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Determination of the D- and L-enantiomers of modafinil in human plasma utilizing liquid–liquid extraction and high-performance liquid chromatography

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Abstract

Modafinil, DL-2-[(diphenylmethyl)sulfinyl]acetamide (Provigil®), which is chiral at its sulfur atom, is a novel wake-promoting agent currently being developed as the racemate in the United States by Cephalon, Inc. In order to characterize the pharmacokinetic properties of each enantiomer, a stereospecific high-performance liquid chromatography (HPLC) method has been developed for simultaneous determination of D- and L-modafinil in human plasma. The analytes are extracted from plasma into a mixture of hexane–methylene chloride–triethylamine (55:45:2, v/v/v) and then resolved on an EM Separations ChiraDex™ β-cyclodextrin column at 12°C using an isocratic mobile phase of 0.020 *M*, pH 3.0 phosphate buffer–acetonitrile (84:14, v/v). D- and L-modafinil, and the internal standard, 3,3-diphenylpropylamine, are monitored by UV detection at 225 nm. The two major circulating metabolites, modafinil acid and modafinil sulfone, have been shown not to interfere with the assay. Using 0.200 ml of plasma for extraction, the quantifiable range of the assay is 0.100 to 15.0 μg/ml for each enantiomer. The utility of the assay for the characterization of D- and L-modafinil pharmacokinetics in humans after single and multiple oral doses of racemic modafinil has been demonstrated.

Keywords: Enantiomer separation; Modafinil

1. Introduction

Modafinil, DL-2-[(diphenylmethyl)sulfinyl] acetamide, is a novel wake-promoting agent discovered by Laboratoire L. Lafon (Maisons Alfort, France) and is currently being developed in the United States by Cephalon, Inc. (West Chester, PA, USA) as Provigil® [1,2]. Modafinil (Fig. 1) has a chiral center at its sulfur atom and the enantiomers are both biologically active. Achiral assays for the determination of modafinil and its acid and sulfone metabolites in human plasma have been previously reported [3,4]. A method using a Chiral AGP (α_1-acid glycoprotein) column to achieve resolution of the enantiomers of modafinil has also been published [5]. The method discussed optimization of chromatographic conditions, but did not address analysis of biological samples. To support the clinical development of modafinil, a stereospecific high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of its

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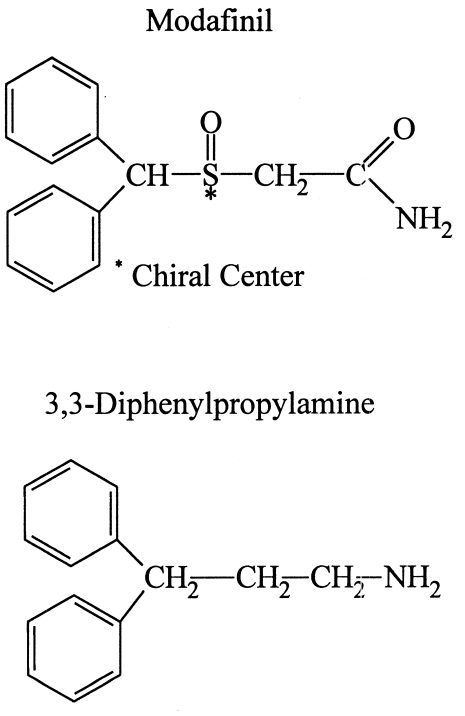

Fig. 1. Structures of modafinil and 3,3-diphenylpropylamine (I.S.).

enantiomers in human plasma. This method was previously presented as an abstract [6].

2. Experimental

2.1. Materials

The D- and L-enantiomers of modafinil were received from Cephalon, Inc. The internal standard (I.S.), 3,3-diphenylpropylamine (Fig. 1), was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Hydrochloric acid, triethylamine, anhydrous monobasic potassium phosphate, orthophosphoric acid (85%), *N,N*-dimethylformamide (DMF), acetonitrile, hexane and methylene chloride were all HPLC grade and were purchased from Fisher Scientific Company (Pittsburgh, PA, USA). Purified water (Milli-Q Plus system, Millipore Corporation, Bedford, MA, USA) was used for preparation of all aqueous solutions. Blank male and female human heparinized plasma was obtained from Rockland, Inc. (Gilbertsville, PA, USA).

2.2. Instrumentation

The analytical system consisted of Perkin Elmer components, including a model 410 pump, a model ISS 200C autosampler and a model 235C photodiode array detector (PE Nelson, Cuperino, CA, USA). The column temperature was maintained using a model 7955 HPLC column heater/chiller (Jones Chromatography, Lakewood, CO, USA). Integration, calibration and quantification were performed using Turbochrom, version 4.03 (PE Nelson).

2.3. Chromatographic conditions

The analytes and the internal standard were separated on an EM Separations ChiraDex™ β-cyclodextrin column (particle size, 5 μm; 250 mm×4.0 mm I.D.; EM Separations, Gibbstown, NJ, USA) at 12°C. The samples were eluted at a flow-rate of 0.60 ml/min with a mixture of 0.020 *M*, pH 3.0 phosphate buffer (pH adjusted with orthophosphoric acid)–acetonitrile (84:16, v/v). The mobile phase was filtered through a 0.45 μm filter and degassed by helium sparging prior to use. The analytes and the internal standard were monitored at a wavelength of 225 nm.

2.4. Sample preparation

Stock solutions (5.00 mg/ml) containing D- and L-modafinil were prepared in DMF. Working solutions were made by serial dilution of these stock solutions with water. Pooled quality control (QC) samples were prepared by diluting working solutions with human plasma. A 0.200 ml aliquot of a QC or test sample was pipetted into a 15 ml screw-cap conical glass centrifuge tube along with 0.040 ml of water and 2.50 μg of I.S. (0.050 ml of a 50.0 μg/ml solution). The sample was extracted into 5 ml of hexane–methylene chloride–triethylamine (55:45:2, v/v/v) by vortex-mixing for 2 min. After centrifugation at 2100 *g* for 10 min, the organic layer was transferred into a clean tube and evaporated to dryness in a Turbovap® LV evaporator (Zymark, Hopkinton, MA, USA) at 30°C under a stream of nitrogen. The residue was reconstituted in 0.250 ml of a mixture of 0.020 *M*, pH 3.0 phosphate buffer–acetonitrile (70:30, v/v). After 10 min of sonication,

followed by vortex-mixing, a 50 μl aliquot was injected onto the HPLC column.

2.5. Quantification

Calibration standards in human plasma were prepared by the concurrent addition of known amounts of D- and L-modafinil to produce final concentrations of 0.100 to 15.0 μg/ml of each enantiomer. Weighted ($1/y^2$) calibration curves were generated using peak-height ratios of the compound to the I.S. regressed against the nominal plasma concentrations.

3. Results and discussion

3.1. Sample preparation

Modafinil can be extracted into a number of organic solvents, including ethyl ether and methylene chloride; however, extraction with either solvent alone results in significant matrix-derived peaks in the chromatograms. Tempering the methylene chloride with hexane minimized extraction of interfering matrix components. Given the potential for pH effects on the ionization state of the I.S., triethylamine was added to the extraction solvent on the assumption that it would improve the consistency of the extraction. However, it has been subsequently determined that triethylamine has no significant effect on the extractability of the I.S.

3.2. Column selection

A previously published method used a Chiral AGP column to achieve resolution of the enantiomers of modafinil [5]. That method was used by Laboratoire L. Lafon for analysis of a limited number of biological samples [7]. However, the chromatographic separation was reported to require extensive method development.

In addition, a small variation from the optimized conditions for chromatographic separation on protein columns frequently results in loss of resolution of enantiomers [8]. Because of potential difficulties in adjusting the chromatographic conditions to account for inter-column variability, the addition of an internal standard and for possible matrix effects, the Chiral AGP column was not deemed suitable for use in routine bioanalysis.

The β-cyclodextrin column was chosen for its versatility. Chromatography on cyclodextrin stationary phases can be developed in the reversed-phase mode. Resolution of the enantiomers of modafinil could be achieved using a broad range of buffers and either acetonitrile or methanol as the organic modifier. The β-cyclodextrin column also demonstrated ruggedness and excellent lot-to-lot reproducibility. No mobile phase adjustments were needed to maintain chiral resolution and only a minor adjustment in mobile phase composition (±2% acetonitrile) was needed to obtain comparable retention of the analytes (within 10%) on columns from three different lots.

The requirements for chiral separation in the reversed-phase mode on a β-cyclodextrin column are the presence of at least one substituent of appropriate size to form an inclusion complex with the cyclodextrin cavity and a substituent on or near the chiral center with the potential to interact with the hydroxyl groups at the mouth of the cyclodextrin cavity [9]. The two aromatic rings of modafinil provided the ability for the inclusion complexation to occur. The sulfinyl oxygen and possibly the amide substituent of modafinil have the potential to interact with the cyclodextrin hydroxyl groups.

3.3. Selectivity

Fig. 2 shows representative chromatograms of: (a) a drug- and I.S.-free pooled human plasma sample, (b) a subject pre-dose plasma, (c) a plasma standard spiked at a concentration of 1.00 μg/ml each of D- and L-modafinil and with 2.50 μg of the I.S. and d) a 2 h post-dose sample spiked with 2.50 μg of the I.S. from a subject administered a 200 mg oral dose of racemic modafinil. There were no interfering peaks observed at the retention times corresponding to the analytes or the I.S. in the purchased blank plasma or in the pre-dose plasma from 20 clinical study subjects. The two major circulating metabolites, modafinil acid and modafinil sulfone, also did not co-elute with the analytes or the I.S. The retention times of the I.S., D-modafinil, and L-modafinil were approximately 9, 19 and 22 min, respectively. The total run time was 26 min.

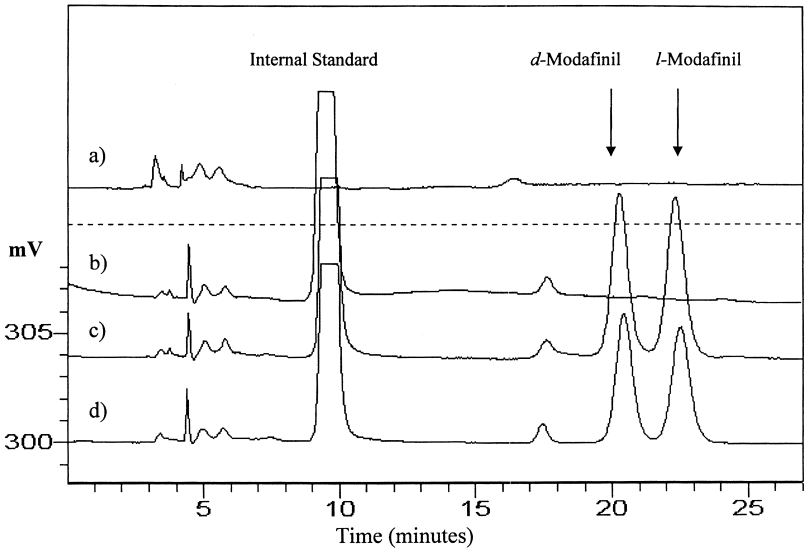

Fig. 2. Chromatograms (all shown at 10 mV full scale) of: (a) a drug-free pooled human plasma sample; (b) a clinical study subject pre-dose plasma; (c) a plasma standard spiked at a concentration of 1.00 μg/ml each of D- and L-modafinil and with 2.50 μg of the I.S.; (d) a 2 h post-dose sample spiked with 2.50 μg of the I.S. from a subject administered a 200 mg oral dose of racemic modafinil

3.4. Lower limit of quantification

The lower limit of quantification (LLQ) was defined as the lowest concentration that could be determined reproducibly. For this method, the LLQ was set at the concentration of the lowest calibration standard, or 0.100 μg/ml. The signal to noise (S/N) ratio at the LLQ was greater than 10.

3.5. Standard curve characteristics

The reproducibility of the response for each analyte was demonstrated by comparison of three sets of calibration standards analyzed on different days. A comparison of interpolated versus nominal concentrations over a range of 0.100 to 15.0 μg/ml is shown in Table 1. The differences of the mean

Table 1
Back-calculated standard values for D-modafinil and L-modafinil in human plasma

	D-Modafinil concentration (μg/ml)					
	0.100	0.500	2.00	5.00	10.0	15.0
Mean (n=3)	0.099	0.517	2.05	5.12	9.65	14.8
SD	0.004	0.030	0.04	0.16	0.65	0.7
%C.V.	4.0	5.8	2.0	3.1	6.7	4.7
%Difference[a]	−1.0	+3.4	+2.5	+2.4	−3.5	−1.3
	L-Modafinil concentration (μg/ml)					
	0.100	0.500	2.00	5.00	10.0	15.0
Mean (n=3)	0.099	0.523	2.05	5.11	9.62	14.7
SD	0.003	0.037	0.06	0.19	0.67	0.7
%C.V.	3.0	7.1	2.9	3.7	7.0	4.8
%Difference	−1.0	+4.6	+2.5	+2.2	−3.8	−2.0

[a] $\%\text{Difference} = \frac{\text{Experimental value} - \text{Nominal value} \times 100}{\text{Nominal value}}$

Table 2
Regression analysis of standard curves for D-modafinil and L-modafinil in human plasma

Run	D-Modafinil			L-Modafinil		
	Slope	Intercept	r^2	Slope	Intercept	r^2
1	0.118	0.00072	0.998	0.105	−0.00025	0.999
2	0.113	0.00149	0.998	0.100	0.00080	0.997
3	0.104	−0.00058	0.994	0.091	−0.00035	0.993
Mean	0.112		0.997	0.099		0.996
SD	0.007		0.002	0.007		0.003
%C.V.	6.3		0.216	7.1		0.301

interpolated values from their nominal values ranged from −3.5% to 3.4% for D-modafinil and from −3.8% to 4.6% for L-modafinil. The precision (%C.V.) of the interpolated values ranged from 2.0% to 6.7% for D-modafinil and from 2.9% to 7.1% for L-modafinil. The slopes, intercepts and correlation coefficients (r^2) for D- and L-modafinil are shown in Table 2. The precision of the slopes over the three analysis days was 6.3% for D-modafinil and 7.1% for L-modafinil. The correlation coefficients for all standard curves were greater than 0.99.

3.6. Precision and accuracy of the assay method

The precision and accuracy parameters of the assay method, determined using the QC samples ($n=6$ per concentration), are summarized for D- and L-modafinil (Tables 3 and 4). For D-modafinil, the within-run precision, expressed as %C.V., was less than or equal to 13.2%. The average within-run precision ranged from 7.3% to 9.4%, and the between-run precision ranged from 4.8% to 12.1%. The mean within-run difference from nominal for the

Table 3
Summary of three-run validation results for D-modafinil

Nominal concentration (μg/ml)		Experimental concentration (μg/ml)[a]			Average within-run precision	Between-run precision (%)	Average difference[b] (%)
		Run 1	Run 2	Run 3	(%)		
15.0	Mean	13.9	15.1	15.2	8.0	4.8	−2.0
	SD	1.4	1.1	1.0			
	%C.V.	10.1	7.3	6.6			
	%Difference	−7.3	+0.7	+1.3			
7.50	Mean	7.09	7.88	7.45	7.3	5.4	−0.3
	SD	0.77	0.50	0.35			
	%C.V.	10.9	6.3	4.7			
	%Difference	−5.5	+5.1	−0.7			
0.300	Mean	0.283	0.305	0.314	8.4	5.3	+0.1
	SD	0.031	0.018	0.026			
	%C.V.	11.0	5.9	8.3			
	%Difference	−5.7	+1.7	+4.7			
0.100	Mean	0.091	0.092	0.113	9.4	12.1	−1.3
	SD	0.012	0.009	0.006			
	%C.V.	13.2	9.8	5.3			
	%Difference	−9.0	−8.0	+13.0			

[a] $n=6$ per concentration per run.

[b] $\%\text{Difference} = \frac{\text{Experimental value} - \text{Nominal value} \times 100}{\text{Nominal value}}$.

Table 4
Summary of three-run validation results for L-modafinil

Nominal concentration (μg/ml)		Experimental concentration (μg/ml)[a]			Average within-run precision (%)	Between-run precision (%)	Average difference[b] (%)
		Run 1	Run 2	Run 3			
15.0	Mean	14.2	15.5	15.7	8.0	4.8	+0.9
	SD	1.6	1.2	1.1			
	%C.V.	11.3	7.7	7.0			
	%Difference	−5.3	+3.3	+4.7			
7.50	Mean	7.30	8.15	7.67	7.6	5.6	+2.8
	SD	0.83	0.53	0.38			
	%C.V.	11.4	6.5	5.0			
	%Difference	−2.7	+8.7	+2.3			
0.300	Mean	0.301	0.317	0.327	8.0	4.1	+5.0
	SD	0.035	0.017	0.023			
	%C.V.	11.6	5.4	7.0			
	%Difference	+0.3	+5.7	+9.0			
0.100	Mean	0.098	0.106	0.108	8.1	4.8	+3.9
	SD	0.012	0.009	0.004			
	%C.V.	12.2	8.5	3.7			
	%Difference	−2.0	+6.0	+8.0			

[a] $n=6$ per concentration per run.

[b] $\%\text{Difference}=\frac{\text{Experimental value-nominal value}\times 100\%}{\text{Nominal value}}$.

three assay runs ranged from −9.0% to 13.0%, and the average difference from nominal ranged from −2.0% to 0.1% (Table 3).

For L-modafinil, the within-run precision, expressed as %C.V., was less than or equal to 12.2%. The average within-run precision ranged from 7.6% to 8.1%, and the between-run precision ranged from 4.1% to 5.6%. The mean within-run difference from nominal ranged from −5.3% to 9.0%, and the average difference from nominal ranged from 0.9% to 5.0% (Table 4).

3.7. Extraction efficiency

The extraction efficiencies of D- and L-modafinil at 0.100 and 15.0 μg/ml and of the I.S. at 2.50 μg/sample were determined by comparing peak heights of extracted plasma standards (n=6) to those of unextracted aqueous standards. Extraction efficiencies at 0.100 μg/ml were 87.7%±6.5% and 83.7%±5.8% for D- and L-modafinil, respectively. At 15.0 μg/ml, the extraction efficiencies were 81.3%±4.4% and 81.6%±5.1% for D- and L-modafinil, respectively. Extraction efficiency of the I.S. at 2.50 μg per sample was 79.8%±6.3%.

3.8. Stability

Stability of the individual enantiomers was not investigated. However, in a separate study, stability of the racemic mixture was demonstrated under several storage conditions. Modafinil was shown to be stable in human plasma at room temperature for at least 51 h, after storage at −20°C for 166 days and after five freeze-thaw cycles. Extracted plasma samples were stable after storage in the autosampler at room temperature for at least 51 h.

3.9. Analysis of clinical samples

The method described has been successfully applied to the quantification of D- and L-modafinil in plasma samples from clinical studies. The time courses of plasma concentrations of D-, L- and total

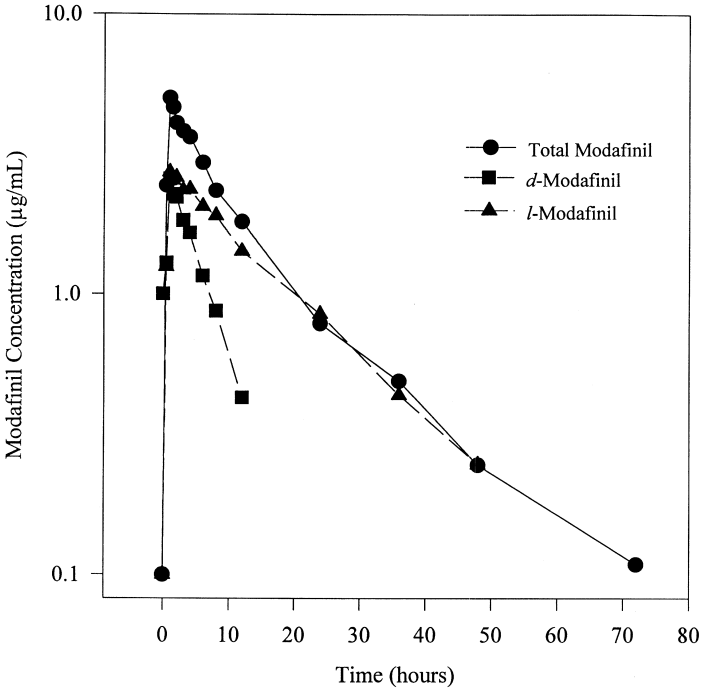

Fig. 3. The time courses of plasma concentrations of D-, L- and total modafinil from a representative clinical study subject administered a 200 mg oral dose of a racemic mixture.

modafinil from a representative subject given a single 200 mg oral dose of a racemic mixture are shown in Fig. 3. The maximum plasma concentration (C_{max}) and the time to achieve the maximum concentration (T_{max}) of the D- and L-enantiomers appear to be the same. However, it can be readily observed that the D-enantiomer is more rapidly cleared from human plasma. This indicates that one or more mechanisms of elimination of modafinil are stereospecific, as suggested by previous results [7].

4. Conclusions

An HPLC method has been described for the determination of D- and L-modafinil in human plasma. This method has been shown to be selective, accurate and precise and has been successfully applied to the analysis of plasma samples from pharmacokinetic studies of modafinil in humans.

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