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Determination of modafinil, modafinil acid and modafinil sulfone in human plasma utilizing liquid–liquid extraction and high-performance liquid chromatography

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Abstract

An assay was developed to determine concentrations of modafinil (*dl*-2-[(diphenylmethyl)sulfinyl]acetamide; Provigil®) and its two major circulating metabolites, modafinil acid and modafinil sulfone, in human plasma. The assay utilized liquid–liquid extraction of the analytes and an internal standard, (phenylthio)acetic acid, from plasma into a mixture of hexane–dichloromethane–glacial acetic acid (55:45:2, v/v). The analytes were resolved isocratically on a narrow-bore phenyl column at a mobile phase flow-rate of 0.3 ml/min and were monitored by UV detection at 235 nm. The method reported herein reduces the required sample volume of previously reported methods from 1.00 to 0.200 ml of plasma while lowering the limit of quantification (LOQ). The linear range of the assay was from 0.100 to 20.0 μg/ml for each of the three compounds.

Keywords: Modafinil; Modafinil acid; Modafinil sulfone

1. Introduction

Modafinil (*dl*-2-[(diphenylmethyl)sulfinyl]acetamide), licensed from Laboratoire L. Lafon (Maisons-Alfort, France), has been developed by Cephalon, Inc., in the United States under the trade name Provigil® for the treatment of excessive daytime sleepiness associated with narcolepsy. During clinical evaluation, a validated assay procedure was required to enable pharmacokinetic characterization of modafinil and its two circulating metabolites, modafinil acid (MA) and modafinil sulfone (MS).

Two methods have previously been reported for analysis of modafinil in human plasma. In the first [1], only modafinil and MA were quantified, using liquid–liquid extraction of 1.00 ml of plasma into diethyl ether, followed by reversed-phase chromatography on a C_{18} column. The quantifiable range of this assay was from 0.130 (0.140 for MA) to 20.0 μg/ml.

The second assay [2] utilized C_{18} solid-phase extraction of 1.00 ml of plasma followed by reversed-phase chromatography on a C_8 column for the determination of modafinil, MA and MS. The practical limit of quantification, defined as the minimum concentration that can be measured routinely with acceptable precision and accuracy, was 0.200 μg/ml for modafinil and 0.250 μg/ml for MA and MS. However, the linear range presented was from 0.500 to 20.0 μg/ml for each analyte.

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Both assays used standard bore columns (3.9–4.6 mm I.D.) with mobile phase flow-rates of ≥1.0 ml/min, and both assays monitored the analytes using UV detection.

An assay procedure that reduced the volume of sample required and reduced the volumes of waste solvents generated was considered to be desirable. In addition, the internal standard utilized in the above assay procedures was not commercially available. In the present assay, the required sample volume was reduced from 1.00 to 0.200 ml while lowering the limit of quantification (LOQ) to 0.100 μg/ml for all three analytes, the volume of waste solvents was reduced by using a narrow-bore column at a lower mobile phase flow-rate, and a different, commercially available internal standard was identified.

The method described herein has successfully been used for the past 8 years for the determination of modafinil, MA and MS concentrations in human plasma samples from pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Modafinil (Fig. 1A), MA (Fig. 1B) and MS (Fig. 1C) were supplied by Cephalon, Inc. (West Chester, PA, USA). The internal standard (I.S.), (phenylthio)acetic acid (Fig. 1D), was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, hexane, dichloromethane, acetic acid, *N′*,*N′*-dimethylformamide (DMF), hydrochloric acid, monobasic potassium phosphate and orthophosphoric acid (85%) were all HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified water (Milli-Q Plus system; Millipore, Bedford, MA, USA) was used for the preparation of all aqueous solutions. Blank human heparinized plasma was obtained from Rockland (Gilbertsville, PA, USA), or Valley Biomedical Products and Services (Winchester, VA, USA).

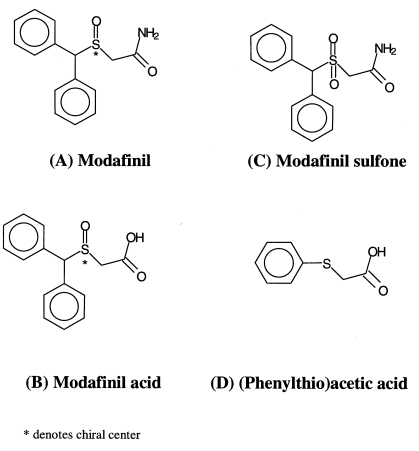

Fig. 1. The structures of modafinil (A), modafinil acid (B), modafinil sulfone (C) and the I.S. (D). An asterisk indicates the location of a chiral center.

2.2. Instrumentation

During validation, the analytical system consisted of Perkin-Elmer components, including a series 200 pump, a model ISS 200C autosampler and a model 235C photodiode array detector (Perkin-Elmer, Norwalk, CT, USA). The column temperature was maintained at 30 °C using a model 7955 HPLC column heater–chiller (Jones Chromatography, Lakewood, CO, USA). Integration, calibration and quantification were performed using Turbochrom version 4.03 (Perkin-Elmer).

2.3. Chromatographic conditions

The analytes and the I.S. were separated on a Hypersil® BDS phenyl column (particle size 5 μm; 2.0 mm I.D.×150 mm; Keystone Scientific, Bellefonte, PA, USA). The samples were eluted at a flow-rate of 0.30 ml/min with a mixture of 0.020 *M*, pH 2.5, phosphate buffer (pH adjusted with orthophosphoric acid)–methanol (64:36, v/v). The analytes and the I.S. were monitored at a wavelength of 235 nm.

2.4. Sample preparation

Stock solutions (500 μg/ml) containing modafinil, MA and MS were prepared in DMF. Working solutions were made by serial dilution of these stock solutions with DMF. Pooled quality control (QC) samples were prepared by mixing working solutions with human plasma. An aliquot of a QC (0.220 ml) or test sample (0.200 ml of plasma mixed with 0.020

ml of DMF) was pipetted into a 15-ml screw-cap conical glass centrifuge tube along with 0.550 μg of I.S. (0.050 ml of an 11.0 μg/ml solution). The samples were acidified with 2.0 N HCl (0.200 ml) and were extracted into 5 ml of hexane–dichloromethane–glacial acetic acid (55:45:2, 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 (0.250 ml) in a mixture of 0.020 *M*, pH 2.50, phosphate buffer–acetonitrile (70:30, v/v). After 10 min of sonication, followed by vortex-mixing, a 25-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 modafinil, MA and MS to produce final concentrations of 0.100–20.0 μg/ml of each analyte. 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

In the present assay, the required sample volume was reduced from 1.00 to 0.200 ml. The reduction of sample volume is significant in several aspects. First, it reduces the volume of blood required. This reduction may positively affect the number of pharmacokinetic samples that can be collected, especially in pediatric studies, or in studies in patients with already low hematocrits (e.g. patients with certain forms of cancer). Secondly, sufficient sample can be readily obtained to allow for analyses of multiple compounds, as might be required in metabolism and/or pharmacokinetic drug–drug interaction studies.

Modafinil, MA and MS can be extracted into a number of organic solvents, including diethyl ether, as in the previously reported method [1], dichloromethane and ethyl acetate. However, with extraction into diethyl ether, dichloromethane or ethyl acetate alone, significant matrix-derived peaks were observed in the chromatograms (data not shown). The analytes were minimally extracted into pure hexane. However, after extraction of blank plasma with hexane, there were no interfering matrix-derived peaks in the chromatograms. In order to minimize extraction of these interfering matrix components, experiments in which dichloromethane and diethyl ether were tempered with hexane were performed. The best chromatographic results were attained after extraction using a mixture of dichloromethane–hexane (45:55, v/v).

Given the potential for pH effects on the ionization state of MA and the I.S., HCl was added to the samples to protonate the carboxylic acid groups. Glacial acetic acid was also included in the extraction solvent on the assumption that it would improve the consistency of extraction of the acids.

3.2. Precision and accuracy of the assay method

The precision and accuracy parameters of the assay method, determined using QC samples ($n=6$ per concentration), are summarized for modafinil, MA and MS in Table 1. For modafinil, the within-run precision, expressed as % C.V., was less than or equal to 12.6%. The average within-run precision ranged from 1.6 to 8.7%, and the between-run precision ranged from 2.5 to 3.7%. The mean within-run accuracy with respect to nominal for the three assay runs ranged from 90.8% to 103%, and the average accuracy ranged from 92.8 to 98.5% (Table 1).

For MA, the within-run precision, expressed as % C.V., was less than or equal to 11.0%. The average within-run precision ranged from 1.8 to 8.8%, and the between-run precision ranged from 2.5 to 10.9%. The mean within-run accuracy with respect to nominal for the three assay runs ranged from 81.8 to 102%, and the average accuracy ranged from 92.3 to 95.2% (Table 1).

For MS, the within-run precision, expressed as % C.V., was less than or equal to 8.5%. The average within-run precision ranged from 2.2 to 5.3%, and the between-run precision ranged from 0.9 to 9.1%. The mean within-run accuracy with respect to nomi-

Table 1
Summary of the precision and accuracy for the determination of modafinil, modafinil acid and modafinil sulfone in human plasma ($n=6$ per concentration per run)

Compound	Nominal concentration (μg/ml)	Average precision (%)		Average accuracy (%)
		Within-run	Between-run	
Modafinil	20.0	1.6	2.5	92.8
	10.0	3.8	2.8	97.2
	0.300	4.6	3.6	98.5
	0.100	8.7	3.7	95.2
Modafinil acid	20.0	1.8	4.1	92.3
	10.0	3.6	4.1	95.2
	0.300	4.1	2.5	95.0
	0.100	8.8	10.9	92.0
Modafinil sulfone	20.0	2.2	3.3	91.5
	10.0	3.6	3.2	94.8
	0.300	3.6	0.9	96.3
	0.100	5.3	9.1	91.6

nal for the three assay runs ranged from 82.0 to 98.3%, and the average accuracy ranged from 91.5 to 96.3% (Table 1).

3.3. Lower limit of quantification

The lower limit of quantification (LOQ) was defined as the lowest concentration that could be determined reproducibly (accuracy within 20% of nominal and C.V. $\leq$20%). For this method, the LOQ was determined to be the concentration of the lowest calibration standard, 0.100 μg/ml. Even though approximately one fifth of the amount of matrix was injected onto the HPLC column, the LOQs were lower than those reported in the previous methods. This was achieved principally by using a narrow-bore (2.0 mm I.D.) column. Theoretically, if the amount of injected solute is kept constant, sensitivity should be inversely proportional to the square of the column diameter [3]. Therefore, an increase in sensitivity, i.e. a decreased LOQ, would be expected. The previous assays injected the equivalent of 0.1 ml of matrix onto the HPLC column, while the method reported herein reduces the on-column injection volume to the equivalent of 0.02 ml of matrix. This decrease in the amount of extracted matrix injected onto the HPLC column was intended to reduce baseline noise attributable to matrix, while maintaining a suitable LOQ.

3.4. Selectivity

During method development, the chromatographic conditions of Moachon et al. [1] were initially used. However, a small matrix-derived peak co-eluted with modafinil. The phenyl column was chosen to change selectivity. Selectivity with respect to matrix-derived peaks was further enhanced by the choice of methanol as the organic modifier. Under the conditions described above, no matrix-derived chromatographic interferences were observed in extracted blank (drug-free) human plasma. At a mobile phase flow-rate of 0.300 ml/min there were no back-pressure-related issues due to the choice of methanol as the organic modifier. The back-pressure was typically ~1600 p.s.i.

The described assay for human plasma has been in use for approximately 8 years. Within-column lot reproducibility of the chromatographic separation has been good. However, over the course of years, inter-lot variability has been observed. Therefore, minor adjustment of the mobile phase by up to ±0.1 pH unit, ±5% methanol and/or ±5 °C has sometimes been required to achieve optimal peak resolution. The chromatography of MA has been especially

sensitive to inter-lot column variability. However, minor adjustment to the pH has always enabled adequate resolution of MA from modafinil and MS. Fig. 2 shows increasing retention of MA with decreasing pH as the ionization of the carboxylic acid group is suppressed. At a buffer pH of 3.6 (figure not shown), MA is eluted before modafinil. However, in some plasma samples there were interfering matrix peaks that co-eluted with modafinil under those conditions, making the higher pH impractical for use.

Fig. 3 shows representative chromatograms of an extracted: (a) human plasma blank; (b) human plasma quality control sample spiked with 0.100 μg/ml each of modafinil, MA and MS and 0.550 μg of the I.S.; and (c) human plasma standard spiked with 1.00 μg/ml each of modafinil, MA and MS and 0.550 μg of the I.S. There were no interfering peaks observed at the retention times corresponding to the analytes or the I.S. in six lots of purchased blank human plasma during validation or subsequently. The retention times of I.S., modafinil, MA and MS were approximately 7, 13, 14 and 16 min, respectively. The total run time was 26 min.

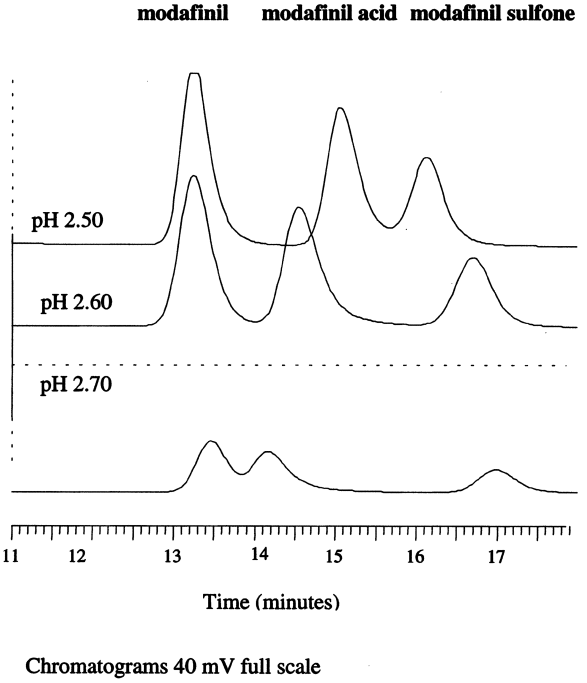

Fig. 2. The effect of pH on resolution of modafinil, modafinil acid and modafinil sulfone for column lot 4606.

3.5. Sample stability

The room temperature stability of modafinil in plasma has been shown to be species-dependent. In a similar, but separate study, there was rapid esterase/amidase-catalyzed degradation of modafinil to MA in rat plasma (data not shown). Addition of DMF (10% by volume) to the rat plasma inhibited the degradation of modafinil (<5.5% loss after 3 h at room temperature).

In light of these observations and to reduce any potential effect of the organic spiking solution on the stability of modafinil, the concentration of the DMF in the human plasma QC samples was decreased to between 0.06 and 3.0% by volume. In those experiments, modafinil, MA and MS exhibited room-temperature stability for >48 h in human plasma.

In other experiments with human plasma, stability was demonstrated after 166 days in storage at approximately −20 °C, after 383 days in storage at approximately −90 °C, and after five freeze–thaw cycles. Extracted, reconstituted plasma samples were stable for least 51 h in the autosampler at room temperature. These observations were based on a difference of <15% between the concentrations determined for stored and freshly prepared samples.

3.6. Standard curve characteristics

The slopes, intercepts and correlation coefficients (r^2) of the calibration curves used to validate the method for modafinil, MA and MS are shown in Table 2. The precision (% C.V.) of the slopes over the three analysis days was 1.1% for modafinil, 1.7% for MA and 1.7% for MS. The correlation coefficients for all standard curves were greater than 0.997.

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 0.100–20.0 μg/ml is shown in Table 3. The accuracy of the mean interpolated values with respect to the nominal concentrations ranged from 98.0 to 105% for

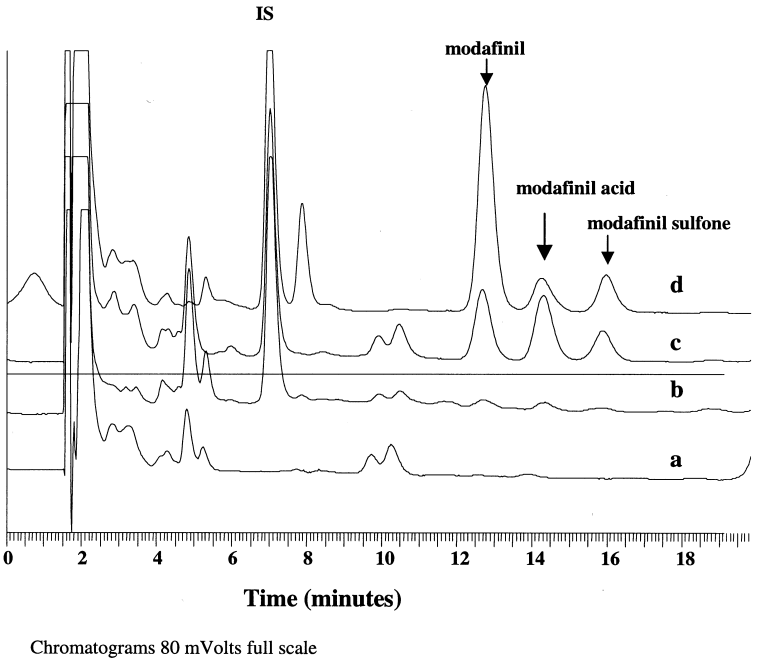

Fig. 3. Representative chromatograms of extracts of: (a) a human plasma blank; (b) a human plasma quality control sample spiked with 0.100 μg/ml each of modafinil, modafinil acid and modafinil sulfone and 0.550 μg of I.S.; (c) a human plasma standard spiked with 1.00 μg/ml each of modafinil, modafinil acid and modafinil sulfone and 0.550 μg of the I.S.; (d) a steady-state trough sample from a subject orally administered 400 mg of modafinil (calculated concentrations: modafinil, 3.09 μg/ml; modafinil acid, 0.495 μg/ml; and modafinil sulfone, 1.24 μg/ml).

modafinil, from 98.5 to 105% for MA and from 97.7 to 105% for MS. The precision of the interpolated values, expressed as % C.V., ranged from 0.6 to 6.0% for modafinil, from 0.2 to 7.1% for MA and from 1.0 to 4.4% for MS.

3.7. Extraction efficiency

The extraction efficiencies of modafinil, MA and MS at 0.100 and 20.0 μg/ml and of the I.S. at 0.550 μg/sample were determined by comparing peak heights of extracted plasma standards ($n=6$) to those of unextracted aqueous standards. Mean extraction efficiencies at 0.100 μg/ml were 91.3, 88.0 and 118% for modafinil, MA and MS, respectively. At 20.0 μg/ml, the respective mean extraction efficiencies were 87.9, 88.6 and 101%. Overall mean extraction efficiency of the I.S. at 0.550 μg per sample, in the presence of 0.100 or 20.0 μg/ml of all three analytes, was 84.1%.

Table 2
Regression analysis for standard curves of modafinil, modafinil acid (MA) and modafinil sulfone (MS) in human plasma

Run	Modafinil			MA			MS		
	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2
1	0.706	0.015	0.999	0.597	0.003	0.999	0.454	0.013	0.999
2	0.717	0.003	1.00	0.616	−0.006	0.999	0.469	0.005	1.00
3	0.722	0.009	0.999	0.605	0.010	0.997	0.465	0.001	0.998
Mean	0.715		0.999	0.606		0.998	0.463		0.999
SD	0.008		0.001	0.010		0.001	0.008		0.001
% C.V.	1.1		0.1	1.7		0.1	1.7		0.1

Table 3
Back-calculated standard values for modafinil, modafinil acid (MA) and modafinil sulfone (MS) in human plasma

	Nominal concentration (μg/ml)							
	0.100	0.200	0.500	1.00	2.00	5.00	10.0	20.0
Modafinil concentration (μg/ml)								
Mean	0.099	0.201	0.507	1.05	1.97	4.96	9.95	19.6
% C.V.	1.0	6.0	0.6	2.9	1.0	1.4	1.3	2.0
% Accuracy	99.0	100	101	105	98.5	99.2	99.3	98.0
MA concentration (μg/ml)								
Mean	0.101	0.198	0.497	1.05	1.97	4.98	9.86	20.2
% C.V.	4.0	7.1	0.2	2.9	1.0	2.8	2.1	2.0
% Accuracy	101	99.0	99.4	105	98.5	99.6	98.6	101
MS concentration (μg/ml)								
Mean	0.099	0.203	0.498	1.05	1.97	4.98	9.77	20.0
% C.V.	1.0	1.5	1.6	2.9	1.0	1.8	4.4	1.0
% Accuracy	99.0	102	99.6	105	98.5	99.6	97.7	100

3.8. Analysis of clinical samples

The method described has been successfully applied to the quantification of modafinil, MA and MS in human plasma samples from more than a dozen clinical studies [4–8]. Fig. 3d shows an example of an extracted steady-state trough sample from a subject orally administered 400 mg of modafinil (calculated concentrations: modafinil, 3.09 μg/ml; MA, 0.495 μg/ml; and MS, 1.24 μg/ml). Fig. 4 shows representative plasma concentration versus time curves for modafinil, MA and MS in a healthy volunteer after administration of a single oral 400-mg dose of modafinil.

Additionally, the method has proven to be especially rugged. Aside from being utilized in-house at Cephalon, it has been transferred to and validated by three different bioanalytical contract research organizations.

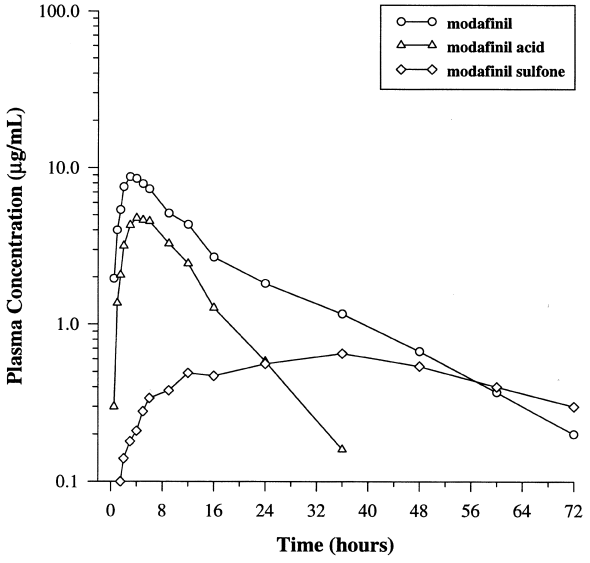

Fig. 4. Representative plasma concentration versus time curves for modafinil, MA and MS in a healthy volunteer after administration of a single oral 400-mg dose of modafinil.

4. Conclusions

An HPLC method has been described for the determination of modafinil and its two circulating metabolites, MA and MS, in human plasma. This method has been shown to be selective, accurate and precise for measurement of concentrations of each compound in the range 0.100–20.0 μg/ml. The method maintains an appropriate quantification range, while reducing the required sample volume relative to that in previously reported methods [1,2]. In addition, the use of a narrow-bore column significantly reduces the volume of waste solvents generated.

Acknowledgements

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