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A method for determination of the plasma levels of modafinil enantiomers, (±)-modafinic acid and modafinil sulphone by direct human plasma injection and bidimensional achiral–chiral chromatography

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Abstract

Coupled-column separation using restricted access media as the first dimension in order to exclude macromolecules and retain micromolecules has been successfully used for a number of biological fluids. This paper describes the first method developed and validated for the analysis in a single run of the enantiomers of modafinil and its two major metabolites. The method was developed using a bidimensional HPLC system by coupling a restricted access medium (RAM) bovine serum albumin (BSA) column (1.0 cm × 0.46 cm i.d.) to an amylose tris[(*S*)-1-phenylethylcarbamate] chiral column. The method was fully validated and showed good linearity, precision, accuracy, sensitivity and selectivity, allowing it to be used for pharmacokinetic studies. The quality of the performance of both columns was maintained with over 280 plasma injections of 100 μl.
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Keywords: Modafinil; Enantiomers; Polysaccharides stationary phases; Plasma samples; Direct injections

1. Introduction

Psychostimulants, such as amphetamine and methylphenidate, are used clinically for treatment of narcolepsy, as well as attention deficit/hyperactivity disorders (ADHD). These compounds, however, have potential risk of dependence and also of tolerance. Thus, as a result, novel nonstimulant treatments for narcolepsy and ADHD has been focus of new researches [1].

Modafinil, (±)-[2-(diphenyl)methanesulfinyl]acetamide (**1**), $C_{15}H_{15}NO_2S$, MW 273 (Fig. 1) is a wake-promoting drug that has, not long ago, emerged for the treatment of narcolepsy. The exact mechanism of modafinil action is still controversial. But, recently, it has been reported that it may indirectly increase wakefulness partly through inhibition of gama-aminobutyric acid (GABA) release via serotonergic mechanisms [1–4].

It is a chiral compound due to the asymmetric sulphoxide function, but it is used as a racemate. Nevertheless, it has been reported that the pharmacological properties of the two enantiomers are different and that the (+)-enantiomer is eliminated three times faster than the (−)-enantiomer [5,6]. Only recently the absolute configurations of both enantiomers have been assigned [7]. Research for elucidating the mechanism of action of the racemate and of each enantiomer in promoting wakefulness and their potential as stimulant in abuse therapeutics demands an efficient analytical method for following the pharmacological profile of the enantiomers and their major metabolites (±)-[2-(diphenyl)methanesulfinyl]acetic acid (**2**) and [2-(diphenyl)methanesulfonyl]acetamide (**3**) (Fig. 1).

There are two published methods for the quantification of the enantiomers of modafinil in human plasma [8,9] and one in serum [10], but none of them enables the quantification of the enantiomers and their metabolites at the same chromatographic run. The aim of this work was to develop an enantioselective HPLC assay that would be reliable for quantification of modafinil enantiomers and their two major metabolites in plasma with on-line sample preparation.

2. Materials and methods

2.1. Equipments and columns

The HPLC system consisted of two Shimadzu LC-10ATVP pumps (Kyoto, Japan) with one of the pumps containing a valve

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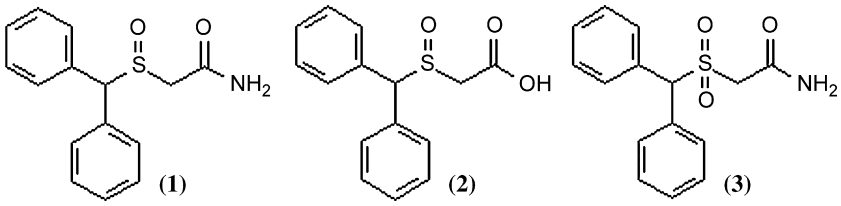


Fig. 1. Chemical structure of modafinil (**1**), (±)-modafinic acid (**2**) and modafinil sulphone (**3**).

FCV-10AL for selecting solvent, an auto injector model SIL 10AVP, a degasser model DGU-14A, a SPD-6AV UV–vis detector (at 240 and 228 nm) and a SCL 10AVP interface. A sample valve HPLC 7000 Nitronic EA (Supelco, St. Louis, MO, USA) was used for the automated column switching. Data acquisition was done on a Shimadzu Class-VP Software. The columns were prepared as described elsewhere [11,12] and consisted of amylose tris[(*S*)-1-phenylethylcarbamate] coated onto APS-Nucleosil (7 μm particle size and 500 Å pore size, 20% w/w, 15 cm × 0.46 cm i.d.) as the chiral column and an octyl BSA (1.0 cm × 0.46 cm i.d., 10 μm) as the restricted access media.

The elution order was determined by injection of (+) and (−)-modafinil at the established separation conditions. The enriched enantiomers were obtained by enantiomeric separation, using a amylose tris[(*S*)-1-phenylethylcarbamate] coated onto APS-Hypersil (5 μm particle size and 100 Å pore size, 15% w/w, 20 cm × 0.7 cm i.d.) as stationary phase, using hexane–ethanol (50:50, v/v) as mobile phase at 3 ml min^{-1}. The optical activities of the separated enantiomers were defined using a Perkin-Elmer Model 241 polarimeter. At analytical and semi-preparative conditions the (+)-modafinil was the first enantiomer to elute.

2.2. Synthesis of modafinil, (±)-modafinic acid and modafinil sulphone [7,13]

The syntheses of modafinil (**1**), (±)-modafinic acid (**2**) and modafinil sulphone (**3**) were initiated with the reaction of benzhydrol and thioglycolic acid in trifluoroacetic acid to afford 2-(benzhydrylsulfanyl)acetic acid (**4**) in 97% yield as a white solid. The reaction of acid (**4**) with thionyl chloride in benzene, followed by treatment of the corresponding acid chloride with concentrated ammonium hydroxide, gave acetamide (**5**) in 80% yield as an off-white solid. Racemic modafinil (**1**) in 92% and modafinil sulphone (**3**) in 80% yields as white solids were obtained by the oxidation of 2-(benzhydrylsulfanyl)acetamide (**5**) using aquo (*N*-oxo of pyridine) and aquo (pyrazol) molybdenum (VI) oxodiperoxo complexes, respectively. The aquo (*N*-oxo of pyridine) molybdenum (VI) oxodiperoxo complex was also used for oxidizing the acid (**4**). (±)-[2-(Diphenyl)methanesulfinyl]acetic acid (**2**) was isolated as a white solid (85% yield). The protocol of oxidations is described elsewhere [13].

2.2.1. (±)-[2-(Diphenyl)methanesulfinyl]acetamide—(*1*)

mp 159–160 °C; IR (υ_{max}, KBr, cm^{-1}): 3361, 1686, 1033, 702. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.49–7.30 (m, 10H); 7.06 (bs, 1H); 5.60 (bs, 1H); 5.20 (s, 1H); 3.50 (d, J = 14.7 Hz, 1H); 3.10 (d, J = 14.7 Hz, 1H). ^{1}H NMR (400 MHz; DMSO-d_6, δ): 7.54–7.31 (m, 10H); 7.06 (bs, 1H); 5.39 (s, 1H); 3.38 (d, J = 13.5 Hz, 1H); 3.21 (d, J = 13.5 Hz, 1H). ^{13}C NMR (200 MHz; DMSO-d_6, δ): 166.5; 137.4; 135.1; 129.9; 129.2; 128.7; 128.1; 69.0; 56.3. Calcd. for $C_{15}H_{15}NO_2S$: %C, 65.91; %H, 5.53; %N, 5.12; %S, 11.73. Found: %C, 66.08; %H, 5.27; %N, 5.02 %S, 11.83.

2.2.2. (±)-[2-(Diphenyl)methanesulfinyl]acetic acid—(*2*)

mp 150 °C; IR (υ_{max}, KBr, cm^{-1}): 3437, 1728, 1270, 1026, 705. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.90–7.31 (m, 10H); 5.40 (s, 1H); 3.56 (d, J = 14.2 Hz, 1H); 3.32 (d, J = 14.2 Hz, 1H). ^{13}C NMR (200 MHz, DMSO-d_6, δ): 167.4; 136.8; 135.0; 129.8; 129.3; 128.7; 128.2; 69.5; 55.6. Calcd. for $C_{15}H_{14}O_3S$: %C, 65.67; %H, 5.14; %S, 11.69. Found: %C, 65.52; %H, 5.20; %S, 12.51.

2.2.3. [2-(Diphenyl)methanesulfonyl]acetamide—(*3*)

mp 196 °C; IR (υ_{max}, KBr, cm^{-1}): 3436, 3195, 1676, 1316, 1130, 702. ^{1}H NMR (400 MHz, CD_3OD, δ): 7.69–7.67 (m, 4H); 7.43–7.40 (m, 6H); 6.49 (sl, 1H); 5.68 (s, 1H); 5.60 (sl, 1H); 3.75 (s, 2H). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.73 (sl, 1H); 7.68–7.63 (m, 4H); 7.51 (sl, 1H); 7.47–7.38 (m, 6H); 6.10 (s, 1H); 3.76 (s, 2H). ^{13}C NMR (200 MHz, DMSO-d_6, δ): 163.5; 133.4; 130.0; 129.0; 128.8; 71.6; 56.0. Calcd. for $C_{15}H_{15}NO_3S$: %C, 62.27; %H, 5.23; %N, 4.84; %S, 11.08. Found: %C, 59.70; %H, 5.27; %N, 4.20 %S, 10.80.

2.3. Sample preparations

2.3.1. Standard solutions

Modafinil (20.0 mg), (±)-modafinic acid and modafinil sulphone (10.0 mg each) stock solutions were individually prepared by dissolving in 10.0 ml of methanol. Then, three stock solutions (500 μg ml^{-1}) were separately prepared by aliquoting 500 μl of each solution and diluting it to 10.0 ml with methanol.

From these stock solutions combined standard solutions in methanol were prepared having individually the concentrations: 2.00, 3.00, 5.00, 10.0, 16.0, 40.0, 70.0 and 100 μg ml^{-1} for modafinil; 1.50, 2.50, 8.00, 20.0, 35.0 and 50.0 μg ml^{-1} for modafinic acid and 5.00, 8.00, 10.0, 20.0, 35.0 and 50.0 μg ml^{-1} for modafinil sulphone. For preparing the quality control sample (QC), four combined standard solutions were also prepared in methanol, at the following concentrations of each analyte: 2.40, 3.60, 50.0 and 80.0 μg ml^{-1} for modafinil; 1.80, 25.0 and 40.0 μg ml^{-1} for modafinic acid and 6.00, 25.0 and 40.0 μg ml^{-1} for modafinil sulphone.

The stability of the stock solutions were assessed by analyzing a set of freshly prepared stock solutions and comparing

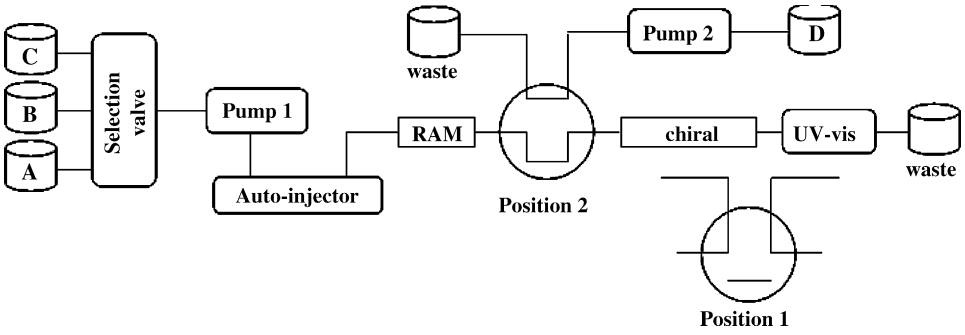


Fig. 2. Schematic diagram of the column-switching system.

the results with the analysis of a set of stock solutions stored at −20 °C for 2 months.

2.3.2. Preparation of spiked human plasma samples

Pooled control human plasma was supplied by the São Francisco University Hospital, Bragança Paulista, SP, Brazil. Heparin was used as anticoagulant and stored at −20 °C until use. For analysis, the plasma samples were thawed at room temperature and centrifuged for 10 min at 10,000 × *g*.

To prepare the fortified plasma samples, aliquots (20 μl) of the appropriate standard solutions were placed into a culture tubes and the solvent was evaporated under a stream of nitrogen. The dried analytes were reconstituted using 200 μl of human plasma. The solutions were mixed by vortex agitation for 15 s. Aliquots of 190 μl were transferred to auto-sampler vials and 100 μl were injected into the column-switching HPLC system.

2.4. Column-switching procedure

The column-switching system used is illustrated in Fig. 2. The position of the column-switching device alternated between positions 1 and 2 and was controlled through the timed events using the Class-VP Software. Initially, the column switching was set to position 1 and the human plasma sample was injected into the RAM column. The time sequence used is listed in Table 1. The six-port valve remained in this position for 5.20 min, while first the macromolecules were discharged for 2.5 min at a flow rate of 1.0 ml min^{-1} using KH_2PO_4 0.01 M pH 2.5:CH_3CN (95:5, v/v) as mobile phase and then, eluting the analytes at 0.8 ml min^{-1} using KH_2PO_4 0.01M pH 5.1:CH_3CN (70:30, v/v). At the same time, the analytical column was conditioned with KH_2PO_4 0.01M pH 5.1:CH_3CN:MeOH (73:22.5:4.5, v/v/v) delivered by pump 2 at a flow rate 0.8 ml min^{-1}. Then, the valve changed to position 2 redirecting the flow from the waste to the analytical column; the analytes were eluted onto the chiral column between 5.20 and 6.80 min at a flow rate 0.8 ml min^{-1}. At the end of this time, the valve was switched back to position 1 and pump 1 remained up to 10 min delivering KH_2PO_4 0.01M pH 5.1:CH_3CN (70:30, v/v), at a flow rate 0.8 ml min^{-1}, for discharging later retained solutes. Before conditioning the RAM column (1.0 ml min^{-1}) with the initial RAM's mobile phase. The RAM column was cleaned for 8 min using KH_2PO_4 0.01 M pH 2.5:CH_3CN (40:60, v/v), also at a flow rate of 1.0 ml min^{-1}. After the transfer of the analytes to the chiral columns, they were analyzed (from 6.8 to 30 min) using the same mobile phase in which the chiral column was conditioned. The absorbance was measured at 228 nm for modafinil and modafinic acid and at 240 nm for the sulphone. Analysis time was 30 min, with no time spent on sample preparation.

2.5. Method validation

Method validation was carried out according to internationally accepted criteria [14]: linearity, selectivity, accuracy and precision, recovery, limit of quantification (LOQ), limit of detection (LOD) and stability.

Using the appropriate standard solution of modafinil, modafinic acid and modafinil sulphone spiked plasma samples were prepared in triplicate at the following calibration concen-

Table 1
Time events for the column-switching procedure and mobile phases

Time (min)	Pump (eluent)	Flow-rate (ml min^{-1})	Event	Valve position
0.00–2.50	1 (A)	1.0	Plasma proteins are excluded by RAM column	1
	2 (D)	0.8	Conditioning of the analytical column	
2.51–5.19	1 (B)	0.8	Elution of the analytes from the RAM	1
5.20–6.80	1 (B)	0.8	Analytes are transferred to the analytical column	2
6.80–30.00	2 (D)	0.8	Analysis of the (±)-modafinil and metabolites	1
6.81–10.00	1 (B)	0.8	Discharging later retained solutes of the RAM column	1
10.01–18.00	1 (C)	1.0	Washing of RAM column	1
18.01–30.00	1 (A)	1.0	Conditioning of RAM column	1

Pump 1 (eluent): (A) KH_2PO_4 0.01 M pH 2.5:CH_3CN (95:5, v/v); (B) KH_2PO_4 0.01 M pH 5.1:CH_3CN (70:30, v/v); (C) KH_2PO_4 0.01 M pH 2.5:CH_3CN (40:60, v/v). Pump 2 (eluent): (D) KH_2PO_4 0.01M pH 5.1:CH_3CN:MeOH (73:22.5:4.5, v/v/v).

trations: 0.100, 0.250, 0.500, 0.800, 2.00, 3.50 and 5.00 μg ml^{-1} for (+)-modafinil; 0.150, 0.250, 0.500, 0.800, 2.00, 3.50 and 5.00 μg ml^{-1} for (−)-modafinil; 0.150, 0.250, 0.800, 2.00, 3.50 and 5.00 μg ml^{-1} for modafinic acid and 0.500, 0.800, 1.00, 2.00, 3.50 and 5.00 μg ml^{-1} for modafinil sulphone. Plasma calibration curves were constructed by plotting the peak area against the concentration of each analyte.

The interference of endogenous compounds was assessed by analyzing drug-free plasma samples and plasma fortified with all analytes.

Inter- and intra-day variability of the method was determined by analyzing replicates of the QC samples (0.120, 2.50 and 4.00 μg ml^{-1} for (+)-modafinil; 0.180, 2.50 and 4.00 μg ml^{-1} for (−)-modafinil; 0.180, 2.50 and 4.00 μg ml^{-1} for modafinic acid and 0.600, 2.50 and 4.00 μg ml^{-1} for modafinil sulphone). Five samples of each concentration were prepared in plasma on three non-consecutive days. The accuracy of the method was evaluated by back-calculation; it was also tested using blinded unknowns, at two different concentrations, which were prepared by a different analyst.

The recoveries of modafinil enantiomers and their metabolite acid and sulphone, from human plasma samples, were measured with the same QC samples used for the determination of intermediate precision. The efficiency of extraction was calculated by comparing the mean peak areas of analytes (QC samples) with the mean of those of similar methanolic standard solutions.

The acceptance criteria for the LOQ were that the precision and the accuracy for three extracted samples be under 20% variability. LOD was calculated taking a signal-to-noise ratio of 3 as criteria. LOQ and LOD were measured by preparing fortified human plasma samples with serial diluted solutions.

The stability of analytes in human plasma samples was evaluated by comparing assay results in fortified samples at different concentrations of QC samples and by analyzing aliquots of the same samples after storages at −20 °C. All stability determinations were assessed by preparing a set of samples from a freshly made stock solution of plasma samples. The chemical stability of the QC plasma samples containing modafinil enantiomers and metabolites and control human plasma samples (matrix) were tested in the following conditions: (a) sitting at room temperature for 4 h (bench-top stability) and 24 h (auto-sampler stability), respectively; (b) stored at −20 °C and exposed to three freeze-thaw cycles; (c) stored at −20 °C for 2 months (long-term stability). The analytes were considered stable if the variation of the concentrations, between the assays, were less than 15% of initial time response.

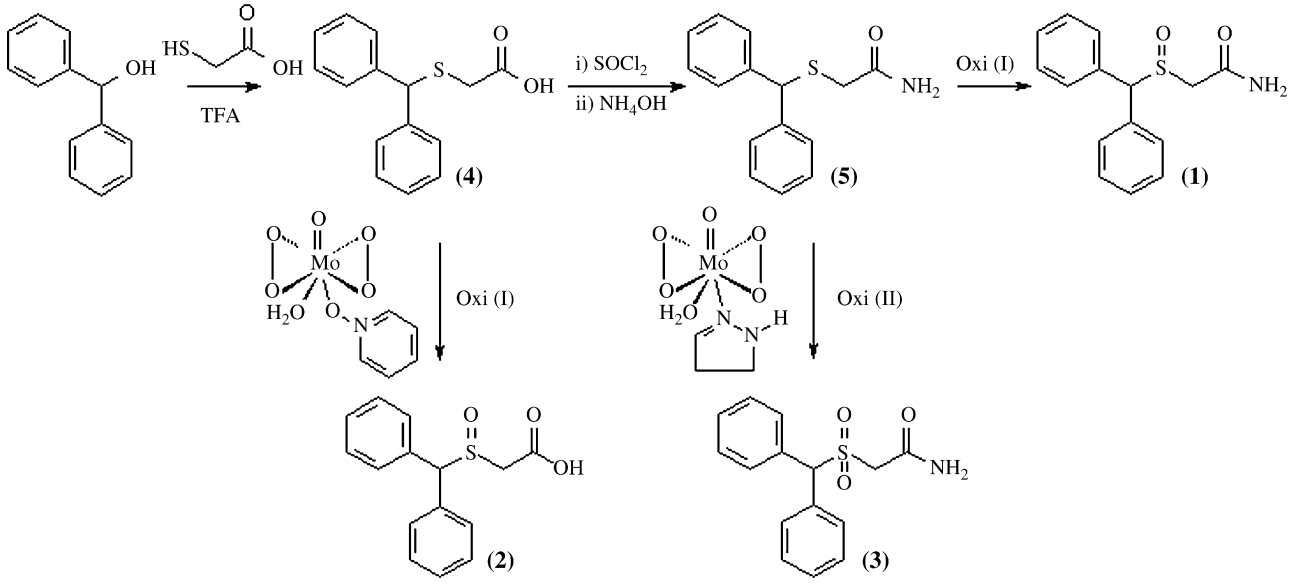
3. Results and discussion

Recently, a wide variety of restricted access media (RAM) columns that allow direct injection of biological fluids have been reported and have generated great interest because of the decreased analysis times [15,16]. To permit direct injection of biological samples, the RAM column must be capable of removing endogenous components with high efficiency. We have previously reported the efficient preparation and application of a series of RAM BSA columns for on-line microsample deproteinization [11,17–21].

Derivatized polysaccharide-based stationary phases have proved to be very efficient in the resolution of a series of chiral suphoxides [12,22–23]. The use of amylose tris[(*S*)-1-phenylethylcarbamate], in the reverse elution mode, showed the best performance in the separation of modafinil enantiomers, as reported before [9], and it was selected as the analytical column.

The reactions for obtaining 2-(benzhydrylsulfanyl)acetamide (**5**) were carried out as described in the work of Prisinzano and collaborators [7]. To obtain modafinil (**1**) and modafinil sulphone (**3**) and to mono-oxidize the intermediate 2-(benzhydrylsulfanyl)acetic acid (**4**) molybdenium (VI) oxo-diperoxo complexes were used [13] (Scheme 1).

The column-switching system used for coupling the RAM to the chiral columns is shown schematically in Fig. 2. In order



Scheme 1. Reaction scheme used for the synthesis of modafinil, (±)-modafinic acid and modafinil sulphone.

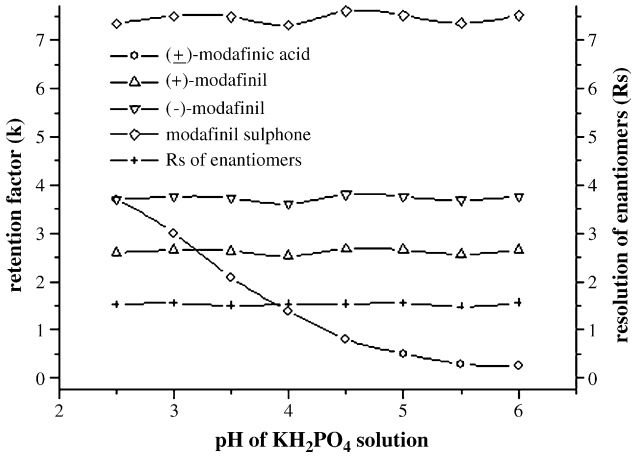


Fig. 3. Influence of the use of phosphate buffer (0.01 M) at different pH with acetonitrile as modifier (75:25, v/v) at 0.5 ml min^{-1} in the retention factors of (±)-modafinic acid (**2**) and on the resolution of the enantiomers of modafinil at the chiral column.

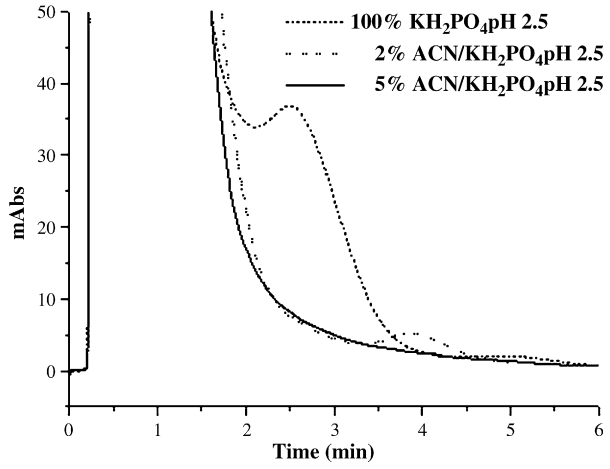


Fig. 4. Typical chromatograms of exclusion profiles of plasma's proteins.

to determine the elution profile and retention times of the analytes in the RAM column, the column was directly connected to the UV detector. Although, the proteins of the plasma could be eluted in the first five minutes using only water as mobile phase, for precise retention of the modafinic acid, it was necessary to evaluate the influence of the mobile phase's pH.

The influence of the pH of phosphate solution (0.01 M) in the elution profile of plasma's proteins at an octyl RAM-BSA column (10 cm × 0.46 cm i.d.) was previously well established

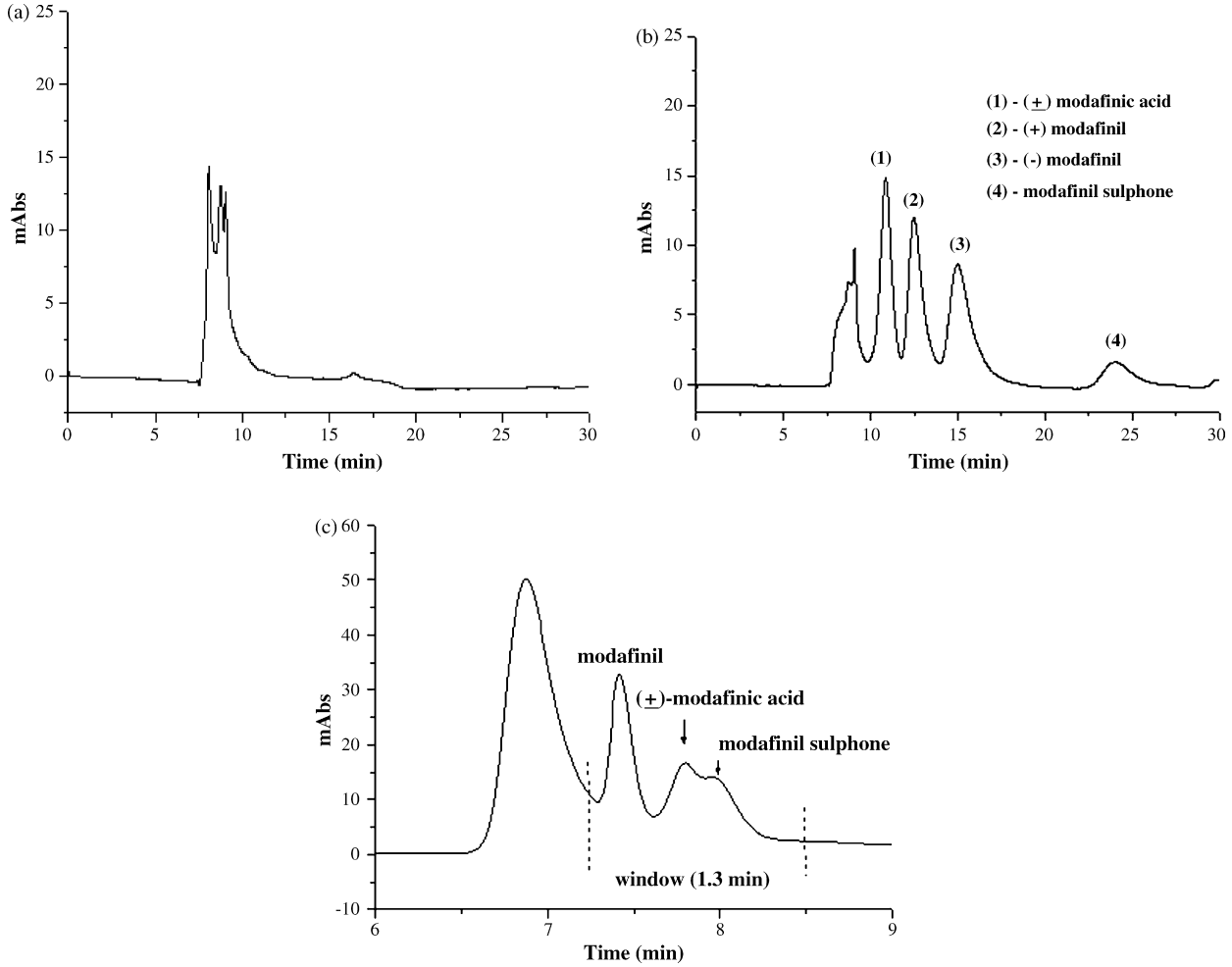


Fig. 5. Chromatograms of: (a) the analysis of human control plasma; (b) spiked plasma with the analytes at 0.25 μg ml^{-1} on the established conditions; (c) show the time-window used for transferring the analytes at 0.25 μg ml^{-1} from the RAM to the chiral column.

Table 2
Accuracy, intra-day ($n = 5$), inter-day ($n = 15$) variability and recovery ($n = 5$) for the assay of modafinil enantiomers and their metabolites acid and sulphone in human plasma

Analyte ($\mu g\,ml^{-1}$)	1st day		2nd day		3rd day		Pooled ($n = 15$)		Recovery
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	(mean ± S.D.)
(+)-Modafinil									
0.120	3.80	101	2.47	90.3	3.43	104	3.23	98.4	101 ± 1.9
2.50	2.35	108	0.750	99.2	1.33	94.0	1.48	100	98.4 ± 1.1
4.00	1.01	110	1.36	102	1.01	96.1	1.13	102	99.5 ± 0.8
(−)-Modafinil									
0.180	2.71	108	6.39	108	1.77	93.2	3.62	103	96.7 ± 2.1
2.50	0.790	111	0.630	99.2	3.13	97.9	1.52	102	98.9 ± 2.3
4.00	0.770	109	1.85	104	2.71	101	5.33	104	100 ± 1.5
Modafinic acid									
0.180	2.68	114	3.75	112	3.03	104	3.15	110	106 ± 3.2
2.50	2.61	103	1.03	99.6	1.38	93.7	1.67	98.7	98.7 ± 2.7
4.00	0.750	102	1.44	97.1	1.37	91.9	1.19	97.0	95.9 ± 3.5
Modafinil sulphone									
0.600	2.86	113	4.23	108	2.84	115	3.31	112	101 ± 2.8
2.50	4.52	92.5	3.02	90.7	1.01	93.4	2.85	92.2	95.3 ± 2.1
4.00	1.76	102	1.04	101	0.910	93.0	1.24	98.6	97.6 ± 1.4

[20]. However, for column-switching, it was necessary to evaluate the influence of pH in the resolution of the four compounds at the chiral column too. For this, a phosphate buffer (0.01 M) at a different pH with acetonitrile as modifier (75:25, v/v) at 0.5 ml min^{-1} was used. The results are showed in Fig. 3. A decrease in retention factor for modafinic acid (**2**) is easily noticed, by the increase in the pH of phosphate buffer (0.01 M), demonstrating that the resolution between the acid and modafinil enantiomers can be tuned by the pH of the mobile phase.

To evaluate the transfer of the analytes from the RAM-BSA column (10 cm × 0.46 cm i.d.) to the chiral column, a series of experiments were carried out. Different percentages of acetonitrile were evaluated, as well as pH values of the phosphate buffer (0.01 M) used as mobile phase. The width of the chromatographic band necessary for transferring the analytes from the RAM column to the chiral column, associated with the poor retention of the acid and high retention of the sulphone by the chiral column, did not yield the necessary resolution for simultaneously quantifying the four analytes. At the best conditions evaluated, the acid had poor retention time and the sulphone a retention time greater than 40 min. To circumvent these problems, a smaller RAM-BSA column was envisaged and thus a 1.0 cm column was prepared.

The recovery of proteins from this new small column was first evaluated as previously reported [11]. Recoveries of human plasma protein in 95.9, 99.8 and 98.3% with CV ($n = 3$) in the range of 0.73–2.6% were achieved in 2 min with 100% of water as mobile phase at flow rate of 1.0 ml min^{-1} for sample injections of 50.0, 100 and 200 μl, respectively. Fig. 4 illustrates the chromatograms obtained when phosphate buffer (with or without acetonitrile) were used as mobile phase at pH 2.5. With 5% of acetonitrile in the mobile phase, deproteinization of the plasma samples were achieved around 2. 5 min, at a flow rate of 1 ml^{-1}, this allowed an early change in strength of the mobile phase for elution and transfer of the analytes.

The achieved bandwidth for transferring analytes, using the RAM-BSA (1.0 cm × 0.46 cm i.d.) column, provided better resolution of modafinil enantiomers and the metabolites at the chiral column. Table 1 shows the time event used for the analytical procedure.

Fig. 5 shows the chromatograms of human control plasma (a) and spiked plasma with modafinil and the two metabolites analyzed at the established conditions (b). The chromatogram at Fig. 5c shows the time-window used for transferring the analytes from the RAM to the chiral column. The chromatograms show that no endogenous compounds interfere with the detection of the analytes. However, no separation of the enantiomers of the modafinic acid was achieved under any of the conditions evaluated, and, therefore, this metabolite was quantified as a racemic mixture.

The calibration curves were linear for the calibration ranges 0.100–5.00 $\mu g\,ml^{-1}$ to (+)-modafinil, 0.150–5.00 $\mu g\,ml^{-1}$ to (−)-modafinil and modafinic acid and 0.500–5.00 $\mu g\,ml^{-1}$

Table 3
Precision and accuracy for the determination of modafinil enantiomers and their metabolites in blinded human plasma samples

Analyte ($\mu g\,ml^{-1}$)	CV (%)	Accuracy (%)
(+)-Modafinil		
1.00	2.89	94.2
0.200	2.50	89.6
(−)-Modafinil		
1.00	3.49	91.6
0.200	4.04	95.7
Modafinic acid		
1.50	0.980	103
0.700	1.06	90.4
Modafinil sulphone		
1.50	2.60	95.2
0.900	2.20	113

Table 4
Stability assays at −20 °C (freeze-thaw cycles and long-term) of spiked samples

Analyte (μg ml^{-1})	Freshly prepared		1st cycle (24 h)		2nd cycle (72 h)		3rd cycle (120 h)		2 months	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
(+)-Modafinil										
0.120	1.21	102	1.28	94.6	2.15	104	1.95	102	3.19	101
2.50	1.14	92.3	1.53	97.3	0.830	104	1.38	100	3.36	106
4.00	2.28	93.4	1.95	96.8	1.54	94.7	2.17	99.1	1.31	110
(−)-Modafinil										
0.180	1.75	105	1.66	108	1.82	102	1.21	105	2.03	107
2.50	2.18	94.5	1.38	99.3	0.850	102	2.57	102	2.11	110
4.00	1.97	98.2	2.64	102	2.24	95.8	2.29	96.1	0.980	109
Modafinic acid										
0.180	2.23	108	2.59	104	2.46	99.5	3.14	97.5	2.98	101
2.50	1.83	96.2	2.35	99.8	1.29	104	1.95	109	1.69	93.2
4.00	1.69	98.7	2.32	96.2	2.84	94.6	1.32	92.7	1.71	91.3
Modafinil sulphone										
0.600	1.54	96.5	1.98	103	1.23	96.5	2.19	89.6	3.33	111
2.50	1.12	101	2.53	106	2.54	101	1.47	100	4.53	90.2
4.00	2.37	103	2.51	101	2.78	105	2.25	102	2.29	102

to modafinil sulphone. The following regression equations and correlation coefficients were obtained: (+)-modafinil ($y = -14656.2 + 273435.0x$) − $r = 0.99988$; (−)-modafinil ($y = -8740.7 + 270655.1x$) − $r = 0.99994$; modafinic acid ($y = -2585.8 + 100835.9x$) − $r = 0.99984$; modafinil sulphone ($y = -17531.8 + 109071.2x$) − $r = 0.99670$. The linear range of the calibrations curves meets the concentrations reported for quantification of the analytes at the therapeutic 400 mg dose administration. Gorman reports [24] that the concentrations found in a subjected that was orally administered 400 mg of modafinil were: modafinil, 3.09 μg ml^{-1}; (±)-modafinic acid, 0.495 μg ml^{-1}; and modafinil sulphone, 1.24 μg ml^{-1}. This paper shows also that at C_{max} the modafinil total concentration is not superior to 10 μg ml^{-1}. The linear range of the concentrations curves meets also the required range reported for the concentrations found for each enantiomer at the therapeutic oral dose of 200 mg of modafinil [8].

The intra- and inter-day precision and accuracy of the method were determined by analyzing five replicates of QC human plasma samples representing the entire range of the calibration curves (low, medium and high concentrations) on 3 non-consecutive days. The results of precision are expressed as coefficients of variation and the accuracies were evaluated by back-calculation and expressed as the percent of deviation, between found and added concentrations for each of the four analytes (Table 2). The extraction and transfer efficiencies were excellent for both enantiomers analyzed and for the two metabolites at the three quality control levels and are given on Table 2. Two blinded samples containing unknown concentrations of the four analytes to the analyst produced accuracies in accordance with the acceptance criteria of the method (Table 3).

The limit of quantification were 0.100 μg ml^{-1} for (+)-modafinil, 0.150 μg ml^{-1} (−)-modafinil and modafinic acid and 0.500 μg ml^{-1} for modafinil sulphone. The limit of detection were 0.050 μg ml^{-1} for (+)-modafinil and modafinic acid while (−)-modafinil and modafinil sulphone had 0.080 μg ml^{-1}.

The stability procedures aimed to evaluate all possible conditions that samples might suffer during collection, handling and analysis.

Stock solutions were stable for 2 months when stored a −20 °C. The comparison of freshly prepared solution with the 2 months stored solutions showed no evidence of analytes degradation.

Under all conditions studied for the plasma samples, modafinil and the metabolites proved to be stable. The results of the stability of three freeze-thaw cycles and long term storage are shown on Table 4.

The lifetime and stability of the RAM BSA and chiral columns proved to be very good. The quality of the performance of both columns was maintained with over 280 plasma injections of 100 μl each. The adsorption of proteinaceous residues on to the stainless steel-frit increased the back pressure of the RAM column during the method development, but a simple cleaning step of the frit decreased the back pressure and restored the column to its original performance level.

4. Conclusions

An automated method for the determination of modafinil enantiomers and their metabolites in human plasma by bidimensional HPLC was developed with success.

The method is fast requiring a total analysis time of 30 min per sample, with no time involved for sample preparation. It is the first method described for the analysis in a single run of the enantiomers of modafinil and its two major metabolites.

Good linearity, precision, accuracy, sensitivity and selectivity were obtained with this method, which can be used for pharmacokinetic studies.

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