

Chiral Analysis of *d*- and *l*-Modafinil in Human Serum: Application to Human Pharmacokinetic Studies

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Summary: Modafinil is a novel stimulant approved by the FDA for use in the management of excessive sleepiness associated with narcolepsy. Utility for other indications includes attention deficit-hyperactivity disorder (ADHD), depression, and management of cocaine dependence. To investigate the pharmacokinetics of modafinil in these patients, the authors improved and validated an HPLC method to separate and quantitate the separate enantiomers of modafinil in human serum. *d*- and *l*-Modafinil and the internal standard 3,3-diphenylpropylamine were extracted from serum, separated by gradient elution on a β -cyclodextrin column, and then detected by UV absorbance at 225 nm. The elution gradient was developed to eliminate interferences by other drugs used to manage narcolepsy, ADHD, and stimulants of abuse, and endogenous substances in human serum. Validation studies included determination of stability, selectivity, precision, accuracy, and recovery. The method was used to investigate the pharmacokinetics of *d*- and *l*-modafinil in a volunteer after receiving 400 mg twice daily of racemic modafinil for 5 days. Interday and intraday assay variability (CV) typically ranged from 3% to 4%. The limits of detection (0.01 $\mu\text{g/mL}$) and quantitation (0.5 $\mu\text{g/mL}$) were well below the concentration expected in serum from patients receiving therapeutic doses of modafinil. The method was free from interference by methylphenidate, cocaine, commonly used antidepressants, and amphetamines. An example of apparent stereoselective disposition is presented as *d*-modafinil was eliminated more rapidly than *l*-modafinil from human serum. The validation data support the use of this method for human pharmacokinetic studies of modafinil in patients with known or suspected use of common antidepressants, psychostimulants, and drugs of abuse. **Key Words:** Modafinil—Pharmacokinetics—HPLC—Enantiomer—Chiral.

Modafinil, *d,l*-2-[(diphenylmethyl) sulfinyl] acetamide, is a novel psychostimulant that increases vigilance and wakefulness but has less propensity for causing central and peripheral side effects associated with

conventional psychostimulants. It appears to have limited abuse liability in humans and is currently marketed for use in the treatment of patients with daytime sleepiness associated with narcolepsy (1). Its pharmacology suggests it may be useful for a variety of additional conditions, including management of attention deficit-hyperactivity disorder (ADHD) and cocaine dependence (2–4). A recent study also reported that modafinil may enhance the efficacy of antidepressants (5). The overlap in symptoms and medications used to manage these conditions and the increasing reliance on combination drug therapy indicate that modafinil may be taken concomitantly with other prescription medications or drugs of abuse.

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Modafinil is marketed as a racemic mixture of its *d*- and *l*-optical isomers (Fig. 1). Both enantiomers are pharmacologically active and are presumed to contribute to the drug's therapeutic effects; however, *d*-modafinil has an apparent steady-state oral clearance approximately threefold greater than that of its antipode (6). Thus, it is important to measure both enantiomers in pharmacokinetic studies. Previous methods for determination of modafinil in plasma or serum have included achiral (7,8) and chiral analyses (9–11). The existing chiral methods are relatively short isocratic high-performance liquid chromatographic (HPLC) separations appropriate for use in normal volunteer studies. More selective methods are needed to eliminate interferences by drugs potentially coadministered with modafinil.

Herein we describe a selective HPLC method to separate and quantitate modafinil's enantiomers in human serum. The method was developed to eliminate potential interferences by cocaine, methylphenidate, and commonly used amphetamines and antidepressants. Validation studies include measurements of selectivity, precision, accuracy, recovery, and stability.

MATERIALS AND METHODS

Materials

The reference standards *d*- and *l*-modafinil were supplied by Cephalon (West Chester, PA). The internal standard 3,3-diphenylpropylamine was purchased from Aldrich (Milwaukee, WI; Fig. 1). Dextroamphetamine,

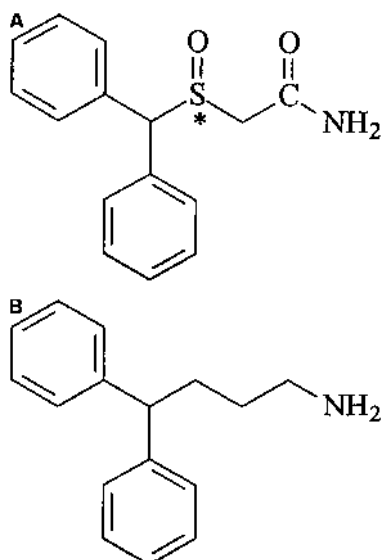


FIG. 1. Structures of (+)-*d*- and (-)-*l*-modafinil (A) and the internal standard 3,3-diphenylpropylamine (B). *Denotes a chiral center.

(-)-methamphetamine, methylphenidate, imipramine, desipramine, and cocaine and its metabolites were purchased from Sigma (St. Louis, MO). Other drugs and metabolites were gifts from their manufacturers. HPLC grade acetonitrile and methanol (Burdick and Jackson Division, Muskegon, MI) and distilled, deionized water were used in mobile phases and preparation of stock solutions. All other reagents were of analytical grade or better. Drug-free human serum was purchased from Quality Assurance Service Corp (Augusta, GA).

Chromatography

A previously described isocratic HPLC separation of modafinil enantiomers was modified for selectivity needed in this study (9). The assay was performed on a Waters 2690 HPLC (Waters Corp., Milford, MA). A gradient elution was performed with a flow rate of 1.0 mL/min. Mobile phase A consisted of 20 mmol/L sodium phosphate adjusted to pH 3.0 with *o*-phosphoric acid. Mobile phase B consisted of 30% acetonitrile in mobile phase A. Phase B was increased from 30% to 60% over a 30-minute period and was then returned to 30% B for 5 minutes. The analytical column was a Cyclobond I-2000 RSP 250 × 4.6 mm (Advanced Separation Technologies, Inc., Whippany, NJ) containing derivatized β-cyclodextrin. The column was preceded by a 10 × 3.2 mm guard column packed with the same material. Detection of modafinil enantiomers occurred by ultraviolet absorption at 225 nm (Waters 2487 detector, Waters Corp.). Chromatographic data were acquired by a computerized integration system (Millenium 3.05, Waters Corp.). *d*- and *l*-Modafinil were identified from their peak retention times through comparisons following direct injections of stock solutions of the enantiomers.

Sample Preparation

Serum samples (200 μL) were spiked to a concentration of 5 μg/mL of the internal standard 3,3-diphenylpropylamine (20 μL of 50 μg/mL solution in 10% acetonitrile). The internal standard and *d*- and *l*-modafinil were extracted with 5 mL hexane, methylene chloride, and triethylamine (55:45:2; v:v:v) and vortex mixed for 30 seconds. The samples were centrifuged at 3000 rpm for 10 minutes, and the supernatant was transferred to a clean tube and evaporated under a stream of nitrogen gas. The remaining residue was dissolved in 200 μL 10% acetonitrile in 20 mmol/L phosphate buffer and analyzed by HPLC as described above.

Quantitation and Validation

To obtain a standard curve for the assay, spiked samples were prepared by serial dilution of stock solutions (1 mg/mL) into 20 mmol/L phosphate buffer containing 10% acetonitrile. Appropriate volumes were added to commercial drug-free serum (200 μ L) to obtain final concentrations of 0.5, 1.0, 5.0, 10.0, 20.0, and 30.0 μ g/mL of *d*- and *l*-modafinil. Concentrations were chosen to create calibration curves covering the expected range of concentrations in subjects receiving modafinil for therapeutic purposes (6). Calibration samples were extracted and analyzed as described above.

The integrated chromatographic peak areas of *d*- and *l*-modafinil and the internal standard were used to construct a standard curve from the peak area ratio versus nominal modafinil enantiomer concentration using unweighted linear regression analysis. The estimated concentrations of modafinil's enantiomers in test samples used the slope of the standard curve generated in the same analytical run. The lower limit of quantitation was defined as the lowest concentration of standard.

Drug-free spiked serum samples at 3 and 15 μ g/mL of *d*- and *l*-modafinil were used as quality control standards. Intraday and interday variations of the assay were assessed by injecting replicate samples at each concentration on the same and subsequent days. Precision, accuracy, and recovery were determined, and stability studies were performed.

The selectivity of the assay was determined by direct injection of potentially interfering compounds at 10 to 50 μ g/mL (Table 1) or by analysis of extracted drug-free serum to which potentially interfering compounds had been added and examination of the resultant chromatograms for interfering peaks.

Application to Human Pharmacokinetic Studies

A healthy male cocaine-dependent subject was administered a racemic mixture of modafinil (400 mg twice daily for 5 days). Two hours after dosing on the sixth day, the subject received a single infusion of 40 mg intravenous cocaine as part an ongoing study to evaluate the potential metabolic interaction of modafinil with cocaine. Serum was collected at 0, 3, 10, 20, 30, 45, 60, 63, 70, 80, 90, and 105 minutes and at 2, 3, 4, 6, 8, 12, and 24 hours after cocaine administration and analyzed for *d*- and *l*-modafinil as described above. Approval was obtained by the Office of Research Integrity at the Medical University of South Carolina, and written informed consent was obtained from the subject.

TABLE 1. Selected drugs that may be used concomitantly with modafinil and their retention times using this HPLC analytical method*

| Compound | Retention time (min) |
|--|----------------------|
| Analytes | |
| <i>d</i> -modafinil | 22.1 |
| <i>l</i> -modafinil | 23.8 |
| 3,3-diphenylpropylamine (internal standard) | 15.7 |
| Stimulants | |
| Dextroamphetamine | ND |
| Methamphetamine | ND |
| Methylphenidate | ND |
| Cocaine | 10.1 |
| Norcocaine | 8.2 |
| Ecgonine | ND |
| Norecgonine | ND |
| Benzylecgonine | 10.3 |
| Benzylnorecgonine | 8.7 |
| Ecgonine methyl ester | ND |
| Selective Serotonin Reuptake Inhibitors | |
| Fluoxetine | 27.6 |
| Norfluoxetine | 26.6 |
| Sertraline | >35 |
| Desmethylsertraline | >35 |
| Nefazodone | 27.1 |
| Venlafaxine | ND |
| Fluvoxamine | 20.3 |
| Tricyclic Antidepressants | |
| Clomipramine | 29.8 |
| Amitriptyline | 32.9 |
| Nortriptyline | 32.3 |
| Desipramine | 23.3 |
| Imipramine | 21.8 |
| MAO Inhibitors | |
| Selegiline | 4.6 |
| Other Antidepressants | |
| Bupropion | ND |
| Citalopram | 13–15 |

* Listed drugs are used to treat narcolepsy, attention deficit/hyperactivity disorder, or depression, or are stimulants of abuse. ND, not detected.

RESULTS AND DISCUSSION

Chiral Column Performance

The β -cyclodextrin column provided baseline separation of the two enantiomers of modafinil and the internal standard. The retention times of the standards shifted by 2 to 4 minutes over the 3-month period but were extremely stable during a single sequence of analytical runs.

Selectivity of Analytical Method

The HPLC method separated the two enantiomers of modafinil and the internal standard from endogenous compounds in commercial drug-free serum. Analysis of serum from a subject receiving modafinil showed that

the two major metabolites of modafinil, modafinil acid and modafinil sulfone, would not interfere in the analysis. Chromatograms of blank serum spiked with internal standard, blank serum spiked with internal standard and 1.0 $\mu\text{g/mL}$ *d*- and *l*-modafinil, and serum from a patient 1 hour after receiving an oral dose of 200 mg of the racemic mixture of modafinil are shown in Figure 2.

The method was also evaluated for interferences by other drugs that may be used concomitantly by patients with narcolepsy, ADHD, depression, or substance abuse problems (Table 1). Stimulants such as amphetamines and cocaine and metabolites displayed very early retention times and would not interfere with this assay. Methylphenidate, though most commonly used manage

ADHD is also FDA approved to treat patients with narcolepsy, also eluted early and would not interfere with modafinil analysis. Tricyclic antidepressants (TCAs) are less likely to be used for the management of depression but are still used to treat patients with cataplexy associated with narcolepsy (12). Of the TCAs, clomipramine appears to be the most commonly used for this purpose, and this drug would not interfere with the analysis. However, imipramine and desipramine eluted in the same region of the chromatogram as *d*- and *l*-modafinil, and gradient conditions would need to be modified if patients were using these drugs with modafinil. Of the selective serotonin reuptake inhibitors (SSRIs) studied, only fluvoxamine demonstrated a retention time near that of the analytes. The monoamine oxidase inhibitor (MAOI) selegiline, also used for the management of narcolepsy, displayed a significantly different retention time from the analytes and would not interfere with the assay.

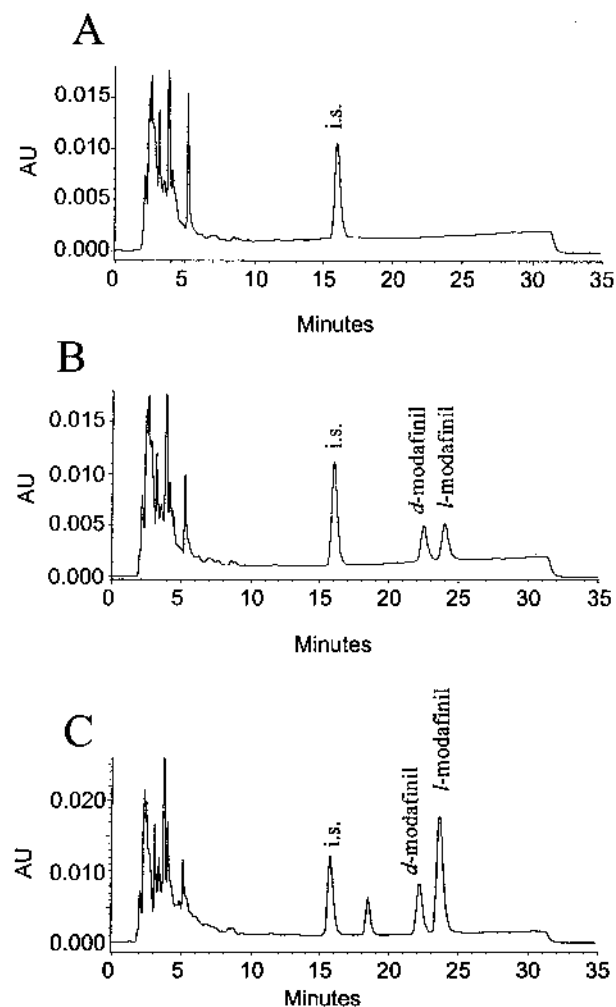


FIG. 2. High-performance liquid chromatograms of blank serum containing the internal standard 3,3-diphenylpropylamine (A), blank serum spiked with 1.0 $\mu\text{g/mL}$ *d*- and *l*-modafinil (B), and a serum sample from a subject who received 200 mg of a racemic mixture of modafinil twice daily for 5 days, 10 minutes after an intravenous infusion of 40 mg cocaine (C).

Limit of Quantitation

The limit of quantitation of this assay is 0.5 $\mu\text{g/mL}$. At this concentration, five replicates that were performed on five separate days had mean \pm SD of 0.45 ± 0.04 for *d*-modafinil and 0.48 ± 0.03 for *l*-modafinil. The S/N ratio was greater than 100.

Linearity

Five standard curves ranging from 0.5 $\mu\text{g/mL}$ to 30 $\mu\text{g/mL}$ were prepared in commercial drug-free serum. They all were linear and had r^2 values of 0.9932 ± 0.0049 for *d*-modafinil and 0.9930 ± 0.0047 for *l*-modafinil.

Precision, Accuracy, and Recovery

All experiments described in the sections below were performed using quality control (QC) samples prepared using commercial drug-free serum. These samples were prepared by spiking 25 mL of serum to a final concentration of either 3.0 $\mu\text{g/mL}$ or 15.0 $\mu\text{g/mL}$ *d*- and *l*-modafinil. Aliquots of 500 μL were prepared and stored at -80°C .

Precision and accuracy experiments were determined by five replicate analyses of each of the QC serum concentrations, 3 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$. Coefficients of variance were 5% and 4% for *d*- and *l*-modafinil at 3 $\mu\text{g/mL}$ and 3% and 4% for *d*- and *l*-modafinil at 15 $\mu\text{g/mL}$. Deviation from nominal values was less than 10% for both enantiomers at both concentrations.

Interday precision and accuracy were determined by

repeating the five replicates of the two QC concentrations on three separate days. Average levels in these samples differed by 7% and 15% for *d*- and *l*-modafinil at 3 $\mu\text{g/mL}$, respectively, and 9% and 7% for *d*- and *l*-modafinil at 15 $\mu\text{g/mL}$, respectively.

Intraday precision and accuracy were determined by repeating the five replicates of the two QC concentrations two times during a single day. Average levels in these samples differed by 4% and 2% for *d*- and *l*-modafinil at 3 $\mu\text{g/mL}$, respectively, and 4% for both *d*- and *l*-modafinil at 15 $\mu\text{g/mL}$.

Recovery experiments were performed by comparing extracted serum samples with standards prepared by serial dilution of stock solutions. Absolute recovery was $91\% \pm 4\%$ and $87\% \pm 4\%$ for *d*- and *l*-modafinil, respectively, at 3 $\mu\text{g/mL}$ ($n = 5$). Absolute recovery was $105\% \pm 3\%$ and $101\% \pm 3\%$ for *d*- and *l*-modafinil, respectively, at 15 $\mu\text{g/mL}$ ($n = 5$).

Stability

Stock solution stability was determined by comparing a freshly prepared stock solution of the analytes with a stock solution that had been refrigerated for 1 month. Samples were diluted to 1 $\mu\text{g/mL}$ in mobile phase and analyzed during the same HPLC run sequence. Peak areas of *d*- and *l*-modafinil in 1-month-old stock solutions differed by 5% and 2%, respectively, from the freshly prepared solution, indicating little or no degradation during the 1-month period.

Autosampler stability was determined by analysis of five replicates of each QC concentration analyzed immediately after extraction and then after storage for 36 hours in the HPLC autosampler. Average calculated values for these samples did not differ by more than 7% after the 36-hour period.

Previous studies have shown that modafinil is stable in plasma when stored at -20°C for up to 6 months; therefore, long-term storage studies were not performed in this study (9). Storage stability was determined by analysis of five replicate samples at both QC concentrations prior to storage and then after storage for 3 or 9 days at -80°C . Average levels in these samples were within the range expected due to interday variance, indicating little or no degradation at this temperature or while thawing. QC serum samples stored for 24 hours at ambient temperature before extraction also showed no measurable degradation.

Pharmacokinetic Application

The method was successfully applied to determine the pharmacokinetics of *d*- and *l*-modafinil in a healthy male

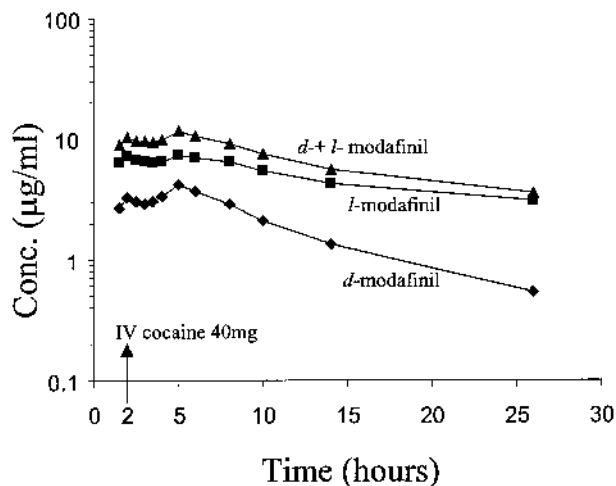


FIG. 3. The 24-hour serum concentration versus time course of *d*-modafinil, *l*-modafinil, and the sum of *d*- and *l*-modafinil. The subject received 400 mg of a racemic mixture of modafinil twice daily for 5 days prior to the final modafinil dose at the zero time point. Intravenous administration of 40 mg cocaine (dissolved in 2 mL volume administered over 1 minute) occurred at 2 hours.

cocaine-dependent subject. As expected, all of the chromatograms from this subject who received cocaine and modafinil were free of interferences by cocaine and its metabolites, which elute earlier in the chromatogram and are at much lower concentrations than the modafinil enantiomers and the internal standard. The peak that eluted at 17.5 minutes is an unidentified substance that was present in this subject's plasma but did not appear to be related to either modafinil or cocaine administration.

The time course of *d*- and *l*-modafinil and the calculated sum of both enantiomers are shown in Figure 3. The concentration of *l*-modafinil predominated in serum at all time points. In addition, the rate of disappearance of *d*-modafinil was apparently more rapid than the rate of *l*-modafinil decline, in agreement with previously published reports of the stereoselective disposition of modafinil (6).

CONCLUSIONS

The described stereoselective HPLC assay is a validated method for detection and quantitation of *d*- and *l*-modafinil for use in pharmacokinetic studies or therapeutic drug monitoring. The gradient elution provides the necessary selectivity to assay samples from patients with suspected or known use of many of the other drugs often taken concomitantly with modafinil.

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