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Short communication

LC–ESI-MS determination and pharmacokinetics of adrafinil in rats

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ABSTRACT

A highly sensitive and specific liquid chromatography/tandem mass spectrometric (LC–MS/MS) method for investigating the pharmacokinetics of adrafinil in rats was developed. Rat serum pretreated by solid-phase extraction (SPE) was analyzed by LC–MS/MS with an electrospray ionization (ESI) interface. The mobile phase consisted of acetonitrile:water:acetic acid (35:65:0.1, v/v/v) in an isocratic elution mode pumped at 1.0 ml/min. The analytical column (250 mm × 4.6 mm i.d.) was packed with Kromasil C_{18} material (5.0 μm). The standard curve was linear from 16.5 to 5000 ng/ml. The assay was specific, accurate (R.S.D. < 2.6%), precise and reproducible (within- and between-day precisions R.S.D. <7.0% and <9.0%, respectively). Adrafinil in rat serum was stable over three freeze–thaw cycles at ambient temperature for 6 h. The method had a lower limit of quantitation of 16.5 ng/ml, which offered high sensitivity for the determination of adrafinil in serum. The method was successfully applied to pharmacokinetic studies of adrafinil after an oral administration to rats.

1. Introduction

Adrafinil (diphenylmethyl)sulfinyl-2 acetohydroxamic acid is one of the psycho stimulants having behavioral activating effects with no adverse side effects, viz. stereotypy and anxiogenesis [1]. It was found to be effective in treating the problems of vigilance, attention, concentration, learning, memory, affective troubles and depressive manifestations [2]. It is generally metabolized to an active form called modafinil (II). Both adrafinil and modafinil serve as alpha-1 adrenergic agonists [3] and increase locomotor activity in mice, rats, monkeys, and dogs [4]. The increase in activity is dose dependent [5,6]. It is important to determine the levels of adrafinil in biological fluids not only for drug development but also for therapeutic monitoring.

A thorough literature search revealed that analytical methods for determination and pharmacokinetics of adrafinil (I) in rat serum were not reported previously. Although, a few analytical methods for the quantification of modafinil (II) in biological samples were available [7–11], but none of them enables the determination of adrafinil (I), modafinil (II) and their acid metabolite (III) in a single chromatographic run. In this paper, for the first time, a simple, rapid, specific, and sensitive HPLC–MS/MS method was developed to determine adrafinil (I) and its two metabolites in rat serum after solid-phase extraction to study its pharmacokinetics in rats. The present method has the advantages of high selectivity, sensitivity, and accuracy. After validation, the proposed method was successfully applied to a pharmacokinetic study of adrafinil following an oral administration of 20 mg/kg adrafinil to rats.

2. Experimental

2.1. Chemicals and reagents

All the reagents were of analytical-grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC-grade acetonitrile (Qualigens Fine-chem. Mumbai, India) and acetic acid (S.D. Fine-chem. Mumbai, India) were used. Adrafinil was purchased from Sigma–Aldrich, USA. II (modafinil) and III (modafinil acid) were synthesized according to reported procedures [12]. Control serum used for calibration curve and validation of the assay was obtained from Wistar rats (Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad, India).

2.2. Animals

Six Wistar rats (200–220 g) were used in the present study. The rats were housed under standard conditions and had ad libitum access to water and standard laboratory diet throughout the experiments. After a single dose by oral administration of 20 mg/kg adrafinil to healthy Wistar rats ($n = 6$), blood samples (1 ml) were

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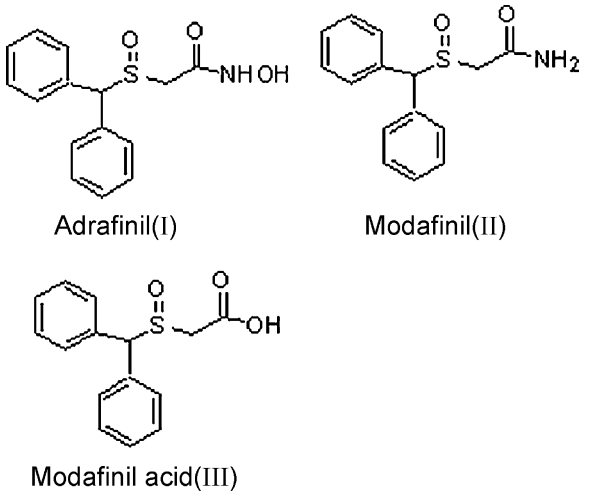

Fig. 1. Chemical structures of adrafinil (I), modafinil (II) and modafinil acid (III).

collected for the determination of adrafinil (I) concentrations. Serial blood samples were collected into the processed test tube at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h post-dose. Serum was separated by centrifugation at 4500 × *g* for 5 min and stored frozen at −20 °C. Specimens were thawed and allowed to reach room temperature, and the concentrations of adrafinil (I), II and III were determined from the calibration curve on the same day. Statistical analysis was performed using Microsoft Excel 2000 while pharmacokinetic software, 'Ramkin', based on non-compartment model was used to calculate the [AUC] from the serum drug concentration vs. time profiles [13].

2.3. Liquid chromatography–ESI-tandem spectrometry (LC–ESI-MS/MS)

Liquid chromatographic separation and mass spectrometric detection were performed using a Finnigan Surveyor LC Pump Plus, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor PDA Plus detector and LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an ESI source. The chromatographic separation was achieved on a Kromasil C_{18} (250 mm × 4.6 mm i.d.; particle size 5 μm) analytical column at a temperature of 30 °C. The final mobile phase comprised acetonitrile:water:acetic acid (35:65:0.1, v/v/v). The flow rate was 1.0 ml/min, the total run time was 20 min, the column was maintained at a temperature of 30 °C and the autosampler temperature was 20 °C. The data acquisition was under the control of a Xcalibur software (Thermo Electron Corporation, USA). The mass spectrometer was operated in positive ion mode. Spray voltage was optimized at 5 kV, transfer capillary temperature at 300 °C, sheath gas and auxiliary gas (nitrogen) pressure at 30 and 8 arbitrary units (set by the LCQ software, Thermo Electron Corporation), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr and collision energies used were 20–40 eV throughout the experimental work.

2.4. Preparation of stock, working solution

The stock solutions of I, II and III were prepared by dissolving the analytes in methanol containing 0.05% formic acid to final concentration of 0.51 mg/ml for I, 0.50 mg/ml for II and 0.49 mg/ml for III. The role of formic acid was to generate protonated mass spectra of the analytes. For the assay of serum samples, working solutions were prepared by appropriate dilution of the stock solution with methanol. Separate solutions were prepared for the calibration curve and quality control samples. Standard solutions were obtained by serial dilutions of stock solutions with methanol. All the solutions were protected from light and stored at 4 °C. The samples were stable for at least 9 days under these conditions. The calibration and quality control serum samples were prepared by addition of standard solutions to drug-free serum in volumes not exceeding 2% of the serum. The serum samples were stored in the freezer at −20 °C and thawed at room temperature before processing of the sample.

2.5. Solid-phase extraction

The solid-phase extraction (SPE) cartridges (Oasis HLB, 6 cm^3/200 mg, Waters, USA) were washed with 1 ml of methanol followed by 1 ml of water. 300 μl of serum spiked with the working solutions of I, II and III with suitable concentrations was pipetted to the polypropylene tube and the tube was shaken briefly. The sample was applied to the SPE cartridge and subsequently washed with 1 ml of water. A clean tube was positioned below the SPE cartridge and the compounds were eluted with 1 ml of acetonitrile. The eluent was transferred to an autosampler vial. 20 μl were injected into the chromatographic system. Same procedure was used for method validation and pharmacokinetic studies.

2.6. Calibration curves

The calibration curve was constructed by varying the initial concentration (IC) from 16.5 (I), 21.0 (II) and 25.4 (III) to final 5000 ng/ml to encompass the expected concentrations of measured samples. The concentrations of individual samples were IC, 30, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using the Microsoft Excel 2000 software. The suitability of the calibration model was confirmed by back calculating the concentrations of the calibration standards.

Table 1
ESI–LC/MS full-scan and CID product ion spectra for adrafinil (I) and its two metabolites (II and III)

Compound	Collision energy (V)	Mass to charge ratio	
		Precursor ions (*m*/*z*)	Product ions (*m*/*z*)
Adrafinil (I)	30	290 (32%) 233 (10%)	233 (100%), 177 (55%) 177 (100%)
II	20	274 (20%) 167 (10%)	167 (100%) 166 (35%), 165 (100%), 152 (80%)
III	28	275 (15%) 167 (14%)	167 (100%) 166 (30%), 165 (100%), 152 (91%)

('-'%): relative abundance.

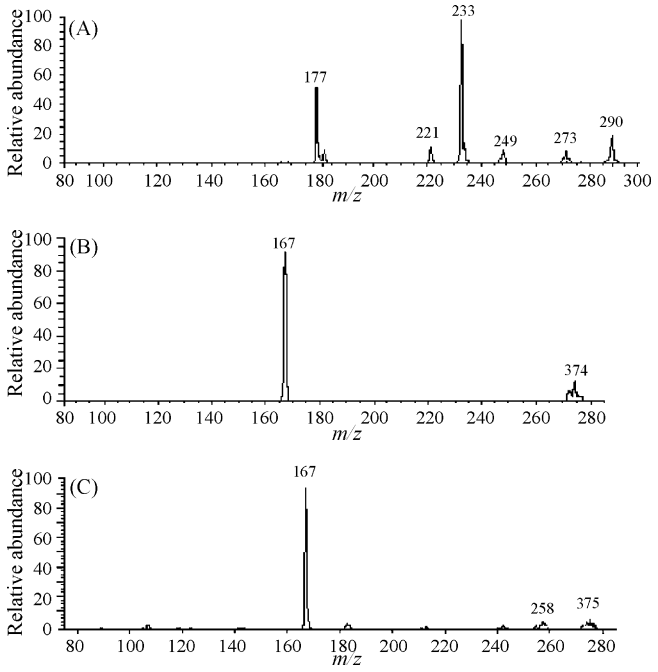

Fig. 2. Mass spectra of (A) adrafinil (I); (B) modafinil (II); (C) modafinil acid (III) (MS conditions: positive ESI mode; ESI probe temperature 300 °C; capillary voltage 5 kV).

3. Results and discussion

3.1. LC–MS/MS conditions

The present study was aimed at developing a chromatographic system capable of eluting and resolving adrafinil and its two metabolites in rat serum. The chemical structures of adrafinil (I), modafinil (II) and modafinil acid (III) are shown in Fig. 1. All the three compounds were subjected to separation by reverse-phase HPLC on a Kromasil C_{18} column using acetonitrile:water:acetic acid (35:65:0.1, v/v/v) as a mobile phase at 30 °C. The tandem mass spectrometer was used in a positive ESI MS mode. The mass spectra and the proposed fragmentation patterns of I, II and III are given in Fig. 2 and Table 1, respectively.

3.2. Validation

A full validation based on the FDA [14] guideline for bioanalytical method validation was required, due to the fact that this method was developed for the first time, and therefore performed.

3.2.1. Calibration curve and lower limit of quantitation

Adrafinil and its metabolites were added to rat serum. After sample clean-up by solid-phase extraction, the extracts were injected into the chromatographic system. The analysis was carried out at decreasing concentrations ranging from 50 to 1.0 ng/ml to determine the minimal concentration with a signal-to-noise ratio of 3:1. The LOD and LOQ values for the samples I, II and III prepared in methanol were found to be 3.5, 5.0, 6.2 and 11.6, 16.5, 20.5 ng/ml, respectively. The serum spiked LOD and LOQ values obtained experimentally for the three compounds I, II, III are given in Table 2. Calibration standards and blanks were prepared and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The calibration curves were constructed and found to be linear having correlations coefficient $r^2 > 0.998$. The results are listed in Table 2.

Table 3
Precision and accuracy of HPLC method in determining the concentration of adrafinil (I), II and III in rat serum

Concentration (μg/ml)		R.S.D. (%)		RE (%)
Theoretical	Calculated	Intraday	Interday	
Adrafinil (I)				
0.50	0.48	6.5	8.4	4.0
5.00	5.16	4.3	7.0	3.2
50.0	49.60	3.8	6.5	0.8
II				
0.50	0.52	4.8	6.9	4.0
5.00	4.92	5.2	6.2	1.6
50.0	50.12	3.7	6.2	0.2
III				
0.50	0.49	5.4	7.2	2.0
5.00	5.31	4.9	6.7	6.2
50.0	51.01	2.9	4.8	2.0

R.S.D.: relative standard deviation; RE: relative error ($n = 6$).

3.2.2. Accuracy and precision

The accuracy and precision of the assay were determined by analyzing samples of adrafinil at the LOQ and QC concentrations in a minimum of 5 replicates in 3 analytical runs together with an independently prepared, triplicate calibration curve. The assay precision was obtained for each test concentration using the coefficient of variation of the measured concentration. Intra-assay precision was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The precision was at most 11.5% and the bias did not exceed 6.5% at all levels. Inter-assay precision and accuracy were evaluated by processing a set of calibration and quality control samples (three levels analyzed twice, results averaged for statistical evaluation) on six separate runs. The samples were prepared in advance and stored

Table 4
The extraction recoveries of I, II and III in rat serum by LC–MS/MS method

Compound	Added concentration (μg/ml)	Extract recovery (%)	R.S.D. (%)
I	50.0	95.10	3.2
	20.0	94.62	4.1
	5.0	92.40	6.7
II	50.0	92.10	4.5
	20.0	91.20	5.0
	5.0	88.40	6.9
III	50.0	90.00	5.2
	20.0	89.82	6.7
	5.0	87.50	8.3

R.S.D.: relative standard deviation ($n = 6$).

Table 2
Calibration curves for adrafinil (I), modafinil (II) and modafinil acid (III) in rat serum

Compound	Regression equation	r^2	Sy,x	LOD (ng/ml)	LOQ (ng/ml)
I	$y = 29.297x - 565.73$	0.9997	502.90	5.9	16.5
II	$y = 33.295x + 53.884$	0.9998	314.03	7.0	21.0
III	$y = 19.791x - 526.33$	0.9982	671.03	8.5	25.4

r^2: coefficient of determination; Sy,x: standard error of estimate.

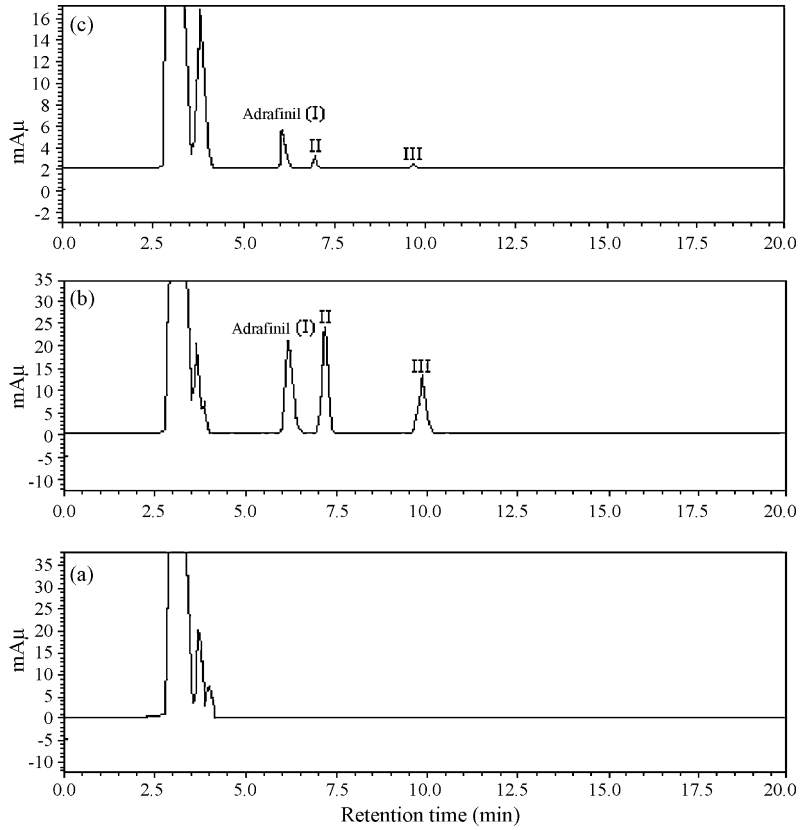

Fig. 3. Chromatograms of (a) blank rat serum; (b) rat serum sample spiked with 5000 ng/ml of adrafinil (I), II and III; (c) rat serum sample after 4 h following a 20 mg/kg oral dose of adrafinil.

at −20 °C. The precision was better than 9% and the inaccuracy did not exceed 8.4% at all levels. The respective data are given in Table 3.

3.2.3. Retention times and specificity

High specificity was found in single ion monitoring mode for the determination of adrafinil in rat serum samples. The typical LC chromatograms of blank serum and spiked serum samples are depicted in Fig. 3. The retention times of adrafinil (I), its two metabolites II and III were about 6.30, 7.25 and 9.90 min, respectively. The chromatogram of an extracted serum sample drawn after oral administration is also shown in Fig. 3. To investigate whether endogenous matrix constituents interfered with the assay, four

Table 5
Stability data of adrafinil in rat serum

Conditions	Matrix	Nominal concentration (ng/ml)	Dev. (%)	CV (%)
Ambient (6 h)	QC samples	50	3.35	1.81
		500	3.04	1.69
	Serum	50	−5.35	3.22
		500	−3.58	2.86
	SPE eluate	50	7.58	4.20
		500	5.41	3.52
3 freeze (−20 °C)–thaw cycles	QC samples	50	6.21	3.32
		500	4.04	2.75
	Serum	50	−6.35	4.12
		500	−4.81	3.16
	SPE eluate	50	6.68	4.32
		500	6.23	3.43
2–10 °C, 3 days	QC samples	50	3.02	2.35
		500	4.25	2.85
	Serum	50	7.35	4.02
		500	4.58	3.74
	SPE eluate	50	5.52	3.45
		500	4.87	2.95
Autosampler stability (24 h)	QC samples	50	3.15	1.67
		500	3.45	1.78
	Serum	50	6.85	4.32
		500	5.25	3.66
	SPE eluate	50	5.58	3.20
		500	4.60	2.98

Dev.: deviation; CV: coefficient of variation; QC: quality control; SPE: solid-phase extraction ($n = 3$).

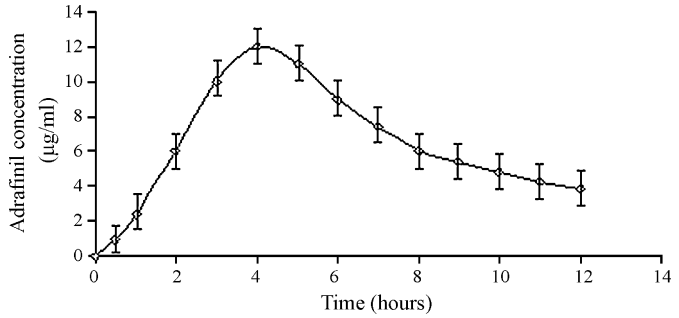

Fig. 4. Concentration vs. time profiles over 12 h of adrafinil (I) in serum of rat ($n = 6$) receiving a single 20 mg/kg dose of adrafinil.

Table 6
Pharmacokinetic parameters for the adrafinil in rat serum ($n = 5$) after oral administration

Parameter	Value
t_{max} (h)	4.0
C_{max} (μg/ml)	60
AUC (μg/ml/h)	402.91
$t_{1/2}$ (h)	4.95

C_{max} (μg/ml): maximum serum concentration; t_{max}: time to C_{max}; AUC: area under serum concentration–time curve; $t_{1/2}$: half-life.

individual batches of control, drug-free rat serum were processed and analyzed according to the described procedures. Responses of adrafinil at the LOQ concentration were compared with the response in the blank samples which were 16.5 and 11.6 ng/ml, respectively.

3.2.4. Extraction recovery

The extraction recoveries of adrafinil and its metabolites from rat serum were determined by comparing peak areas from serum samples spiked before extraction with the corresponding standard solutions without extraction. The results (Table 4) showed that extraction recoveries of I, II and III from rat serum, determined at three concentrations, were ranging from 87.50% to 95.10% ($n = 6$), respectively.

3.2.5. Stability

The stability of adrafinil was investigated in the stock, working solutions, serum and extracts during storage and processing. The results are presented in Table 5. In rat serum the stability of adrafinil was examined during storage and after three freeze–thaw cycles at −20 °C with a minimal interval of 24 h. The quality control samples that had been frozen and thawed three times were compared with freshly prepared quality control samples and found stable. Adrafinil was stable in rat serum during processing for 6 h at room temperature and in the final SPE eluate for 3 days at 2–10 °C. The reinjection reproducibility after storage of the samples in glass vials in the autosampler for 24 h was also determined. Stability experiments were performed on at least two concentrations (50 and 500 ng/ml) in triplicate. Adrafinil was considered to be stable in stock and working solutions having the mean recoveries 98–102% of the original concentration. Whereas in biological matrices recoveries were 88–102% of the initial concentration.

3.3. Application of analytical method in pharmacokinetic studies

The utility of the validated HPLC method was demonstrated in the in vivo conditions. Each rat was administered an oral dose of 20 mg/kg of adrafinil. Blood samples were collected at a regular time intervals. The samples were centrifuged and the separated serum samples were frozen at −20 °C. Serum concentration–time profile of adrafinil in rats was plotted (Fig. 4). The pharmacokinetic parameters of adrafinil are listed in Table 6. Adrafinil was absorbed rapidly with peak concentration at around 4 h. It could be seen that the concentrations of adrafinil in rat serum were detectable for at least 7 h after the oral administration.

4. Conclusion

A highly sensitive and specific method for the determination of adrafinil and its two metabolites was developed using high-performance liquid chromatographic separation with tandem mass spectrometric detection. It was shown to be sensitive and rapid for the simultaneous determination of adrafinil (I), modafinil (II) and acid metabolite (III) in rat serum. The analysis can be completed in 12 min. Under optimized conditions, the method can detect 5.9 ng/ml of adrafinil, 7 ng/ml of II and 8.5 ng/ml of III. The extraction recoveries at three different concentrations (low, medium and high) of the method were 93.5% for adrafinil, 90.2 for II and 88.8 for III. The method is rapid and practically applicable to the pharmacokinetic studies of adrafinil in rats.

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