-grade helium was used as the carrier gas. The gas flow-rate through the column was 0.75 ml/min at 295°C, the flow-rate through the split vent was set at 60 ml/min and the septum purge rate was 2.4 ml/min. A 3-μl injection volume was used with a 1.6-min splitless period. The injection port was set at 290°C. The column oven was held at 190°C for 1.6 min after injection, then ramped from 190 to 295°C at 30°C/min and held at 295°C for 8.9 min. The total temperature program time was 14 min. The GC–MS transfer line was set at 300°C and the ion source was heated by conductance to approximately 170°C.

The detector was tuned daily using an automated program designed to optimize performance for the ion fragments of perfluorotributylamine (PFTBA) at 69, 219 and 502 daltons. The electron multiplier was set 400 V above the setting determined for the PFTBA tune. All other detector component settings optimized by the tune program were used unchanged. The detector was turned on from 9 to 14 min after sample injection. Ions at *m/z* 322 and 340 were monitored from 9 to 11 min using a peak dwell time of 150 ms for each ion. Ions at *m/z* 314 were monitored from 11 to 14 min with a peak dwell time setting of 0.30 s.

*Data acquisition and quantification*

*HPLC assay.* Collection and analysis of the detector output (1 v/A.U.) was performed with a Microvax computer (Digital Equipment, Maynard, MA, USA) using VG Multichrom software

(VG Laboratory Systems, Altrincham, U.K.). The mean relative weight response (RWR) [(peak-height ratio of analyte/I.S.) × (I.S. concentration/analyte concentration)] was calculated for the plasma standard calibration curve. Assay results were calculated by multiplying the peak-height ratio (analyte/I.S.) by the I.S. concentration and dividing by the mean RWR. This mode of calibration was selected as a result of the linearity of response and relatively constant relative standard deviation (R.S.D.) observed over the validation range. This approach is roughly equivalent to using a weighted ($1/x$) linear model with forced zero intercept.

*GC–MS assay.* Collection and analysis of the detector output was performed with the HP Series 300 computer using MS-MSD operating system software (Hewlett-Packard). Non-linear dependence of response upon concentration was consistently noted for both analytes and was presumably due to adsorptive losses in spite of silanization of glassware. Therefore regression for both analytes was performed using a power fit of the form $y = bx^m$ where $y$ = peak-height ratio (analyte/I.S., $x$ = analyte concentration, $b$ = a multiplicative factor and $m$ = exponent of the fitted curve. Concentrations for unknowns ($x$) were calculated by entering the peak-height ratios ($y$) into the equation along with values obtained for $m$ and $b$ from the non-linear regression analysis of the calibration curve. Data acquisition, integration and quantification were all performed in an automated operating mode. Calibration standard response, and assay precision and accuracy were evaluated for each of the four validation runs.

*Specificity*

Heparinized plasma was obtained from twenty drug-free, apparently healthy human volunteers. The samples were stored at approximately −20°C until they were assayed in duplicate to screen for endogenous analyte interferences.

Analytical standards were prepared at a concentration of 1000 ng/ml for the sulfoxide, sulfone, N-dealkylated, 7-hydroxy-N-dealkylated and N-ethanol derivatives of I. In addition, 1000 ng/ml analytical standards of the potential concomitant drugs chloral hydrate, diphenhydramine and benztropine mesylate were analyzed.

*Assay cross-validation study*

Thirteen plasma samples from a clinical study using single 25-mg oral doses of I in normal volunteers were assayed by both HPLC and GC–MS. The goal of this experiment was to examine agreement of the two methods across their common dynamic range.

## RESULTS

*High-performance liquid chromatography*

Typical HPLC within-run retention times (and R.S.D.) for II, I and IV were 4.0 min (0.4%), 9.7 min (0.5%) and 11.9 min (0.5%), respectively. Injection of over 250 plasma extracts did not produce a significant change in system back-pressure or retention times. Inter-day differences in mobile phase composition, as well as chromatography using two lots of column packing material did not significantly alter peak resolution or sensitivity.

*Capillary GC–MS*

Typical GC–MS retention times for the trimethylsilyl (TMS) ether derivatives of the analytes were 9.9, 10.1, 12.6 and 13.0 min for IV, I, III and II, respectively. Intra-day retention time reproducibility was typically within ±1% of the mean ($n$ = 64). Comparable chromatographic efficiency, resolution and retention times have been obtained using three different DB-1 columns. The system was demonstrated to be free from injection carry-over problems. Automated sample injection and integration for a typical clinical run of 60 samples can be completed in 20 h. Mass spectra of the TMS ether derivatives of I and IV formed using BSTFA are presented in Fig. 2. The molecular ions for the I and IV derivatives were present at $m/z$ 455 and 473, respectively. Mass spectra of the TMS ether derivatives of II and III are presented in Fig. 3. The presence of molecular ions at $m/z$ 543 indicates that these analytes are doubly derivatized with BSTFA at both the ring and terminal hydroxyl positions. All four derivatives produced characteristic ions corresponding to the tricyclic portion of the molecule (I = 210, IV = 228, II and III = 298) and an N-ethyl fragment (I = 322, IV = 340, II and III = 410). The ions selected for quantification were at $m/z$ 322 for I, $m/z$ 340 for IV and $m/z$ 314 for II and III.

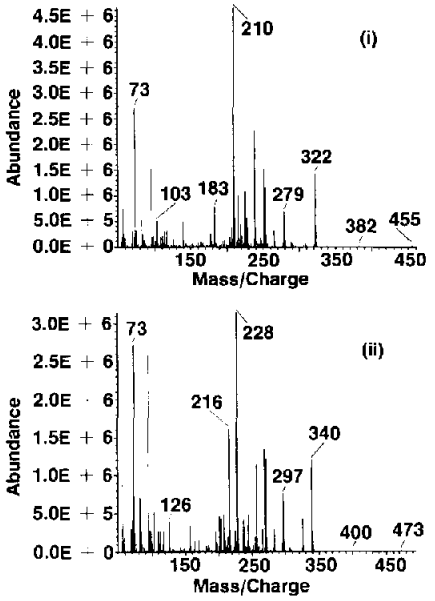



Fig. 2. Electron-impact mass spectra of TMS ether derivatives of I (i) and IV (ii).

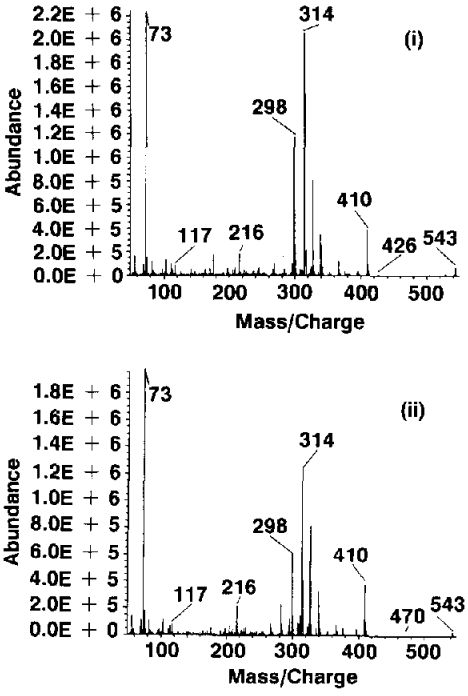



Fig. 3. Electron-impact mass spectra of TMS ether derivatives of II (i) and III (ii).

TABLE I

POOLED HPLC SPIKED PLASMA CALIBRATION STANDARD RESULTS

Spiked plasma calibration standards were assayed in duplicate at each level during four separate validation runs. Assay values were calculated using the mean relative weight response calculated across the range of the calibration curve.

| Spiking concentration (ng/ml) | Mean assay concentration (ng/ml) | R.S.D. (%) | Relative recovery (%) |
|---|---|---|---|
| *Compound I* | | | |
| 16.0 | 18.1 | 3.1 | 113 |
| 32.1 | 32.9 | 2.6 | 103 |
| 64.1 | 63.0 | 3.1 | 98 |
| 128 | 123 | 2.9 | 96 |
| 257 | 246 | 2.8 | 96 |
| 513 | 496 | 3.0 | 97 |
| 1030 | 1010 | 3.5 | 98 |
| *Compound II* | | | |
| 20.5 | 19.0 | 10 | 93 |
| 40.9 | 39.2[a] | 6.3 | 96 |
| 81.8 | 83.1 | 4.8 | 102 |
| 164 | 167[a] | 5.5 | 102 |
| 327 | 319 | 8.5 | 98 |
| 654 | 686[a] | 2.8 | 105 |
| 1310 | 1410[a] | 4.3 | 108 |

[a] $n = 7$, one assay result was rejected by the Q-test at the 90% confidence level.

*Calibration standard response, precision and accuracy*

*HPLC.* The mean absolute recovery across four HPLC validation runs was found to be 93.2 and 84.6% for I and II, respectively. Validation results for I and II HPLC calibration standards are presented in Table I. The pooled estimate of calibration standard precision for I from 16 to 1000 ng/ml was 6.4% R.S.D. The signal-to-noise ratio for I at the quantification limit of 15 ng/ml was consistently three times greater than coeluting endogenous plasma components. The pooled estimate of calibration standard precision for II from 21 to 1300 ng/ml was 7.5% R.S.D. The signal-to-noise ratio of II at 20 ng/ml was consistently greater than 10:1. Chromatograms of a plasma blank and two calibration standards are shown in Fig. 4.

*GC–MS.* Results for I and II GC–MS calibra-

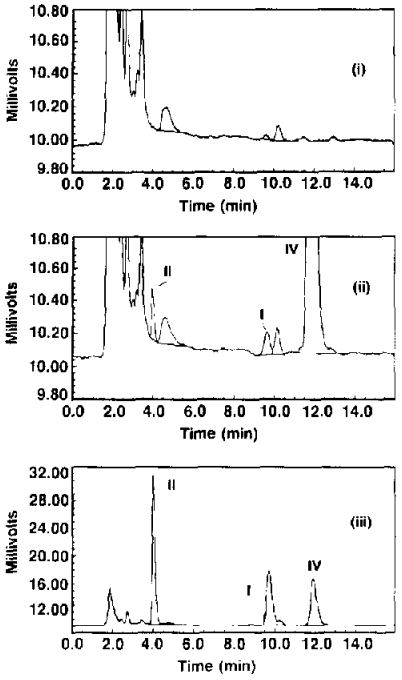



Fig. 4. Representative HPLC profiles of a plasma blank (i), a plasma calibration standard spiked at 16 ng/ml for I and 21 ng/ml for II (ii) and a plasma calibration standard spiked at 1000 ng/ml for I and 1300 ng/ml for II (iii).

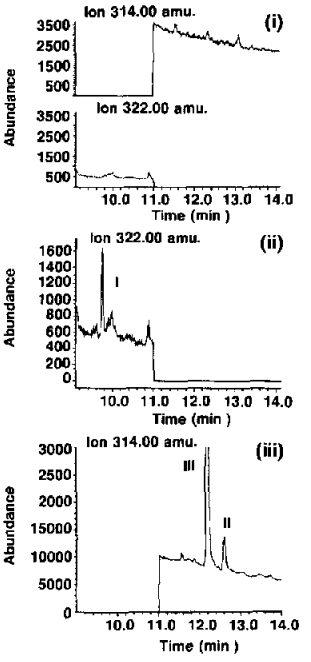



Fig. 5. Representative GC–MS profiles of a plasma blank (i), a 2 ng/ml plasma calibration standard of I (ii) and a 5 ng/ml plasma calibration standard of II (iii).

TABLE II

POOLED GC–MS SPIKED PLASMA CALIBRATION STANDARDS

Spiked plasma calibration standards were assayed in duplicate at each level during four separate validation runs. Data were analyzed using a power fit curve of the form $y = bx^m$.

| Spiking concentration (ng/ml) | Mean assay concentration (ng/ml) | R.S.D. (%) | Relative recovery (%) |
|---|---|---|---|
| *Compound I* | | | |
| 2.12 | 2.28 | 17 | 108 |
| 5.30 | 5.25 | 15 | 99 |
| 10.6 | 9.29 | 8.6 | 88 |
| 21.2 | 19.6 | 9.2 | 92 |
| 53.0 | 54.8 | 13 | 103 |
| 106 | 114 | 8.5 | 108 |
| *Compound II* | | | |
| 5.10 | 5.05 | 8.4 | 99 |
| 10.2 | 10.0 | 10 | 98 |
| 20.4 | 19.4 | 5.3 | 95 |
| 51.0 | 50.5 | 7.0 | 99 |
| 102 | 98.4 | 4.5 | 96 |
| 204 | 217 | 5.2 | 106 |

tion standards are presented in Table II. Pooled estimates of standard precision for II over the entire calibration range were $\leq 10\%$ R.S.D., with mean recoveries ranging from 95 to 106%. The pooled estimate of standard precision for I was $\leq 15\%$ R.S.D. with the exception of the 2 ng/ml standard (17% R.S.D.). Mean recoveries ranged from 88 to 108%. A signal-to-noise ratio of $\geq 5{:}1$ was consistently maintained for both analytes at their respective quantification limits. Representative mass chromatograms of a plasma blank, a 2 ng/ml plasma standard of I and a 5 ng/ml plasma standard of II are shown in Fig. 5.

*Quality control sample precision and accuracy*

*HPLC.* HPLC assay precision and accuracy for I and II were evaluated by assaying pooled plasma quality control samples (Table III). Pooled plasma quality control sample mean relative recoveries for II at 41 and 330 ng/ml were $\geq 96\%$ ($\leq 6\%$ R.S.D.). The pooled estimate of relative recovery for I at 32 and 260 ng/ml was 91% (2% R.S.D.).

*GC–MS.* GC–MS assay precision and accuracy for I and II were also evaluated by assaying

TABLE III

POOLED PLASMA QUALITY CONTROL SAMPLE PRECISION AND ACCURACY

For HPLC, $n = 3$ for 1 day. Duplicate quality control samples were run on subsequent days in light of the good precision observed on day 1. For GC–MS, $n = 2$ for all values reported.

| Spiking concentration (ng/ml) | Mean assay concentration (ng/ml) | | | | Pooled estimate of precision and recovery |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| *I by HPLC* | | | | | |
| 32.1 | 29.2 | 28.8 | 29.5 | 30.0 | 29.3 |
| % R.S.D. | 1.6 | 2.0 | 1.6 | 1.2 | 2.0 |
| % Recovery | 91 | 90 | 92 | 94 | 91 |
| 257 | 234 | 226 | 237 | 240 | 234 |
| % R.S.D. | 1.4 | 2.1 | 0.1 | 0.3 | 2.4 |
| % Recovery | 91 | 88 | 92 | 93 | 91 |
| *II by HPLC* | | | | | |
| 40.9 | 41.1 | 38.4 | 37.9 | 38.2 | 39.1 |
| % R.S.D. | 2.0 | 1.1 | 2.5 | 0.5 | 4.1 |
| % Recovery | 100 | 94 | 93 | 93 | 96 |
| 327 | 343 | 316 | 299 | 327 | 324 |
| % R.S.D. | 1.8 | 1.1 | 9.6 | 0.1 | 6.4 |
| % Recovery | 105 | 97 | 91 | 100 | 99 |
| *I by GC–MS* | | | | | |
| 5.00 | 4.30 | 4.43 | 5.18 | 4.76 | 4.67 |
| % R.S.D. | 2.0 | 4.3 | 3.3 | 18.1 | 10.7 |
| % Recovery | 86 | 89 | 104 | 95 | 93 |
| 50.0 | 53.7 | 51.0 | 46.8 | 43.9 | 48.8 |
| % R.S.D. | 23.2 | 8.5 | 8.6 | 1.0 | 13.5 |
| % Recovery | 107 | 102 | 94 | 88 | 98 |
| *II by GC–MS* | | | | | |
| 5.13 | 6.15 | 6.16 | 5.34 | 5.63 | 5.82 |
| % R.S.D. | 0.7 | 2.6 | 10.3 | 6.8 | 7.8 |
| % Recovery | 120 | 120 | 104 | 110 | 113 |
| 51.3 | 50.9 | 55.4 | 50.5 | 46.9 | 50.9 |
| % R.S.D. | 0.7 | 3.4 | 8.0 | 3.3 | 7.2 |
| % Recovery | 99 | 108 | 98 | 91 | 99 |

pooled quality control samples (Table III). Pooled plasma quality control sample mean relative recovery for I was 93% (11% R.S.D.) at 5 ng/ml and 98% (13% R.S.D.) at 50 ng/ml. Pooled plasma quality control sample mean relative recovery for II was 113% (7.8% R.S.D.) at 5 ng/ml and 99% (7.2% R.S.D.) at 50 ng/ml.

*Stability*

There was no significant change in concentration of quality control samples assayed by HPLC after storage for twelve months at either −20°C or −70°C. There was no change in potency for either analyte by GC–MS after three months of storage at −20°C. Stability of spiked plasma samples carried through three freeze-thaw cycles has been demonstrated for both assays.

*Specificity*

No quantifiable analyte interference peaks were present in plasma samples obtained from twenty drug-free, human volunteers. Both methods were demonstrated to be specific against interference from caffeine, nicotine, acetaminophen, ibuprofen, acetylsalicylic acid and any related metabolites formed within 1 h of ingestion. Plasma harvested from hemolyzed blood was also free of analyte interference peaks.

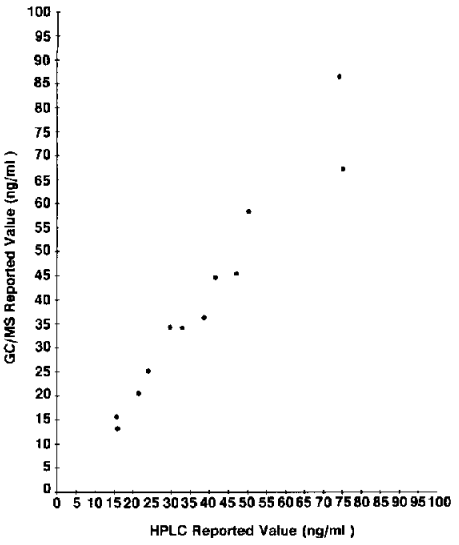



Fig. 6. HPLC *versus* GC–MS cross-validation assay data for I from clinical plasma samples following a single 25-mg oral dose.

Potential metabolite and concomitant drug analytical standards were also analyzed. None of the standards that were screened produced detectable analyte interference peaks in the HPLC system. The sulfoxide and sulfone derivatives of I apparently underwent 1–2% conversion to a form which was indistinguishable from I by GC MS. This finding prompted cross-validation experiments in actual clinical samples to determine if these derivatives produced a significant assay bias for I. None of the other standards that were screened produced detectable analyte interference peaks by GC–MS.

*Assay cross-validation study*

A comparison of GC–MS and HPLC assay cross-validation results is presented in Fig. 6. Evaluation of the data using the paired Student's *t*-test showed that no difference between the methods could be demonstrated for determination of I at a 0.05 significance level ($p = 0.46$). None of the samples contained quantifiable levels of II by either technique.

## DISCUSSION

The ion fragments selected for GC–MS analysis of both analytes and their respective internal standards corresponded to the fragment of the greatest abundance at >300 daltons in order to provide maximum signal relative to endogenous background. It was critical that the ions monitored for I ($m/z$ 322) and IV ($m/z$ 340) were unique for each compound, since the two peaks were not adequately resolved. The baseline resolution for II and III enabled collection of the same ion fragment for both analytes ($m/z$ 314). This permitted the use of a longer dwell time setting, which resulted in an improved signal-to-noise ratio for both peaks.

Validation results over the respective assay calibration ranges indicated that HPLC assay precision was superior to GC–MS overall, and that GC–MS assay precision for II was superior to that for I. Possible sources for these trends are adsorptive losses to glassware, the derivatization reaction, lower analyte concentrations and the shorter dwell time setting used for I. Apparently the I.S. used for II is better able to compensate for these sources of variability. III is a positional isomer of II, whereas the I.S. for I is a fluorinated derivative of the latter.

Of the potential metabolites and concomitant drugs that were screened, only two, the sulfoxide and sulfone derivatives of I, produced interference peaks. Injection temperature-dependent conversion of sulfoxide metabolite to parent drug has been reported for other antipsychotic drugs assayed by GC techniques [3]. Clinical samples were assayed by both HPLC and GC/MS to verify if the conversion demonstrated with analytical standards produced an assay bias in plasma samples from subjects receiving I. Since the cross-validation results failed to demonstrate bias this conversion to parent is apparently not a problem in real samples.

## CONCLUSIONS

HPLC and GC–MS human plasma assays for I and II were evaluated for response, precision, accuracy and specificity, and demonstrated adequate performance with respect to these parameters. The GC–MS method offers a significant reduction in the limit of quantification over the HPLC technique (7.5-fold increase for I and 4-fold increase for II). An experienced operator can prepare 60 plasma extracts in 8 h for automated

overnight chromatographic analysis by either technique. The assays have proven to be rugged and reproducible during the performance of over 700 clinical assays by HPLC and 600 assays by GC–MS. The GC–MS assay was used to characterize the key pharmacokinetic parameters [time of maximum concentration ($T_{max}$), maximum concentration level ($C_{max}$), area under the concentration–time curve (AUC), and terminal elimination half-life ($t_{1/2}$)] of I after a single 25-mg oral dose in man (unpublished work). The 7-hydroxy metabolite II was not present in plasma at quantifiable levels (> 5 ng/ml) in samples at this dose level. The HPLC assay will be the method of choice in studies conducted at higher-dose levels in patients because of its extended linear range as well as its simpler sample preparation and maintenance requirements.

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