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Original Article

Liquid chromatography tandem mass spectrometry method for the estimation of modafinil in human plasma

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ABSTRACT

A rapid, selective and sensitive high performance liquid chromatography–tandem mass spectrometry method (LC-MS/MS) was developed and validated for the estimation of modafinil in human plasma. Modafinil and the internal standard (ISTD), Modafinil-D5, were extracted from plasma samples using solid phase extraction with Agilent® Bond Elut Plexa cartridges. Chromatographic separation was performed on a Ascentis® C18 column (150mm×4.6mm, 5µm) using methanol: 2mM ammonium acetate: glacial acetic acid (35:65:0.1% v/v/v) as the mobile phase at a flow rate of 1.0 mL/min. Detection of modafinil and modafinil-D5 was achieved by tandem mass spectrometry with an electrospray ionization (ESI) interface in positive ion mode. The calibration curves were linear over the range of 30.8 to 8022.1 ng/mL. The method has a lower limit of quantitation (LLOQ) of 30.8 ng/mL and the limit of detection (LOD) achieved of 1 ng/mL for modafinil, based on a signal to noise ratio of 10. The intra- and inter-day precisions were within 3.1%, while the accuracy was within ±3.3% of nominal values. No matrix effect was observed in this method.

Keywords: Modafinil; LC-MS/MS; Solid phase extraction; Method development; Method validation.

INTRODUCTION

Modafinil [*d*, *l*-2-[(diphenylmethyl) sulfinyl] acetamide) (Fig. 1)], a α1-adrenergic agonist, is a memory-improving and mood-brightening psychostimulant, commonly prescribed in the treatment of narcolepsy and hypersomnia [1]. It gets readily absorbed upon oral administration and reaches its maximum plasma concentration between 2-4 hours. It gets extensively metabolized, mainly in liver, through hydrolytic deamidation followed by S-oxidation via the cytochrome P450 (CYP) 3A4/5 enzymes with less than 10% of the parent compound excreted in the urine [2]. Modafinil is moderately bound to plasma protein (about 60%), primarily to albumin and has the apparent terminal half-life of ~11-14 hours [3].

Relatively very few analytical methods have been reported for the quantification of modafinil and/or its metabolites in biological matrix. The reported methods commonly used HPLC with UV detection to measure modafinil and/or its metabolites in plasma [4-8]. Subsequently published analytical methods utilized single quadrupole liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS) technique to measure modafinil and/or its metabolites from biological matrix [9-10]. These methods are relatively susceptible to matrix effect, require intensive sample purification steps, have low selectivity and sensitivity. In last two decades liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been emerged as the preeminent analytical tool for quantification of drugs and/or their metabolites in biological matrix. To the best of our knowledge, only one liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been published to determine adrafinil, modafinil with modafinil acid in rat serum using solid phase extraction [11]. This published LC-MS/MS method has relatively long analytical run time (~20 min) that does not meet the requirement of high throughput biosamples analysis required during industrial research. The work presented here has several merits over the reported methods such as improved selectivity, higher sensitivity, and simpler extraction technique with relatively shorter analytical run time.

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The purpose of this study is to develop an improved, sensitive and high throughput bioanalytical method for quantification of modafinil in human plasma by LC-MS/MS, in order to apply the methodology to assess the pharmacokinetics of modafinil in forthcoming bioavailability/bioequivalence studies.

EXPERIMENTAL

Chemicals and materials: Modafinil (99.89% purity, CAS No.: 68693-11-8) and its isotope modafinil-D5 (100.00% purity, CAS No.: 1133712-38-5) as internal standard (ISTD) were procured from Clearsynth (Mumbai, India) and Vivan Life Sciences (Mumbai, India), respectively. Two metabolites of modafinil, modafinil sulfone and modafinil carboxylate, were purchased from Vivan Life Sciences (Mumbai, India). HPLC grade methanol was obtained from Qualigens (Mumbai, India). Glacial acetic acid (≥99.0% purity), and ammonium acetate (≥99.0% purity) were of LC-MS grade and were purchased from Fluka (Sigma-Aldrich, Steinheim, USA). Blank human plasma was obtained from Yash Laboratories (Mumbai, India). Bond Elut Plexa cartridges (30 mg/1cc) for sample extraction were obtained from Agilent (Lakeforest, USA). All water was distilled, deionized, and further purified via Milli-Q® gradient A10 (Millipore, Moscheim Cedex, France). Other chemicals were purchased from standard sources and were of the highest quality available.

LC-ESI-MS/MS instrumentation and analytical conditions: The liquid chromatographic separation was performed using a Shimadzu scientific instruments (Shimadzu Corporation; Kyoto, Japan) consisting of two LC-20ADvp delivery pumps, a SCL-10Avp system controller, an on-line DGU-20A3 prominence solvent degasser, a SIL-HTc Shimadzu autosampler and a CTO-20A prominence column oven. The chromatography separation of the analytes was performed using an Ascentis® C18 analytical column (150 mm × 4.6 mm, 5 µm; Supelco, CA, USA). The column oven and autosampler temperature were maintained at 35 ± 1 ºC and 10 ± 1 ºC respectively. Analytes were eluted with an isocratic mobile phase (methanol: 2mM ammonium acetate: acetic acid; 35:65:0.1% v/v) at a flow rate of 1.0 mL/min for 4.5 minutes and splitting ratio was set at 4:10. Detection was performed on API-3200 LC-MS/MS System (MDS Sciex®; Toronto, Canada) equipped with a Turbo IonSpray® source (TIS; thermally and pneumatically assisted electrospray). The optimized conditions of MS/MS were set as follows: ion spray source temperature at 400 °C, curtain (CUR) gas at 40, gas 1 (GS1) at 65, gas 2 (GS2) at 40, ionspray voltage (IS) at 5500 V, and collision-activated dissociation (CAD) at 6 units; declustering potential (DP) at 20 V, entrance potential (EP) at 6 V, collision cell entrance potential (CEP) at 12 V, collision energy (CE) at 23 V, and collision exit potential (CXP) at 2 V. The mass spectrometer was interfaced to a computer workstation running Analyst software (Version 1.4.1, Applied Biosystems, Foster City, CA) for data acquisition and processing. Data acquisition was performed at unit of both Q1 and Q3 resolution, in positive multiple-reaction monitoring (MRM) mode, monitoring the transition of modafinil parent ion m/z 274.2 to product ion m/z 167.0, and of ISTD parent ion m/z 279.1 to the product ion m/z 172.2 (Fig. 1).

Preparation of stock solution, standard and quality control samples: Stock solutions of modafinil in duplicate, one for calibration curve standards and the other for quality control (QC) samples, and modafinil-D5 were prepared by dissolving accurately weighed standard compounds in methanol to yield for each compound a concentration of 1 mg/mL. The prepared stock solutions were stored between 1 and 10 °C; protected from light.

Working solutions of modafinil (ranging from 1.54 to 401.11 µg/mL) were prepared by serial dilution of the stock solution in methanol-water (50:50, v/v). A 200 µL aliquot of each working solution was added to blank K_3EDTA plasma to yield spiked calibration standards at eight different concentrations ranging from 30.8 to 8022.1 ng/mL. Quality control (QC) samples at four concentrations (31.0, 84.9, 2424.8 and 6062.1 ng/mL) were prepared in the similar manner as the calibration standards. Spiked calibration standards and quality control samples were stored at around -20 °C until assayed or used for validating the analytical method. The ISTD working solution (500.0 ng/mL) for routine use was prepared by diluting the modafinil-D5 stock solution in methanol-water (50:50, v/v) and stored at room temperature until use.

Sample preparation: Plasma samples stored at around -20 °C were thawed on the day of extraction at room temperature followed by vortexing to ensure homogeneity. A plasma sample (100 µL) was pipette into polypropylene tubes, with addition of 50 µL ISTD working solution and 500 µL of water. The mixture was vortexed-mixed for 30s and loaded onto the Bond Elut Plexa cartridges, which had been conditioned with 0.5 mL of methanol followed by 0.5 mL of water. The cartridges were washed twice with 1 mL of water and then eluted with 1 mL of methanol in

respective labeled polypropylene tubes. The eluents were then evaporated to dryness at 50 °C using 20 psi of nitrogen. The dried extracts were reconstituted with 500 µL mobile phase, and transferred into autosampler vials for analysis. 10 µL was injected into the chromatographic system.

Method validation: The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability of analyte during both short-term sample processing and long-term storage as per the guidelines set by USFDA [10]. The selectivity of the method towards endogenous plasma components, metabolites, and concomitant medications was assessed in eight lots of blank K_3EDTA human plasma (six normal, one lipemic and one haemolysed). They were processed and analyzed using the proposed extraction protocol and the set chromatographic conditions for modafinil at the LLOQ level.

The linearity of the method was determined by analysis of standard plots associated with an eight point standard calibration curve. Calibration curves from accepted precision and accuracy batches (n=3) were used to establish linearity. Peak area ratios of analyte/ISTD obtained from MRM were utilized for the construction of calibration curves; using weighted ($1/X^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios. The correlation coefficient $r > 0.99$ was desirable for all the calibration curves. The lowest standard on the calibration curve was to be accepted as the LLOQ, if the analyte response was at least five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with accuracy within ±20.0% and a precision ≤20.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%.

Intra-day precision and accuracy were assessed by analyzing six replicates of the quality control samples at four levels during a single analytical run. The inter-day precision and accuracy were assessed by analyzing 18 replicates of the quality control samples at each level through three precision and accuracy batches runs on 2 consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except LLOQ QC, for which it should not be more than 20.0%. Similarly, the mean accuracy should not deviate by ±15.0% except for the LLOQ QC where it should be ±20.0% of the nominal concentration.

The relative extraction recoveries for analyte and ISTD at low, middle and high QC concentration levels were determined by measuring the mean peak area response of six replicates of extracted quality control samples against the mean peak area response of six replicates each of three neat solutions containing analyte and a neat solution containing ISTD at concentrations equivalent to those obtained in the final extracted concentration for analyte and ISTD in the quality control samples.

Matrix effect was assayed at two concentration levels (LLOQ QC and HQC). The matrix effect is validated to be nullified if the accuracy and precision do not deviate by ±15.0% for HQC and ±20.0% for LLOQ QC of the nominal concentration. The matrix factor (MF) is defined as the peak response in the presence of matrix ions versus the peak response in the absence of matrix ions. Since this method involved terminal drying step, biological matrix samples were prepared by reconstituting the post-extracted blank plasma samples with three neat solutions containing modafinil and modafinil-D5 at concentration representing the final extracted concentration for analyte (low, medium and high QC concentration) and ISTD. The control samples were the same neat solutions prepared in mobile phase. Matrix factor was evaluated by measuring the respective mean peak area response and mean analyte/ISTD peak area ratio (ISTD normalized MF) of biological matrix sample against the mean peak area response and mean analyte/ISTD peak area ratio of neat solutions.

Stability of modafinil in matrix was evaluated at low and high QC levels by analyzing four replicates of QC samples. All stability exercises were performed against freshly prepared calibration standards processed along with freshly prepared four replicates of QC samples at low and high concentrations which act as comparison samples for determining the % absolute stability of modafinil. Bench-top stability was assessed after exposure of the plasma samples to room temperature for 6.8 h, which exceeds the residence time of the sample processing procedures. The freeze-thaw stability was evaluated after undergoing three freeze (at around -20˚C) and thaw (room temperature) cycles. The autosampler storage stability was determined by keeping the reconstituted QC samples for 71 h in autosampler at 10°C before being analyzed. Long-term stability was assessed after storage of the test samples at around -20˚C for 48 days. The working solutions and stock solutions of modafinil and ISTD were also evaluated for their stability at room

temperature for 9 h and at refrigerator temperature (between 1 and 10°C) for 23 days, respectively.

RESULTS AND DISCUSSION

Method Development: During the early stage of method development, both ESI and atmospheric pressure chemical ionization (APCI) sources were investigated for detection of modafinil in positive ion mode. The signal intensity obtained in the positive mode with ESI interface is much higher than that of APCI and was thus chosen as the ionization source. Both analyte and internal standard formed protonated molecules $[M+H]^+$ under acidic condition due to the addition of proton to the acetamide functional group. The Q1 full scan spectra were dominated by protonated quasimolecular ion $[M+H]^+$ at m/z 274.2 for modafinil and 279.1 for modafinil-D5 and no other additive or fragments ions were observed (data not shown). The MS/MS parameters, including declustering potential (DP), collision cell exit potential (CXP), gases (GS1, GS2, CAD) and collision energy (CE) were optimized to identify the most stable and intense product ion for analyte and ISTD. Fig. 1 shows the product ion spectra of modafinil and modafinil-D5. The product ion at m/z 167.0 for modafinil and at m/z 172.2 for modafinil-D5, formed by the loss of 2-sulfinyl-acetamide side chain, was observed with higher abundance and greater stability. Deuterated ISTD shared similar fragmentation patterns with its non-labeled counterpart. During method development different additives of varying strength were added to the mobile phase, so as to obtain higher abundance of protonated parent ion of analyte and ISTD. Use of ammonium acetate with acetic acid in mobile phase enhances the occurrence of $[M+H]^+$ and eventually improved peak area response for analyte and ISTD.

After optimizing mass parameters liquid chromatographic conditions were tuned. To select the starting conditions towards optimizing the LC parameters, we have paid attention to the previous works. A number of reversed-phase C18 columns, such as Zorbax SB C18, Novapak C18, Discovery® C18 and Ascentis® C18 were tested to obtain optimal response, suitable retention time and good peak shapes for analyte and ISTD. The Ascentis® C18 column was selected since it provided good peak shape and high intensity with greater signal to noise (S/N) ratio. Later, mobile phase composition was optimized so as to achieve symmetric peak shape, good sensitivity and a shorter run time for the analysis. The use of 2mM ammonium acetate solution and glacial acetic acid in aqueous phase improved sensitivity for modafinil and ISTD. Moreover use of 0.1% glacial acetic acid in mobile phase separates its metabolites like modafinil carboxylate and modafinil sulfone from peak of interest i.e. modafinil (data not shown). Methanol revealed a higher mass spectrometric response than acetonitrile and was chosen for the organic phase. Thus a mobile phase consisting of methanol: 2mM ammonium acetate solution: glacial acetic acid (65:35:0.1, v/v/v) was used in the experiment. The retention time for both modafinil and ISTD was 2.80 min as shown in Fig. 2.

During method development different options were evaluated to optimize sample cleanup so as to eliminate possible matrix interferences, concentrate the sample and obtain as clean a sample as possible to preserve the life of the analytical column. To select the starting conditions towards optimizing sample cleanup, we have paid attention to previous works, relating to the extraction of modafinil and/or its metabolites from biological matrix. Firstly, the simplest and fastest protein precipitation (PPT) method for preparing samples was carried out; unfortunately, it does not result in a very clean extract, produces higher background noise with poor sensitivity. Secondly, liquid-liquid extraction (LLE) evaluated as proposed in previous work [4, 6-8] towards isolation of analytes from biological matrix. LLE rather produces clean extracts compared to PPT, but the procedures involved are cumbersome and have multiple pitfalls. Cleanest sample was obtained using SPE principle as compared with solvent extraction and protein precipitation technique. Taking this into account, we employed Agilent® Bond Elut Plexa disposable cartridge to extract analytes from plasma samples.

Method Validation: Selectivity was ascertained in different lots of human plasma by comparing the chromatograms of blank plasma samples with the corresponding spiked LLOQ plasma samples. Fig. 2 shows the typical chromatograms of a double blank, blank spiked with ISTD, a spiked plasma sample with modafinil at LLOQ and ULOQ level. As can be seen no interfering peaks from endogenous compounds were observed at the retention times of the analyte and ISTD. The chromatograms presented in Fig. 2 indicated selectivity of the method.

The method was validated using the above criteria and found linear over the concentration range of 30.8 to 8022.1 ng/mL. The intercept with the y-axis was not significantly different from zero. A typical regression equation was $y = 4.42\times10^{-3}x + 4.18\times10^{-3}$ with a correlation coefficient (r) of 0.9998, where y represents the peak area ratio of modafinil to that of ISTD and x represents the plasma concentration of modafinil. The lower limit

of quantification for this assay was 30.8 ng/mL in plasma. Eighteen replicates of the QC samples from three consecutive validation runs were used to evaluate precision and accuracy at each concentration level. The intra- and inter-day precision and accuracy values of the QC samples are summarized in Table 1. The intra- and inter-day precision and accuracy values were within the acceptable range. The method was thus judged to be accurate and reproducible.

At three QC concentration levels low, middle and high the percent mean extraction recoveries of modafinil obtained from plasma were 91.7%, 86.7% and 90.8%, respectively, whereas the mean recovery for modafinil-D5 at the concentration employed was 93.0%.

Matrix effect results shows that no additional variations in plasma concentration due to the use of different plasma lots were observed as percentage of nominal concentrations for modafinil at LLOQ QC and HQC level were 105±5.0% and 110±2.0%, respectively. The average absolute matrix factor values at low, middle and high QC concentrations from six lots of plasma samples are 0.986, 0.957, and 0.968, respectively, whereas the ISTD normalized matrix factor values at low, middle and high QC concentrations are 0.996, 1.000, and 0.995, respectively. The precisions of absolute and ISTD normalized matrix factor from six lots of plasma samples were ≤1.5%. These results showed that ion suppression or enhancement from the plasma matrix was negligible under the stated conditions.

The stability results summarized in Table 2 showed that, modafinil spiked into human plasma was stable for at least 6.8 h at room temperature, in an autosampler post extraction for 71 h at 10°C, in plasma stored at around -20°C for 48 days and in plasma after three freeze-thaw cycles (-20°C to room temperature). The stock solutions of modafinil and ISTD were found stable at refrigerator temperature (between 1 and 10°C) for 23 days and the working solutions of modafinil and ISTD were found stable for 9 h at room temperature.

CONCLUSION

A rapid, sensitive and selective LC–MS/MS method for the determination of modafinil in human plasma was developed and validated. Solid phase extraction methodology was adopted in plasma sample preparation that provides consistent extraction recovery with minimal endogenous interference and matrix effect. According to the validation parameters, the developed method could be useful for modafinil pharmacokinetic studies and routine therapeutic drug monitoring with desired precision and accuracy. An added advantage over the earlier methods was the proposed solid phase extraction procedure was simple, efficient and easy to automate.

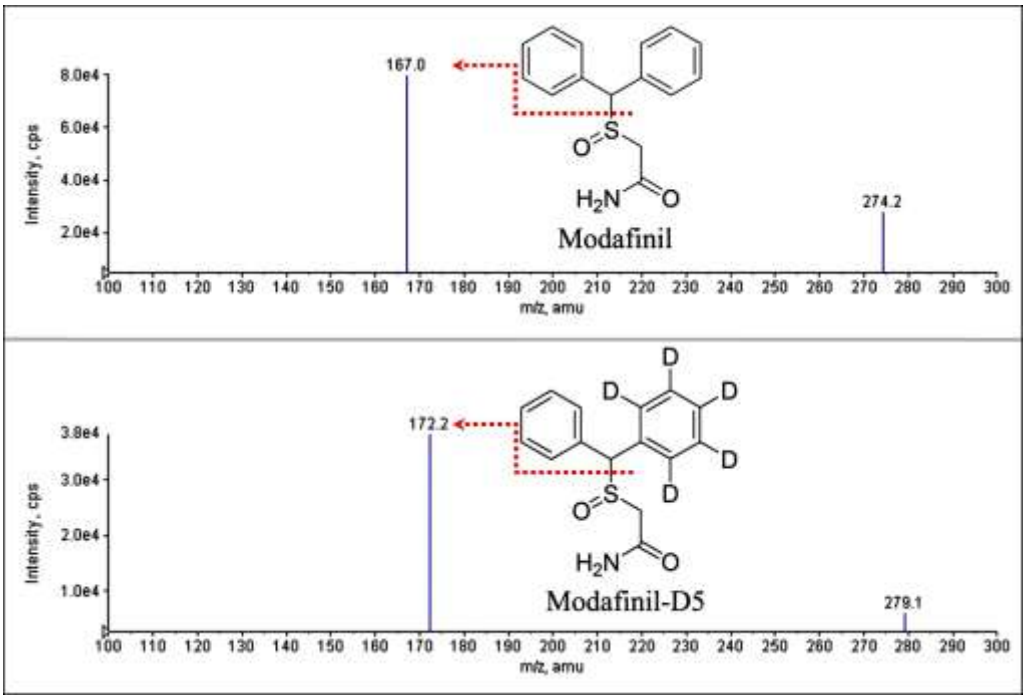

Fig.1. The product ion spectra of modafinil and modafinil-D5

Table 1 Intra- and inter-day precision and accuracy data for the determination of modafinil in human plasma.

Spiked Concentration (ng/mL)	Intra-day (*n*=6)			Inter-day (*n*=18)		
	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
31.0	29.97	96.7	1.7	30.57	98.6	3.1
84.9	86.17	101.5	1.9	87.01	102.5	1.8
2424.8	2503.3	103.2	1.9	2497.73	103.0	2.3
6062.1	6168.67	101.8	1.1	6190.81	102.1	1.5

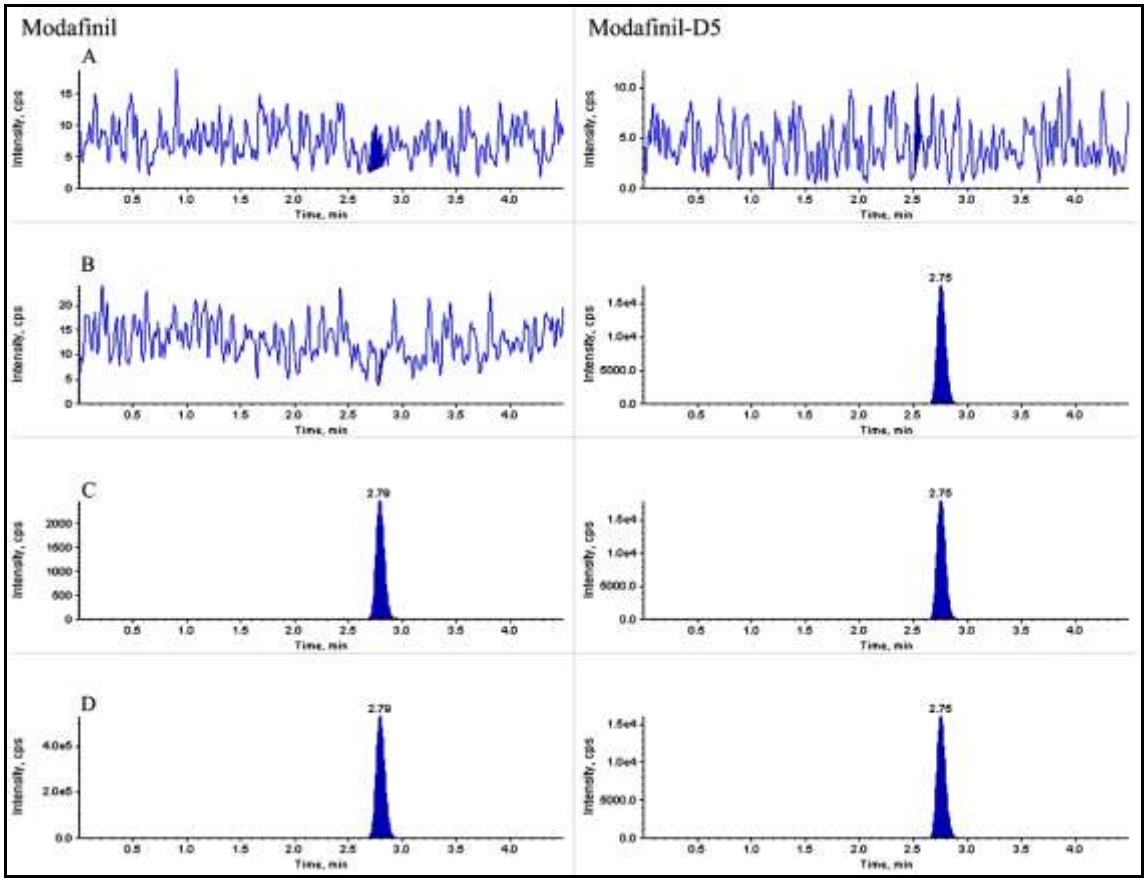

Fig.2. Representative chromatograms in human plasma: (A) double plasma blank; (B) plasma blank with ISTD; (C) LLOQ, 30.8 ng/mL; and (D) ULOQ 8022.1 ng/mL. Modafinil (left panels, A-D) and it's ISTD-modafinil-D5 (right panels).

Table 2 Stability data for modafinil in human plasma under various conditions (n = 4)

Stability	Sample	Spiked concentration (ng/mL)	Average±S.D (ng/mL)	Accuracy (%)	CV (%)	Absolute stability (%)
Freeze/thaw stability (three freeze/thaw cycles at-20°C)	Comparison Samples	84.8	88.30±1.24	104.1	1.4	N/AP
		6056.6	6248.95±60.02	103.2	1.0	N/AP
	Stability Samples	84.9	85.93±0.38	101.2	0.4	97.2
		6062.1	6305.63±27.68	104.0	0.4	100.8
Bench top stability (6.8 h at room temperature)	Comparison Samples	84.8	78.78±0.73	92.9	0.9	N/AP
		6056.6	6258.48±105.81	103.3	1.7	N/AP
	Stability Samples	84.9	79.83±1.59	94.0	2.0	101.2
		6062.1	6320.72±98.75	104.3	1.6	100.9
In-injector stability (71 h)	Comparison Samples	84.8	81.60±0.62	96.2	0.8	N/AP
		6056.6	6314.08±132.27	104.3	2.1	N/AP
	Stability Samples	84.9	80.08±1.57	94.3	1.9	98.0
		6062.1	6217.13±89.83	102.6	1.4	98.4
Long-term stability (48 days)	Comparison Samples	85.0	84.85±2.43	99.8	2.87	N/AP
		6069.4	6250.50±173.95	103.0	2.78	N/AP
	Stability Samples	84.9	82.48±3.71	97.1	4.49	97.3
		6062.1	6138.60±132.38	101.3	2.16	98.3

% Absolute stability=(average concentration of stability samples/average concentration of comparison samples×C.F.)×100.

C.F.= concentration of stability sample/concentration of comparison sample.

N/AP= Not Applicable

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