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„Simultaneous Quantification of Modafinil, its main metabolite Modafinil acid and the novel analog CE-123 in rat plasma by LC-HRMS“

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TABLE OF CONTENTS

1. Zusammenfassung/Abstract

1.1. Zusammenfassung

Dopamin-Wiederaufnahmehemmer, wie Modafinil und seine Analoga, sind nützlich bei der Behandlung von Narkolepsie**, „**excessive shift work sleep disorder“, obstruktiven Schlafapnoe-Syndrom, Aufmerksamkeitsdefizit-/Hyperaktivitätsstörung, Depression, Schizophrenie sowie Kokain- und Amphetaminabhängigkeit. CE-123 ist ein vielversprechendes Analog von Modafinil, welches intensiv als potentieller Arzneistoff getestet wird. Obwohl CE-123 sowohl präklinische Effektivität ohne neurologische Nebenwirkungen demonstriert hat, als auch eine höhere Spezifität für Dopamin-Transporter besitzt, ist eine detaillierte Charakterisierung seiner pharmakokinetischen Eigenschaften wichtig. Während der chromatographischen Analyse der Standardlösungen – welche *R*-Modafinil und CE-123 beinhalten sowie von der Uppsala Universität vorbereitet wurde – wurde der Hauptmetabolit von *R*-Modafinil, Modafinilsäure, entdeckt. Dadurch war es notwendig eine Methode, um die Metabolisierung von *R*-Modafinil in Rattenblutplasma zu verhindern sowie eine LC-HRMS Methode für die simultane Quantifizierung von Modafinil. seinem Hauptmetabolit Modafinilsäure und seinem neuen Analog CE-123 in Rattenblutplasma zu entwickeln. Die Probenvorbereitung bestand aus Proteinfällung mit eisgekühltem Acetonitril, welches 0,2 % Ameisensäure beinhaltet hat. In dieser Arbeit wurde eine konstante Menge von CE-114, einem weiteren Modafinilderivat, als interner Standard verwendet. Die Trennung wurde an einer Kinetex Phenyl-Hexyl (50 x 2,1 mm, 2,6 µm) Säule durch eine mehrschrittige, zusammengesetzt lineare Gradientenelution durchgeführt. Als mobile Phase wurde Wasser mit 0,1 % Ameisensäure und Acetonitril bei einer Durchflussrate von 0,6 mL/min verwendet. Die Bestimmung wurde mit positiver Elektrospray Ionisierung im Bereich von *m/z* 50–2500 optimiert und durchgeführt. Lineare Kalibriergeraden für Modafinil und CE-123 wurden in einem Konzentrationsbereich von 10 – 2500 ng/mL und für Modafinilsäure im Bereich von 25 – 200 ng/mL erstellt. Die Methode wurde verwendet, um präklinische pharmakokinetische Studien in Rattenplasma zu untermauern.

1.2. Abstract

Dopamine transport blockers such as modafinil and its analogs are considered to be useful for treating narcolepsy, excessive shift work sleep disorder, obstructive sleep apnea/hypopnea syndrome, attention deficit hyperactivity disorder, depression, schizophrenia, and addiction to cocaine and amphetamines. CE-123 is the most promising analog of modafinil, which is intensively tested as a potential drug. While CE-123 has shown preclinical effectiveness without neurological adverse side effects, and has a higher specificity for the dopamine transporter, it is important to provide a detailed characterization of pharmacokinetic properties. During the chromatographic analysis of standard solutions containing *R*-modafinil and *S*-CE-123 prepared by the Uppsala University, the main metabolite of *R*-modafinil, modafinil acid, was detected. Thus it was required to develop a method to inhibit a metabolization of *R*-modafinil in rat blood plasma and an LC-HRMS assay for simultaneous quantification of modafinil, its main metabolite modafinil acid and the novel analog CE-123 in rat plasma. Sample preparation consisted of protein precipitation with ice-cold acetonitrile containing 0.2 % formic acid. In this study a constant amount of another modafinil analog, CE-114, was applied as an internal standard. Separation was performed on a Kinetex Phenyl-Hexyl (50 x 2.1 mm, 2.6 µm) column by a multiple steep steps linear gradient elution with mobile phase consisting of water containing 0.1 % formic acid and acetonitrile, pumped at a flow rate of 0.6 mL/min. The determination was optimized and carried out with positive electrospray ionization in the range of *m/z* 50–2500. Linear calibration curves for modafinil, CE-123 and modafinil acid were obtained in the concentration range of 10 – 2500 ng/mL for modafinil as well as CE-123 and 25 – 200 ng/mL for modafinil acid, respectively. The method was utilized to support preclinical pharmacokinetic studies in rat plasma.

2. Introduction

2.1. Modafinil and its analogs

2.1.1. Modafinil

Modafinil, (±)-[2-(diphenylmethyl)-sulfinyl] acetamide (**Figure 1)**, is a synthetic molecule approved in the EU and USA for the treatment of narcolepsy and has been demonstrated to be effective and well-tolerated [1]. Modafinil is also recommended for patients suffering from excessive shift work sleep disorder, obstructive sleep apnea/hypopnea syndrome, attention deficit hyperactivity disorder (ADHD), depression, schizophrenia, and addiction to cocaine and amphetamines [2].

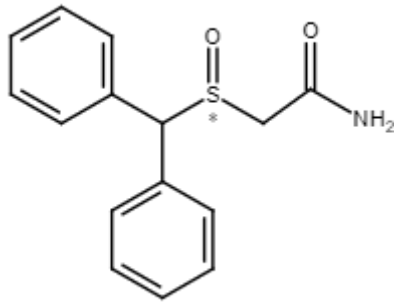

Figure 1: The structure of modafinil with a molecular weight (MW) of 273.35 g/mol and a sum formula of $C_{15}H_{15}NO_2S$.

In Austria modafinil is sold with a valid doctor prescription under the brand name Modasomil® or Provigil® [3, 4]. Modafinil is a mild, wake-promoting stimulant that is pharmacologically distinct from other central nervous system stimulants [5]. Modafinil is classified as a mild psychostimulant because it is less potent and efficacious than other stimulants (e.g., amphetamine, methamphetamine and cocaine) [6].

Despite numerous clinical uses of modafinil, its mechanism of action in the brain was for a long period of time unclear: modafinil interacts with multiple targets in the central nervous system — dopamine (DA), norepinephrine, serotonin and orexin receptors — associated with modafinil‘s clinical and behavioral effects [5].

According to Zolkowska *et al*. [7] modafinil displayed measurable potency only at dopamine transporters (DAT), inhibiting [^{3}H] DA uptake, with an IC_{50} value of 4.0 µM. This finding was later supported by a number of studies [5, 6, 9, 18] demonstrating that

the pharmacological target is the DAT, which is inhibited by modafinil with mediocre affinity.

Using *in vitro* binding assays, Shanmugasundaram *et al*. [8] have determined that the IC_{50} values for modafinil was 11.11 µM for DAT. The serotonin transporter (SERT) and the noradrenaline transporter (NET) were blocked with even lower affinities, with IC_{50} values of 1547 µM and 182 µM, respectively. However, the mechanism of action of modafinil is based on an increase of the dopamine concentration in the synaptic cleft.

Modafinil, by definition, comprises the *R*-(−) – and *S*-(+) – enantiomers [9]. It is known that for chiral compounds, each enantiomer possesses its own chemical and pharmacological behavior. *R*-Modafinil possesses about three times higher affinity than its *S*-enantiomer at blocking DA uptake at the DAT. *R*-Modafinil is also metabolically more stable and has higher plasma concentrations in comparison to the *S*-enantiomer [8]. As a result, the interval between doses can be enhanced and therefore the compliance increases.

In plasma (*in vitro*) modafinil is moderately bound to proteins; approximately 60% to mainly albumin. Only two metabolites reached significant concentrations in plasma – modafinil acid and modafinil sulfone (**Figure 2**), which have no pharmacological effect. The major route of elimination is metabolism primarily by the liver with subsequent renal elimination of its metabolites [10].

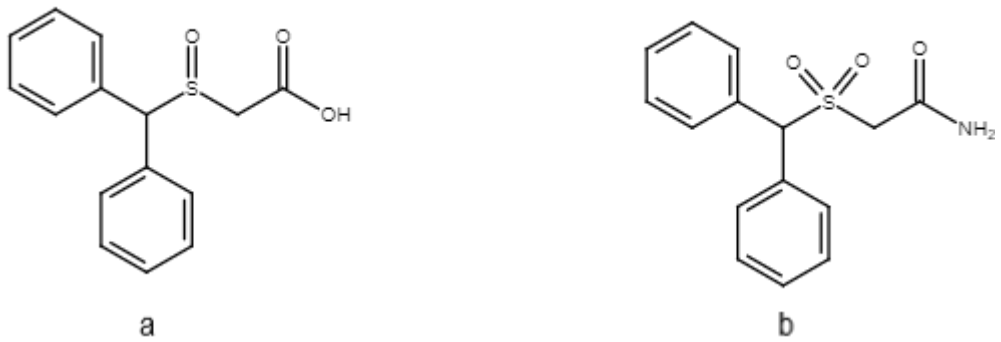

Figure 2 : Chemical structures of modafinil acid (a) with a MW of 274.33 g/mol and a sum formula of $C_{15}H_{14}O_3S$ and modafinil sulfone (b) with a MW of 289.40 g/mol and a sum formula of $C_{15}H_{15}NO_3S$.

2.1.2. Pharmacokinetic profile of modafinil

Modafinil is a unique wake-promoting agent that is clinically and pharmacologically distinct from central nervous system (CNS) stimulants [11]. The clinical and nonclinical pharmacokinetic profile of modafinil was previously described by Wong *et al.* [12]. In 1999, healthy young males and females, as well as healthy elderly males, participated in an open-label, single-center, single-dose, parallel-group study. Fasted (overnight) patients received single oral doses of 200mg modafinil and then, blood and urine samples were collected at various times up to 72 hours post dose to examine the influence of age and gender on the pharmacokinetics of modafinil. Modafinil was rapidly absorbed after oral dosing and slowly cleared ($t_{1/2}$ ~ 11-14 hours) from the body. Two major metabolites of modafinil, modafinil acid and modafinil sulfone, were also detected in plasma and urine samples. The total amount of modafinil acid detected in the plasma was similar between young males and females. However, a significantly higher amount of the dose was excreted in urine as modafinil acid in young males (51%) compared to females (35%) [12]. The modafinil acid has been reported to be further metabolized to modafinil sulfone, presumably mediated by CYP3A4 [13]. Modafinil sulfone were near the lower limit of quantification (LLQ; 0.1 mg/L) in this single-dose study. There were certain age and gender effects on modafinil clearance processes. The clearance rate of modafinil in males decreased slightly with age. Young females cleared modafinil at a slightly faster rate than young males [12].

The pharmacokinetics of a single dose and multiple doses are similar [14]. The pharmacokinetics of modafinil, *S*-modafinil and *R*-modafinil were studied by Wong *et al.* [15] after treating of 32 healthy male volunteers. Multiple doses of modafinil (200, 400, 600 or 800 mg/day for 7 days) were given randomly to patients. The 800 mg/day dose was discontinued after 3 days because of the development of clinically relevant cardiovascular changes (intolerable hypertension as well as tachycardia). In a study of pharmacokinetics in young males is demonstrated that maximum plasma concentration (C_{max}), area under the plasma concentration-time curve from time zero to infinity (AUC_{∞}) and area under the plasma concentration-time curve for one dose administration interval (AUC_{0-t}) for modafinil and its enantiomers are dose dependent. The pharmacokinetics of modafinil, *R*-modafinil and *S*-modafinil at the steady state were characterized by a t_{max} of 1.7 to 2.7 hours, 1.8 to 2.5 hours and 2.0 to 2.9 hours, respectively, and were similar. The $t_{½}$ of modafinil and *S*-modafinil

is 14-17 hours and 13-16 hours, respectively. The *R*-modafinil was eliminated faster than *S*-modafinil and the $t_{½}$ of *R*-modafinil is around 4-5 hours. The approximate volume of distribution (V_d) of modafinil is 0.8 L/kg, which is larger than total body water, suggesting that it is able to penetrate into tissues. The approximate V_d for both *R*-modafinil and *S*-modafinil is 0.5 L/kg. The renal clearance accounted for approximately 5% of the plasma clearance. During the course of the sample analysis, additional peaks of metabolites, modafinil acid and modafinil sulfone, appeared in HPLC chromatograms, which are excreted by the kidneys [15].

Furthermore, another study [13] reported that the absorption of modafinil by patients with hepatic impairment is delayed, causing a slight increase in t_{max}. The C_{max} and AUC are also increased after acute and chronic administration, the C_{max} of modafinil acid is decreased and the $t_{½}$ of modafinil is doubled. The drug dose should be halved in patients with renal as well as hepatic failure. In severe renal impairment (creatinine clearance 16.6 ± 0.7 mL/min), plasma modafinil concentrations are increased slightly but plasma concentrations of modafinil acid (due to impaired renal clearance) are considerably higher (C_{max}= 5.4 vs 2 mg/mL). The safety of such modafinil acid concentration is unknown [13].

2.1.3. Modafinil analogs

Kalaba [16] developed a series of novel modafinil analogs with higher affinity for the DAT than the parent compound. Several modafinil analogs demonstrate a higher selectivity for DAT *versus* NET/SERT and are not causing efflux of dopamine (an amphetamine-like effect). Furthermore, they demonstrate no general *in vivo* neurotoxicity that may render it suitable for further preclinical development.

According to Saroja *et al.* [17] the modafinil analog 4-(diphenyl-methanesulfinylmethyl)-2-methyl-thiazole (code: CE-111) selectively inhibited DAT-mediated rather than NET- or SERT-mediated dopamine uptake. It also improves memory performance of rats in the radial arm maze (RAM).

S-CE-123 displayed much higher specificity for DAT in male rats in an attentional set-shifting task (ASST) that proves for cognitive flexibility and a 5-choice serial-reaction time task (5-CSRTT) assessing visuospatial attention and impulsivity [18].

Table 1 shows the IC_{50} values (half maximal inhibitory concentration – the efficacy of a comp inhibiting a target or a specific biological/biochemical process by half) of chosen modafinil analogs. According to the Food and Drug Administration (FDA), IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro* [19].

Table 1: Data for uptake inhibition at the monoamine transporters for modafinil and it is analogs (DAT, NET, and SERT) (n=3, mean ± SD) [8, 16-18].

Compound	IC_{50} (μM) ± SD		
	DAT	**NET**	**SERT**
***R*-modafinil**	11.11 μM	1547.00 μM	182.30 μM
CE-111	3.25 μM ± 0.23	174.00 μM ± 15.50	272291.00 μM ± 3453.00
***S*-CE-123**	2.70 μM ± 0.30	137.70 μM ± 15.50	NA
CE-114	69.10μM ± 4.10	30.00 μM ± 8.00	NA

* NA: not active

2.1.4. CE-123

The modafinil analog, 5-((benzhydrylsulfinyl) methyl) thiazole (code: CE-123), is the most promising analog, which is intensively tested as a potential drug. In comparison to the patent compound, CE-123 is an apparently very selective DAT inhibitor with negligible binding to NET and SERT. The highly selective inhibition of the DAT may be the reason for absence of neurological adverse side effects [20].

CE-123 has a thiazole functional group attached on position 5 *via* a methylene bridge to the sulfoxide moiety (**Figure 3**) instead of carboxyl-amide group on position 5 in modafinil. A substitution of the carboxyl-amide group with the thiazole group could result in increased metabolic stability of the compound [20].

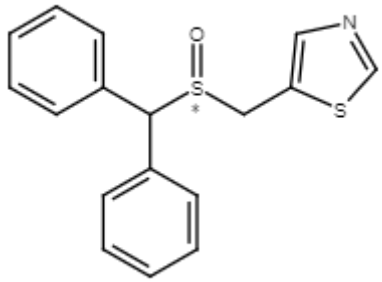

Figure 3: The structure of CE-123 with a MW of 314.07 g/mol and a sum formula of $C_{17}H_{16}NOS_2$.

In vivo studies provided by Kristofova *et al.* [20] indicated that the elimination rate constant is significantly higher for CE-123 than for *R*-modafinil. Sprague Dawley rats were treated with CE-123 or *R*-modafinil at a dose of 10 mg/kg. Plasma, cerebrospinal fluid (CSF) and brain samples were taken at 15 min, 1 h and 7 h after the dose and measured *via* HPLC-MS. The results of measurements are demonstrated in **Table 2**.

Table 2: Plasma, CSF and brain levels of CE-123 and *R*-modafinil in Sprague Dawley rats after a single intraperitoneal administration of 10 mg/kg (n=3, mean ± SD) [20].

Time, min	Plasma (µg/g)		CSF (µg/g)		Brain (µg/g)	
	CE-123	*R*-modafinil	CE-123	*R*-modafinil	CE-123	*R*-modafinil
15	7.7 ± 1.1	2.3 ± 1.0	0.3 ± 0.1	0.5 ± 0.2	4.4 ± 0.5	1.1 ± 0.3
60	3.0 ± 0.9	0.2 ± 0.1	0.3 ± 0.04	0.1 ± 0.03	2.0 ± 0.4	0.1 ± 0.0
420	s.d.	0.0	s.d.	s.d.	s.d.	0.0

*s.d.: still detectable

2.1.5. CE-114

5-(Benzhydrylsulfinylmethyl)-4-isopropyl-1, 2, 3-thiadiazole (code: CE-114) is a modafinil analog and has on position 5 an isoproyl-thiadiazole substituent (**Figure 4)** instead of carboxyl-amide group on position 5 in modafinil.

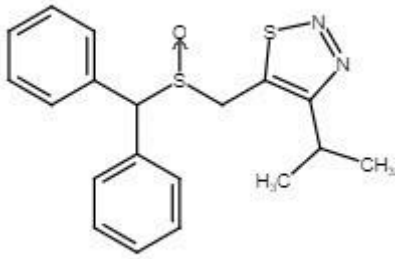

Figure 4: The structure of CE-114 with a MW of 356.50 g/mol and a sum formula of $C_{19}H_{20}NOS_2$.

In *in vitro* binding assays the IC_{50} value for CE-114 is determined and was found to be 69.10 µM ± 4.10 for DAT. Substitution of the amide group with a substituted thiadiazole group led to a decrease of DAT reuptake inhibition compared to modafinil [16].

In the present study, CE-114 was used as an internal standard (IS) for quantification of *R*-modafinil and *S*-CE-123 in rat plasma samples. When an IS is used for quantification, the standard which is usually an analog of the analyte should be added at the earliest step possible during sample preparation and extraction [21]. CE-114 shows a higher intensity and is not overlapping with the peaks of the plasma.

2.2. Liquid chromatography – high resolution mass spectrometry (LC-HRMS)

Liquid chromatography–high resolution mass spectrometry (LC-HRMS) has seen huge advances in recent years and is now a well-established analytical tool applied in the identification and quantification of drugs in plasma or other biological materials. Owing to its high selectivity and sensitivity it is used in pharmacokinetic trails in the course of development to support *in vitro* and *in vivo* studies.

2.2.1. High performance liquid chromatography (HPLC)

In the modern pharmaceutical industry, high performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development and production.

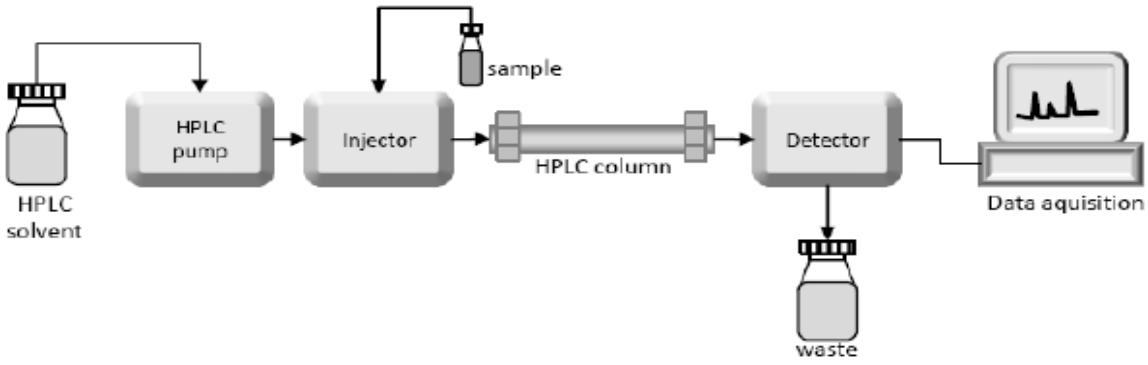

Figure 5: Schematic diagram of the HPLC system (S. Czaplicki, 2013, p.102) [22].

Typical HPLC system – described in **Figure 5** – consists of the following components:

- ***Pumping system.*** The main pumping system consists of pump(s) able to deliver a constant flow of solvent through the injector, chromatographic column, and through the detector(s). The pumps must be able to generate a high pressure, which is needed mainly to overcome the resistance to flow of the chromatographic column [23].
- ***Solvent reservoirs.*** The solvent supply provides the solvent(s), which are necessary as a mobile phase for the HPLC and it is consisted of one or more reservoirs.
- ***Injector.*** This allows adding in the mobile phase a small, precisely measured volume of a solution containing the sample. The injection must be done reproducibly and accurately.

- ***Column.*** The chromatographic column is designed for performing the separation in HPLC. Separation is based on the transport of the mobile phase with dissolved analytes through the stationary phase. The stationary phase retains stronger or weaker different molecular species and releases them separately in time, back into the mobile phase. The stationary phase used in HPLC is in the form of column packed with very small porous particles (1-5 μm in diameter). Columns used for ultra-high performance liquid chromatography (UHPLC) have an inner diameter of 1-2 mm, a length of 3-15 cm with particles size of 1.7-1.9 μm in diameter and using typical flow rates of 100-1000 μL/min [24].
- ***Detector.*** The most common detector used in pharmaceutical analysis is ultraviolet (UV). With UV detector it is possible to monitor and acquire the UV absorbance from 190 to 400 nm. Also, the registration of the absorbance at a selected wavelength or over a span of wave lengths (diode array detection) is allowed [23].
- ***Data acquisition and control system.*** All parameters of HPLC instrument are controlled by a computer-based system. Afterwards, signals are transferred to the computer, where the data is evaluated [23].

2.2.2. Mass spectrometry (MS)

Mass spectrometry (MS) is a powerful and sensitive instrumental method used in quantitative biological analysis. In combination with chromatographic separation techniques, in form of liquid chromatography, MS has become the principle method of mixture analysis in pharmaceutical research and development [25].

The basic principle of MS is to measure the mass to charge ration (*m/z*) of ions. A fundamental requirement is that atoms or molecules are ionized and analyzed as gas phase ions which characterized by their mass (*m*) and charge (*z*) [26].

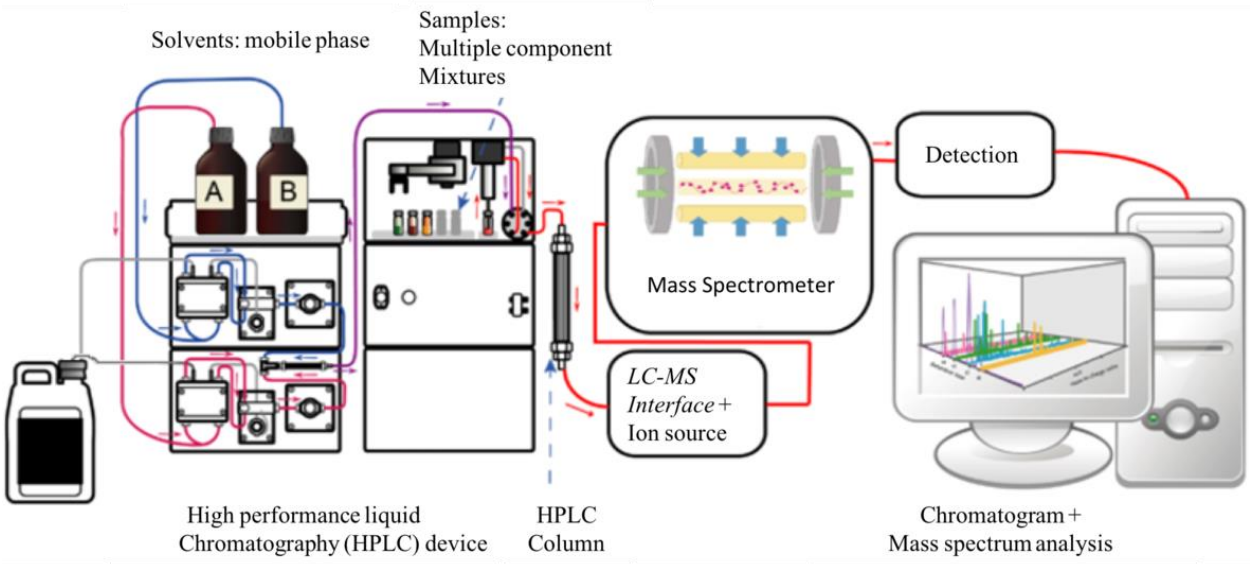

Figure 6: Schematic diagram of an LC-MS system [27].

Generally, a mass spectrometer – described in **Figure 6** – consists of the following components:

- ***Sample introduction device.*** In contrast to the mass spectrometer, the HPLC system analyzes a liquid phase and operates under high pressure, whereas mass spectrometer analyzes a gas phase and operates under high vacuum or at low pressure. For this reason a sample introduce device is needed, which couples two instruments. There are two different principles of intake systems: direct inlet and liquid interface. The direct samples inlet allows analyze solid and liquid samples, while liquid interface is suitable for liquid samples [26].

- ***Ion source.*** In mass spectrometry, the performance of the MS system is dependent in the method of ion generation. There is no single ion source for all applications. Specific ionization techniques are required depending on polarity

and molecular weight of molecule [28]. Numerous ionization methods have been developed, including electron impact (EI), chemical ionization (CI), desorption ionization (DI), matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) [23]. In this study, an ESI interface was applied, which ionizes molecules with no or only little fragmentation. ESI method is an ionization method in which mobile phase solution is introduced into a capillary and a high voltage is applied between the capillary and the opposing electrode [29]. In the ion source, positive or negative ions are produced. ESI is suitable for our study, because in positive ion mode such ion types as $[M+H]^+$, $[M+Na]^+$ or $[M+NH_4]^+$ can be formed [30].

- ***Mass analyzer*** is the component of the mass spectrometer that measures the mass-to-charge ratios of ions and provides a means of separating the ions. The operating principles of mass analyzers depend on interactions of charged particles with electrical or magnetic fields. Commonly used mass analyzers are quadrupole, time-of-flight (TOF), magnetic sector, electrostatic sector, and Fourier transform (FT) analyzers. For LC-HRMS measurements Qq-TOF mass analyzer was applied [26].

- ***Quadrupole mass analyzer.*** The main function of quadrupole mass analyzer is scanning of ions and allowing ion transmission. It consists of four parallel cylindrical metal rods inside a vacuum chamber. A direct current and radiofrequency are applied to the quadrupole, so that only the ions with the target m/z successfully pass through the quadrupole and get to the detector [31]. The quadrupole is small and relatively inexpensive [23].

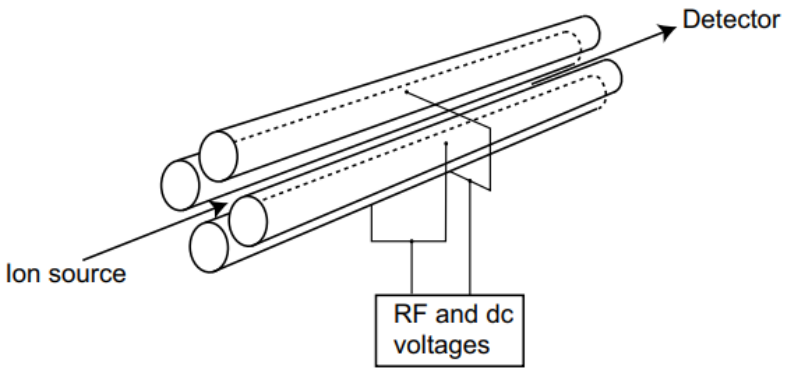

Figure 7: Quadrupole mass spectrometry (S. Hansen, 2012, p. 251) [32].

- ***Time-of-flight analyzer.*** In contrast to quadrupole analyzer, TOF is pulsed and non-scanning mass analyzer. It has a simple construction and contains an accelerator, a field-free region, a reflection and detector inside a high vacuum chamber. TOF separates and detects ions of different m/z by measuring the time taken for the ions to travel through a field-free region.

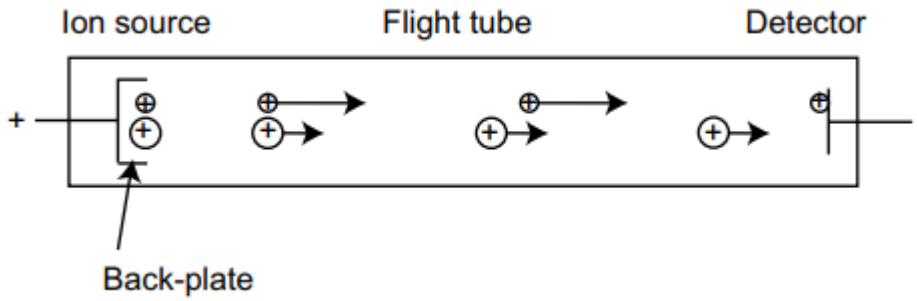

Figure 8: Time of flight mass spectrometry (S. Hansen, 2012, p. 252) [32].

- ***Detector.*** The detector converts an ion signal into an electrical signal.

2.3. Bioanalytical method

High performance liquid chromatography coupled with high resolution mass spectrometry is one of the most selective and sensitive analytical techniques in the determination of drugs in different sample matrices and hence can be used and further developed to determine modafinil and CE-123 [33].

Rat blood samples were collected and analyzed for this study. Erythrocytes, leucocytes, thrombocytes, and plasma are the four primary components of blood. Plasma is an aqueous solution containing about 8% of proteins and trace amounts of other substances such as dissolved nutrients and waste products, electrolytes, lipoprotein particles, immunoglobulins and blood clotting factors [32]. Drugs in blood exist in two forms: unbound and bound to plasma proteins and erythrocytes. Modafinil is highly lipophilic, and approximately 60% bound to plasma proteins, primarily albumin [34].

There are three reasons why samples may will need to be processed before being analyzed by LC-MS. The primary purpose of sample preparation is to isolate one or more analytes from the rest of the sample mixture's components (matrix). If co-components of the matrix are not removed prior to analysis, it can influence the quantitation of target analyte(s) during LC-MS experiments [35]. Removing these matric components allows to avoid clogging the chromatography column and prevents damage to the column and the build-up of excessive pressure within the LC system. Secondly, an improvement of chromatographic performance. The quantitation limits, selectivity and robustness of the assay can be modified by the volume, pH, organic solvent, buffer and aqueous composition of the liquid injected into the LC system. To solve these issues, complex biofluids often need to be exchanged for an injection solution compatible with the LC method prior to injection. In addition, the precision, accuracy of the method and the long-term stability of the LC-MS instrument response, is improved by selectively depleting the biological matrix to increase the analyte-to-matrix ratio [36].

Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte [37]. This effect is generally caused by exogenous (all substances introduced during sample processing and analysis) or endogenous (*e.g.* metabolites of the target analyte, proteins or lipids) compounds. There are different methods to eliminate or reduce matrix effects, for example, optimization of the sample

preparation or chromatographic separation. With proper sample preparation it is possible to strongly reduce many matrix effects [35].

Protein precipitation (PPT) is the most common technique used to remove proteins from plasma prior to LC-MS analysis. The PPT procedure is based on mixing the samples with a water-miscible solvent such as acetonitrile (ACN), methanol (MeOH), or a mixture of ACN with water in the ratio 1:3-5 (ACN: water, v: v). The organic solvents destroy the hydration layer of proteins, reduce the repulsive forces between protein molecules, and significantly lower the solubility of the proteins, resulting in proteins precipitating [38]. Before research analysis, the precipitate should be removed by centrifugation or filtration. According to Zhao *et al.* [38] using ACN in a ratio of 3:1 (ACN: plasma, v:v) for PPT can provide a clear supernatant compared to methanol. In our research work ACN was applied to all samples to achieve efficient protein removal.

There are three methods commonly used for quantitative analysis in HPLC:

- ***External standard method****.* This method involves the use of a series of standard solutions containing known concentrations of the reference substance and is normally preferred when few sample preparation steps are necessary such as in protein precipitation from serum or plasma. The peak area or the height of the sample and the standard used are compared [32].
- ***A standard addition method*** is used when sufficient amount of the samples is available. A known quantity of the standard compound is added to the sample solution to be determined.
- ***Internal standard method****.* In this method, a known amount of an IS is added to the known amount of samples and homogenized with it. The aim of procedure is to compensate for the loss of the analytes during pretreatment of the sample. Loss of the component of interest will be accompanied by the loss of an equivalent fraction of the IS [39]. This was the method applied to the study *R*-modafinil and *S*-CE-123 as analytes and CE-114 as IS. Quantification is based on standard curves set up after analysis of standard solutions. Standard solutions of plasma analysis are prepared in drug-free plasma. Prior to analysis stock solutions of all compounds should be prepared in water or in water miscible organic solvents. For this study ACN was used. After analysis of standard solutions and sample solutions, the detector response ratio (peak height or peak area) of the analytes and IS is calculated. When the same

amount of IS is added to standard solutions and to sample solutions the calibration curve is a plot of response ratio against concentration. Unknown concentrations are determined from the standard curve [32].

2.4. Quantification

The concentration of unbound, pharmacologically active drug in blood (plasma) is used to establish pharmacokinetics-pharmacodynamics (PK-PD) relationship by applying the so-called free drug hypothesis [40]. The blood-brain barrier (BBB) is the principle route for the entry of most drugs into the CNS and remains one of the greatest challenges for the discovery as well as development of treatments for CNS disorders [41]. The rate of CNS penetration relates to the speed at which a compound enters the CNS, independent of how much drug will enter the brain or to the degree of CNS penetration. The rate of CNS penetration is controlled by two factors: the cerebral blood flow (CBF), which controls the amount of drug delivered to the brain, and the permeability of the compound across the BBB [42]. The presence of the BBB often leads to asymmetry in drug BBB transport which does not allow the use of plasma unbound-drug concentration as a surrogate of intracerebral target-site drug concentration [43]. The pharmacokinetics of a drug in the brain (**Figure 9**) can be described based on just a few key compartments.

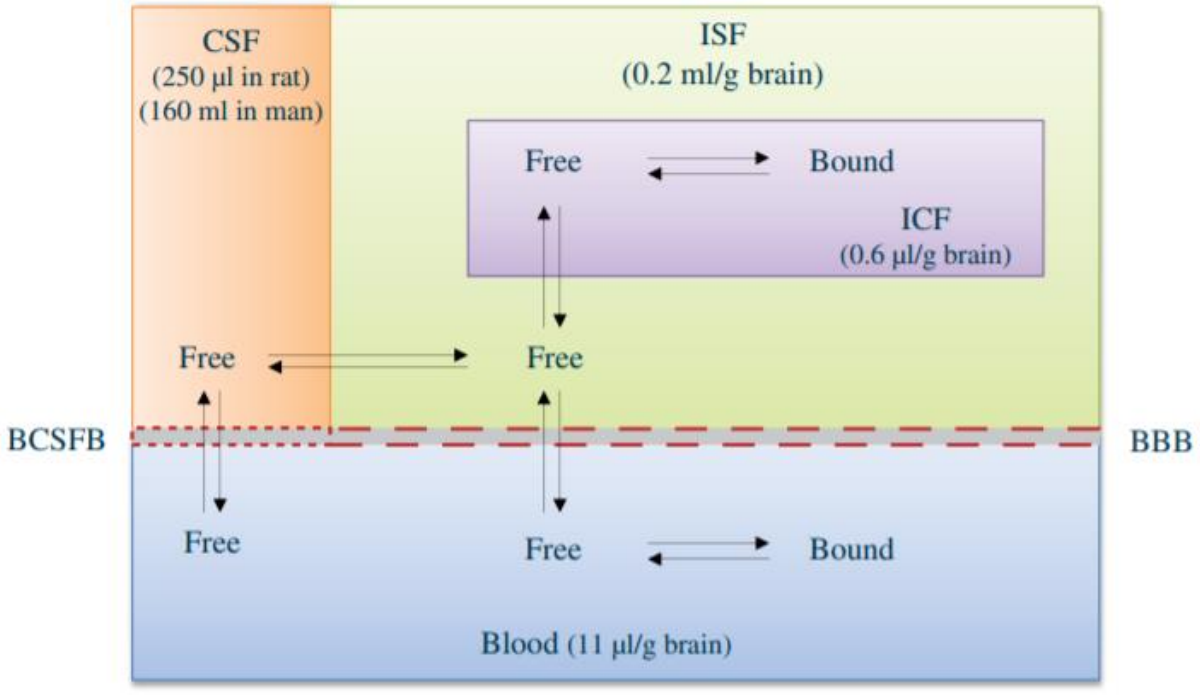

Figure 9: A schematic drawing of the four key compartments within the CNS commonly used to describe the NeuroPK of compounds, including typical physiological volumes: CSF, cerebrospinal fluid; ICF, intracellular fluid; ISF, interstitial fluid; BCSFB, blood– cerebrospinal fluid barrier. Plasma concentrations include both bound and unbound drug, and total brain

concentrations include bound and unbound drug both in brain ISF and intracellularly (N. Hoboken, 2015, p. 17) [42].

Three parameters can be used to comprehensively describe the drug delivery to the brain: $K_{p,uu}$ (concentration ratio of unbound drug in brain to blood), CL_{in} (permeability clearance into the brain), and $V_{u,brain}$ (intra-brain distribution). The permeability of the BBB is less relevant to drug action within the CNS than the extent of drug delivery, as most drugs are administered on a continuous (repeated) basis [43].

The unbound partition coefficient ($K_{p,uu}$) is the ratio of unbound drug concentration in brain ISF to unbound drug concentration in blood. According to Hammarlund-Udenaes *et al.* [43] $K_{p,uu,brain}$ can be described by:

$$K_{p,uu,brain} = \frac{K_{p,brain}}{V_{u,brain} \cdot f_{u,plasma}}$$

where $K_{p,brain}$ is the amount or concentration of drug present in the brain at steady state in relation to that in blood, $V_{u,brain}$ is the unbound drug volume of distribution in brain and $f_{u,plasma}$ is the fraction of unbound drug in plasma.

The unbound fraction of the drug in plasma can be measured *in vitro* using equilibrium dialysis (ED) [44]. ED is the gold standard method to measure the amount of free and bound ligand to a macromolecule [45]. The advantages provided by ED include the automation, because it can be implemented using simple liquid handling techniques and the minimization of nonspecific binding of drug-like compounds. The development of a high throughput plasma protein binding (PPB) assay, based on a new 48-well format rapid equilibrium dialysis (RED) has been reported by van Liempd *et al*. [46] (**Figure 10**).

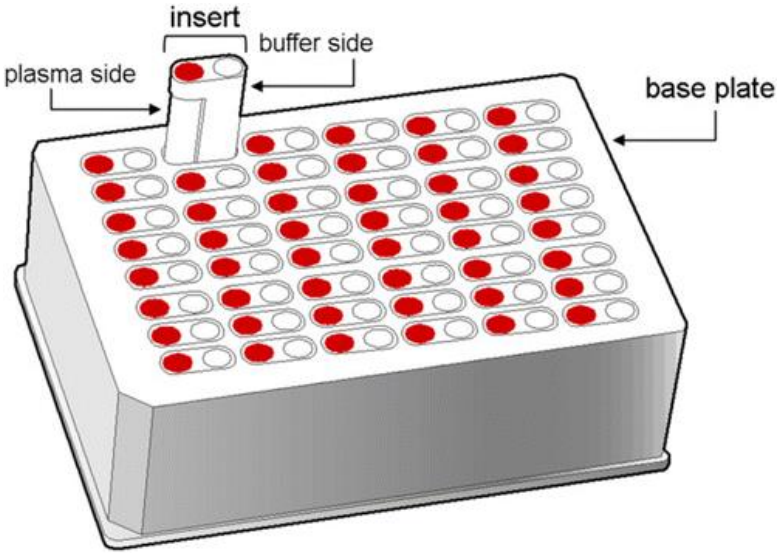

Figure 10: The rapid equilibrium dialysis device base plate and inserts (van Liempd et al., 2011, p.57) [46] .

The RED device is constructed from a 48-well Teflon base plate with disposable inserts. Each dialysis insert has a buffer and a plasma compartment separated by a semipermeable membrane, which prevents plasma proteins and other high molecular weight compounds from crossing from one compartment to the other, but does allow equilibrium of the drug between both compartments. The drug was previously spiked to the plasma and then left to equilibrate between the buffer and plasma compartments. After the incubation period of the RED device, the drug concentrations of plasma and buffer samples are collected and stored at —80 °C until LC-HRMS analysis [46].

2.5. Aim of the research

In the past, according to the literature survey, few analytical methods [47, 48] were developed to analyze CE-123 in rat plasma samples using LC-HRMS. However, these methods faced limitations in terms of recoveries. This required the development of a rapid and reliable method to analyze modafinil and CE-123 in plasma samples.

The presented thesis was done in cooperation with M.D., PhD Irena Loryan and Josefin Keife from the University of Uppsala. Loryan *et al.* [49] proposed and tested a screening toolbox for the assessment of central CNS exposure of new chemical entities (NCEs). The combinatory mapping approach - described in **Figure 11** - was used for the assessment of the extent of blood-brain and cellular barriers transport via estimation of unbound-compound brain ($K_{p,uu,brain}$) and cell ($K_{p,uu,cell}$) partitioning coefficients. The screening toolbox comprises *in vivo*, *in vitro* and *in silico* methods. *In vivo* measurements of total drug brain and plasma exposure ($AUC_{tot,brain}$ and $AUC_{tot,plasma}$) are required for the assessment of the brain partitioning coefficient $K_{p,brain}$. Drug plasma and brain tissue binding properties ($f_{u,plasma}$ and $f_{u,brain}$) are necessary for the determination of $K_{p,uu,brain}$ and $K_{p,uu,cell}$ parameters. For *in silico* calculation of drug subcellular distribution ($K_{p,uu,cyto,pred}$ and $K_{p,uu,lyso,pred}$), the compound-specific pK_a values in combination with the physiological estimates of pH (pH_i) are used. Physiological volumes (V_i) of brain interstitial fluid, cytosol and lysosomes with $K_{p,uu,cyto,pred}$ and $K_{p,uu,lyso,pred}$ are used for calculation of $K_{p,uu,cell,pred}$. Assessed neuroPK parameters in conjunction with relevant pharmacodynamics readouts are recommended to be used for evaluation and selection of NCEs [49].

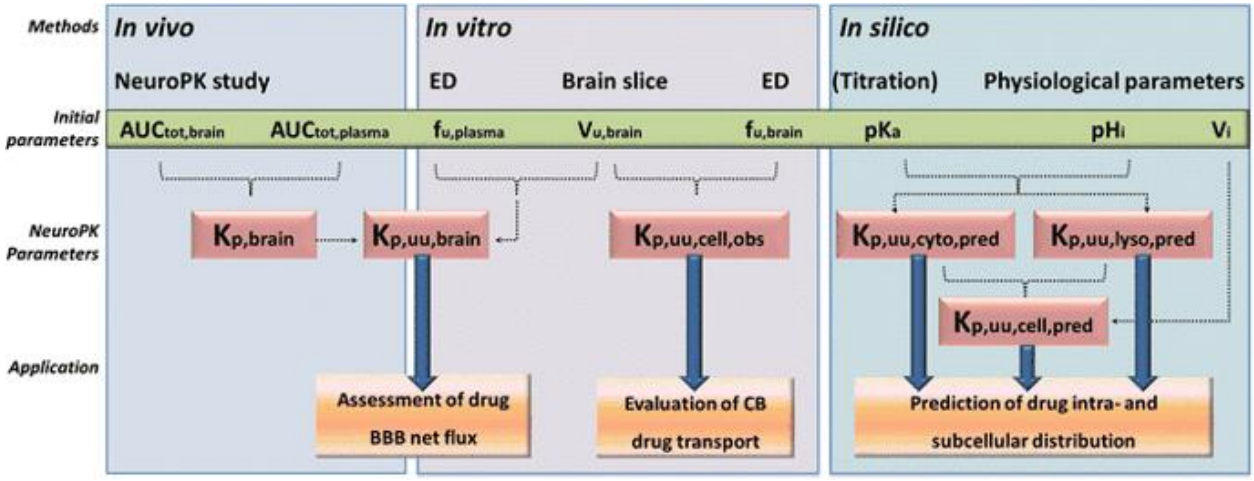

Figure 11: The combinatory mapping for the evaluation of unbound-drug CNS exposure (Loryan et al., 2014, p. 2216) [49].

The aim of the present research work was the assessment of plasma protein binding properties ($f_{u,plasma}$) of modafinil and CE-123 in rat blood sample using high performance liquid chromatography coupled to mass spectrometer. A simple and rapid analytical method for the quantitative determination of modafinil and CE-123 in rat plasma samples was developed.

3. Materials and methods

3.1. Chemicals and materials

R-Modafinil, *S*-CE-123 and CE-114 were synthesized by Dr. Predrag Kalaba at the University of Vienna. The chemicals used were water (LC-MS grade, Merck KGaA, Darmstadt, Germany), acetonitrile (ACN) (LC-MS grade, Merck KGaA, Darmstadt, Germany), dimethylformamide (DMF) (for liquid chromatography, Merck KGaA, Darmstadt, Germany) and formic acid (FA) (98 %, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Control rat plasma was provided by Jovana Malikovic (Himberg, Medical University of Vienna). Treated rat plasma was supplied by Irena Loryan, M.D., PhD (Uppsala University, Department of Pharmaceutical Biosciences, Translational PKPD Group, Sweden). Rat plasma samples were stored at -80 °C in 96-well assay plates and delivered to the University of Vienna.

3.2. Equipment

The Nexera XR ultra high performance liquid chromatography (UHPLC) system (serial NO: L203054, 62310, Shimadzu, Japan) was applied for chromatographical measurements. The UHPLC system consists of DGU-20A5R degassing unit, DGU-20A5R degassing unit, LC- 20AD XR liquid chromatography pump, SIL-20AD XR autosampler, SPD-M20A prominence diode array detector and CTO-20AC prominence column oven. Two channels were used for gradient elution with LC-MS grade ACN and LC-MS grade water containing 0.1 % FA at a flow rate set to 600 µL/min. For separation a 50 x 2.1 mm Kinetex Phenyl-Hexyl column (Phenomenex, Inc., Torrance, CA, United States) packed with 2.6 µm diameter particles was used.

For the LC-HRMS measurements an UltiMate 3000 RSLC-series system (Dionex; Thermo Fisher Scientific, Inc., Gemering, Germany) paired with a maXis electrospray ionisation double quadrupole mass analyser time-of-flight (HD ESI Qq-TOF) mass spectrometer (Bruker Corporation, Bremen, Germany) was used. The column used for LC-HRMS analysis was the same.

The powder of each drug was weighed on an analytical balance (MC210P, Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany). An centrifuge (5804 R, Eppendorf AG, Hamburg, Germany) was used for plasma samples preparation. Data were analysed using Compass DataAnalysis 4.2 (Bruker Corporation).

3.3. Preparation of standard solutions

Stock solutions of the drugs *R*-modafinil, *S*-CE-123 and CE-114 in ACN with a concentration of 1 mg/mL were prepared, respectively. The powder of each drug was weighed on an analytical balance, transferred into a 10 mL volumetric flask and filled with ACN. Exactly 10.01 mg *R*-modafinil, 10.03 mg *S*-CE-123 and 10.02 mg CE-114 were measured in 10 mL volumetric flask. ACN was used to fill in the flask up to 10 mL. Thus, the stock solution concentrations of *R*-modafinil, *S*-CE-123 and CE-114 prepared were respectively 1.001 mg/mL, 1.003 mg/mL and 1.002 mg/mL. This method was used for all stock solutions that were prepared for this pharmacokinetic study. The exact weight of each sample was considered when calculating the concentrations. The three solutions containing approximately 1 mg/mL of each drug were then diluted in order to obtain working solutions with a concentration of 100 µg/mL. 1000 µL of each solution were transferred into a 10 mL volumetric flask and filled with ACN.

Furthermore, a blank sample, containing 1 mL mobile phase B (ACN), was prepared. The measurement of a blank sample is necessary to differentiate between the signal of the drug and the background.

3.4. Method development

3.4.1. HPLC conditions

The Nexera XR UHPLC system (Shimadzu, Japan) was used for HPLC measurements and UltiMate 3000 RSLC-series system (Dionex/Thermo Scientific, Inc., Gemering, Germany) coupled to maXis HD ESI-Qq-TOF mass spectrometer (Bruker Corporation, Bremen, Germany) for LC-HRMS measurements. Chromatographic separation of *R*-modafinil, *S*-CE-123 and CE-114 was achieved using a Kinetex Phenyl-Hexyl column at a constant flow rate of 0.6 mL/min and at a temperature of 40 °C. Mobile phase A was water containing 0.1 % FA and mobile phase B was ACN. The injection volume was 20 µL and a wavelength of 254 nm was chosen for absorbance evaluation.

The chromatographic conditions are shown in **Table 3**.

Table 3: Parameters of high-performance liquid chromatography.

Parameter	Values
Column + inventory number	Kinetex Phenyl-Hexyl 2.6 µm
Column dimension	50 x 2.1 mm I.D
Flow	0.6 mL/min
Mobile phase A	0.1% FA water
Mobile phase B	ACN
Injection volume	20 µL

The gradient 1 (**Table 4**) used was as follows: 0–1.5 min, 95% A, 5% B; 1.5–6.5 min, linear gradient to 70% A, 30% B; 6.5–6.6 min, linear gradient to 30% A; 6.6–8.0 min, a washing step at 5% A, 95% B; 8.0–10.0 min, column re-equilibration with 5% B. The chromatographic parameters were the same for both the HPLC and LC-HRMS analysis.

Table 4: HPLC and LC-HRMS gradient 1.

Time, min	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
1.5	70	30
6.5	30	70
6.6	5	95
8	5	95
10	95	5

For HPLC, different wavelengths were checked for better sensitivity. The signals were recorded with a diode array detector, observing wave lengths of 190 nm, 215 nm and 254 nm. After comparing the measurements of the wavelengths, 254 nm was selected as the one with the best outcome for the three substances. The comparison of the ultraviolet (UV) chromatograms (254 nm) of *R*-modafinil (100 µg/mL), *S*-CE-123 (100 µg/mL) and CE-114 (100 µg/mL) measured with gradient 1 is given in **Figure 12**.

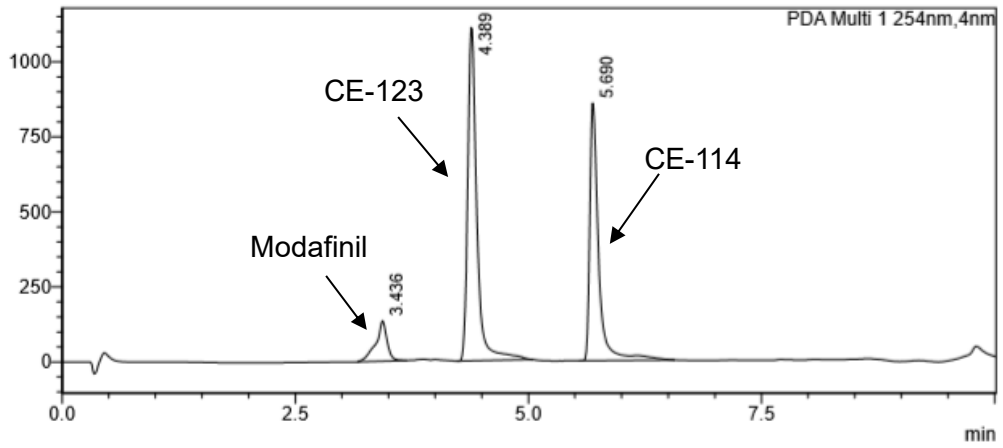

Figure 12: UV chromatogram (254 nm) of the mixture of *R*-modafinil, *S*-CE-123 and CE-114. The concentration of each drug was 100 µg/mL.

3.4.2. LC-HRMS conditions

Franschitz [48] developed an LC-HRMS method for measuring the concentration of *S*-CE-123 in rat plasma. This method was chosen and optimized for analysis of drugs in the present work.

As already mentioned, liquid chromatography was performed using an UltiMate 3000 RSLC-series system coupled to a maXis HD ESI-Qq-TOF mass spectrometer. The settings of the ESI ion source were as follows: capillary voltage: 3.5 kV; nebulizer: 0.8 bar N_2; dry gas flow rate: 7.0 L/min N_2; and dry temperature: 200°C. Full scan mass spectra were recorded in the range of *m/z* 50–2500 in the positive-ion mode. The optimized parameters in the positive mode setting are summarized in **Table 5.**

Table 5: Acquisitions parameters of MS.

Parameter	Values
Source Type	ESI
Focus	Active
Scan Begin	50 m/z
Scan End	2500 m/z
Ion Polarity	Positive
Set Capillary	3500 V
Set Nebulizer	0.8 bar
Dry Heater	200 °C
Set End Plate Offset	-500 V
Set Charging Voltage	2000 V
Set Corona	0 nA
Set Dry Gas	7.0 l/min
Set Divert Valve	Source
Set APCI Heater	40 °C

It was operated with the same chromatographic parameters and the same gradient as displayed in **Tables 3** and **4**. The LC-HRMS data was analyzed using the software

Compass DataAnalysis 4.3. For quantification, extracted ion chromatograms (EIC) were generated according to specific *m/z* values for *R*-modafinil and *S*-CE-123 as analytes, and CE-114 as IS. The calculated *m/z* values of modafinil, CE-123 and CE-114 positively charged with H^+, their diphenylmethyl (DPM) cations and respective retention times are shown in **Table 6** below.

Table 6: Overview of calculated *m/z* values of modafinil, CE-123 and CE-114 positively charged with H+ , their DPM cations and respective retention times. The *m/z* values used in this study are highlighted in bold.

Test item	Sum Formula	Molecular weight (g/mol)	*m/z* $[DPM]^+$	*m/z* $[M+H]^+$	RT (min)
***R*-modafinil**	$C_{15}H_{15}NO_2S$	273.35	**167.0848**	274.0878	3.1
***S*-CE-123**	$C_{17}H_{15}NOS_2$	314.07	167.0848	**314.0646**	3.6
CE-114	$C_{19}H_{20}NOS_2$	356.50	167.0848	**357.1065**	4.7

The DPM cation is demonstrated in **Figure 13**.

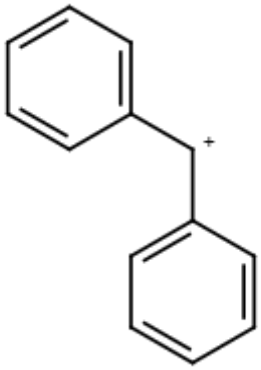

Figure 13: The structure of the DPM cation with a MW of 167.23 g/mol and a sum formula of $C_{13}H_{11}^+$.

3.4.3. Optimization of internal standard

Franschitz [48] developed an LC-MS method for measuring the concentration of *S*-CE-123 in rat plasma. This method was chosen and optimized for analysis of drugs in our work. Diazepam with the concentration of 10000 ng/mL was found to be suitable as IS. In this work, we tested diazepam as a potential IS and concluded that diazepam is not suitable as IS because there is no baseline separation between modafinil and CE-123. It was operated with the same chromatographic parameters as displayed in **Table 3**. The HPLC was performed in the gradient mode as given in **Table 7***.*

Table 7: HPLC gradient 2 using for testing of new IS. The gradient was adapted from the LC-HRMS method described by Franschitz [48].

Time, min	Mobile phase A (%)	Mobile phase B (%)
0	95	5
5	95	5
5	70	30
10	55	45
12	30	70
13	3	97
14.5	3	97
14.5	95	5
15.5	95	5

The total ion chromatograms of *R*-modafinil (10 µg/mL), *S*-CE-123 (10 µg/mL) and diazepam (10 µg/mL) measured with gradient 2 is given in **Figure 14.** Baseline separation was not achieved. Thus, another IS was applied. For that purpose, CE-114 was chosen which is an analog of modafinil.

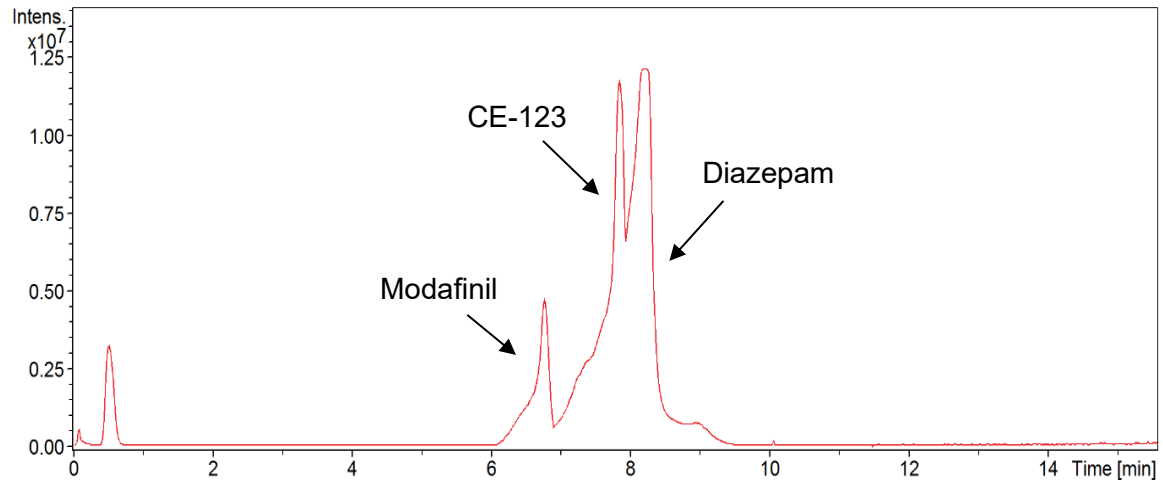

Figure 14: Total ion chromatogram of *R*-modafinil, *S*-CE-123 and diazepam. The concentration of each drug was 10 µg/mL.

CE-114 was tested as the IS for HPLC measurements. The peak of CE-114 appears at minute 8.9 (concentration of 10 µg/mL in ACN for each drug) showing a baseline separation with modafinil and CE-123 (**Figure 15**) and was chosen to be suitable as IS for the project.

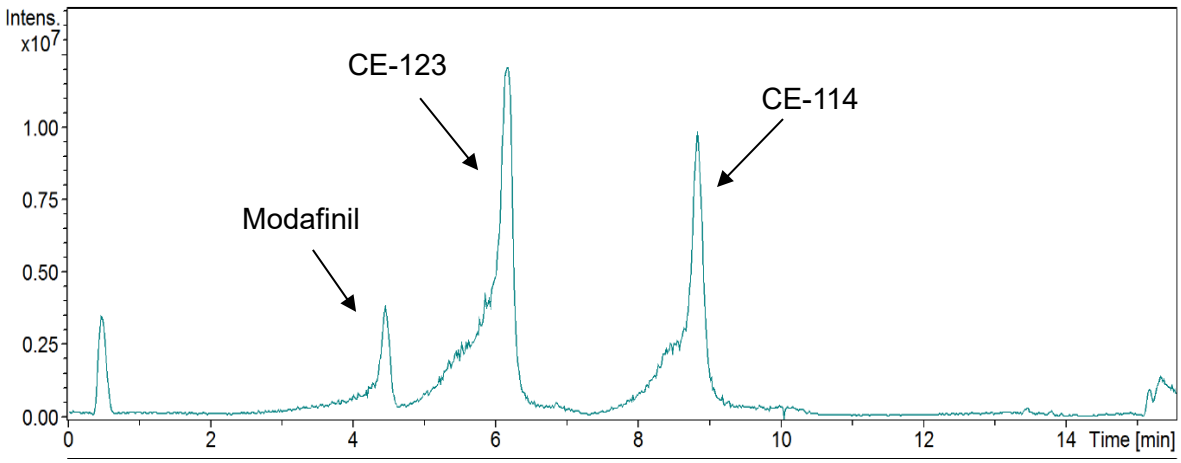

Figure 15: Total ion chromatogram of *R*-modafinil, *S*-CE-123 and CE-114. The concentration of each drug was 10 µg/mL. The retention time of *R*-modafinil was 4.3 minutes, *S*-CE-123 had a retention time of 6.1 minutes and CE-114 had a retention time of 8.9 minutes.

3.5. Measurements of Uppsala samples

3.5.1. Sample preparation

Pooled rat plasma was obtained from the blood of Sprague Dawley male 250–300 g rats [49]. The samples were prepared by the method of van Liempd *et al*. [46] with slight modifications. The fraction of unbound-compound in species-specific plasma ($f_{u,plasma}$) was determined using high-throughput equilibrium dialysis. Briefly, fresh heparinized blood was collected from male rats and centrifuged to obtain plasma. The system was manually filled with the same volume of plasma spiked with test compound and phosphate-buffered saline (PBS). Modafinil and CE-123 were used as test compounds. After the RED device was loaded with sample and buffer, 96-well plates were incubated at 37°C for 6 and 24 h in a revolving incubator. Then, the plasma samples were stored at a -80°C until analyzed.

The rat plasma samples of unknown concentrations of modafinil and CE-123 and the standard solutions for calibration curve containing both drugs at 9 different initial concentrations (10 – 2500 ng/mL) were provided by Irena Loryan M.D.,PhD and delivered from the University Uppsala. The samples were preserved at - 80°C in 96-well assay plate. All samples were thawed and vortexed for one minute. The sample preparation protocol included protein precipitation with ice-cold ACN + 0.2 % FA. Rat plasma was spiked with ice-cold ACN + 0.2 % FA solution 1:3 (v:v) containing CE-114 (1000 ng/mL) as IS. After spiking the samples were vortexed for one minute and centrifuged for 10 minutes at 4500 x *g* at 4 °C. The supernatant was transferred into HPLC vials and mixed 1:2 (v:v) with water containing 0.1 % FA. All samples were vortexed for 1 minute to assure proper homogeneity of solutions before the LC-HRMS analysis.

The stock solution of CE-114 was prepared by weighing exactly 10.02 mg CE-114 in a 10 mL volumetric flask and filling up with ACN. Thus, the stock solution concentrations of CE-114 prepared was respectively 1.002 mg/mL. The next step was making of working solution (WS 100) with a concentration of 100 µg/mL. Exactly 100 µL of CE-114 stock solution was taken and added to 900 µL ACN. The last step was taking 100 µL working solution of CE-114 and spiking it with 9900 µL ACN + 0.2 % FA containing aqueous solution. Newly prepared spiking solution are stored and chilled for 30 minutes at -20 °C in a refrigerator until time for analysis. A solution of 100 µL of

ice-cold ACN containing IS is added to each plasma sample, except for the blank to which 100 µL of pure ACN is added, to facilitate protein precipitation.

3.5.2. Calibration curve in plasma

The standard solutions contain *R*-modafinil and *S*-CE-123 at nine different initial concentrations (10 – 2500 ng/mL) prepared from the Uppsala University were preserved at a temperature of -80°C in Eppendorf tubes. Each standard sample was prepared following the procedure described in section **3.5.1** and is shown in **Table 8.**

Table 8: Preparing the standard solutions samples from the Uppsala University.

Standards (ng/mL)	Volume of the samples used for spiking (µL)	Spiking solution volume (µL)	Volume left (µL) + water volume (µL)	Total volume (µL) used for LC-HRMS
2500	50	100	=50+50	100
1000	50	100	=50+50	100
600	50	100	=50+50	100
500	50	100	=50+50	100
300	50	100	=50+50	100
200	50	100	=50+50	100
100	50	100	=50+50	100
50	50	100	=50+50	100
10	50	100	=50+50	100

During the LC-HRMS run a new compound was detected as DPM cation (*m/z*=167.0848) at a retention time of 3.2 minutes (**Figure 16**). It is operated with the gradient 2 as displayed in **Table 7** and the same LC-HRMS conditions are used.

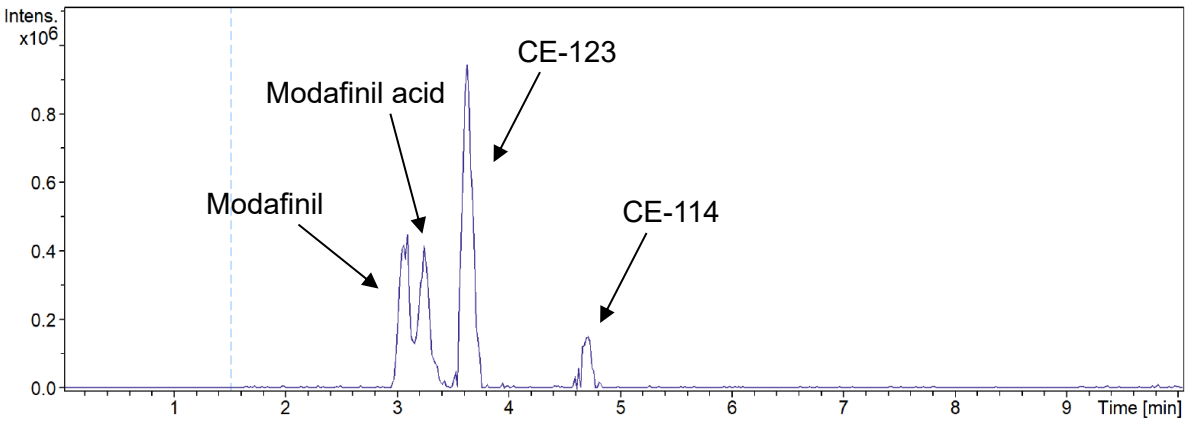

Figure 16: EIC of *R*-modafinil (*m/z*=167.0848) and modafinil acid (*m/z*=167.0848) in standard 2500 ng/mL. As it can be seen, there is no base peak separation between *R*-modafinil and its metabolite. The standard solutions of *R*-modafinil could not be used for the quantification in blood plasma samples and the new statistically significant calibration curves of *R*-modafinil and modafinil acid were necessary.

Modafinil and modafinil metabolites, modafinil acid and modafinil sulfone, are reported to ionize as DPM cation [50]. According to this hypothesis the two metabolites were synthetized by colleagues at the University of Vienna. Synthesized metabolites were measured with direct infusion in HRMS and both were detected as DPM cation (*m/z*=167.0848) and sodium adduct (*m/z*=297.0524 modafinil acid and *m/z*=312.0639 modafinil sulfone. The DPM cations of modafinil and modafinil acid show a significant higher intensity, in contrast to its sodium adducts (data not shown). Using these values and EIC we can confirm that new detected compound is modafinil acid (**Figure 17**).

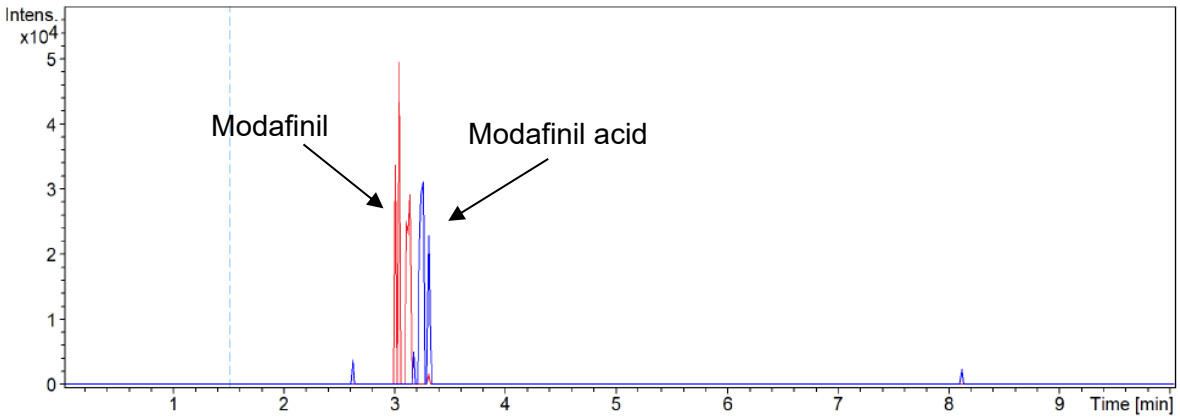

Figure 17: EIC of *R*-modafinil (*m/z*=167.0848) and modafinil acid (*m/z*=167.0848) in standard with concentration of 2500 ng/mL.

As there is no baseline separation between *R*-modafinil and modafinil acid (both are detected as DPM cation) we were not able to quantify *R*-modafinil in rat plasma of this

samples. After the detection of modafinil acid in all standard solutions samples, it was decided to optimize the method of sample preparation and LC-HRMS parameters to achieve separation of all compounds. Furthermore, it was necessary to develop a method to inhibit the metabolization of *R*-modafinil to its metabolite in rat blood plasma. Furthermore, the new statistically significant calibration curves of *R*-modafinil and modafinil acid in plasma were needed to calculate concentrations of *R*-modafinil in different blood plasma samples. The comparison of the concentrations of two substances between blood plasma samples and brain tissue samples can indirectly give information about the pharmacokinetics of our substances and the percentage amount of the substances which is able to pass the blood brain barrier.

3.5.3. Optimization of LC-HRMS gradient

There are several published methods for the quantification of the metabolites of *R*-modafinil in human plasma or in serum, but none enables the quantification of the metabolites in rat plasma [51, 52]. The aim of this work was to develop a selective HPLC assay that is reliable for quantification of *R*-modafinil and its major metabolite, modafinil acid, in rat plasma.

In order to achieve baseline separation between analytes and IS, the HPLC gradient was optimized. The procedure of standard samples preparation is described in section **3.3.** The first LC-HRMS measurement of the prepared samples was performed using the gradient 3 shown in **Table 9**.

Table 9: HPLC gradient 3. The following multi-step gradient was applied: 5 % solvent B at 0.01 min, 23 % solvent B at 3.50 min, 35 % solvent B at 5.50 min, 70 % solvent B at 9.50 min, a washing phase at 97 % solvent B from 9.51 to 11.50 min and column re-equilibration with 5 % solvent B from 11.51 to 13.00 min.

Time, min	Mobile phase A (%)	Mobile phase B (%)
0.01	95	5
1.50	95	5
3.50	77	23
5.50	65	35
9.50	30	70
9.51	5	95
11.50	5	95
11.51	95	5
13.00	95	5

The gradient started with 5 % ACN in order to elute the hydrophilic components, then the amount of ACN was increased eluting the analyses, followed by a washing phase (95 % ACN) to elute the hydrophobic elements and ended with column re-equilibration (5 % ACN). A long washing step is necessary to remove all interfering components from the column. The gradient elution was: min 0 with 5% solvent B to min 1.50; 1.50-3.50 min, linear gradient from 5 to 23 % B; 3.50-5.50 min, linear from 23 to 35% B; 5.50-9.50 min, increasing 70% B; a washing phase at 95% B from 9.51 to 11.50 min and column re-equilibration with 5 % B from 11.51 to 13.00 min. As shown in

Figure 18, baseline separation between *R*-modafinil and its metabolite was not achieved. The baseline separation between modafinil and its metabolite is necessary, because for all evaluations conducted later in the study only the more prominent adduct of the two analytes was used, which was DPM cation for modafinil and modafinil acid. DPM cations have a significantly higher intensity than modafinil and modafinil acid positively charged with H^+ (**Table 6**). The reason for using the more prominent adducts is that the quantification of lower concentrations of the less strong adducts was not possible.

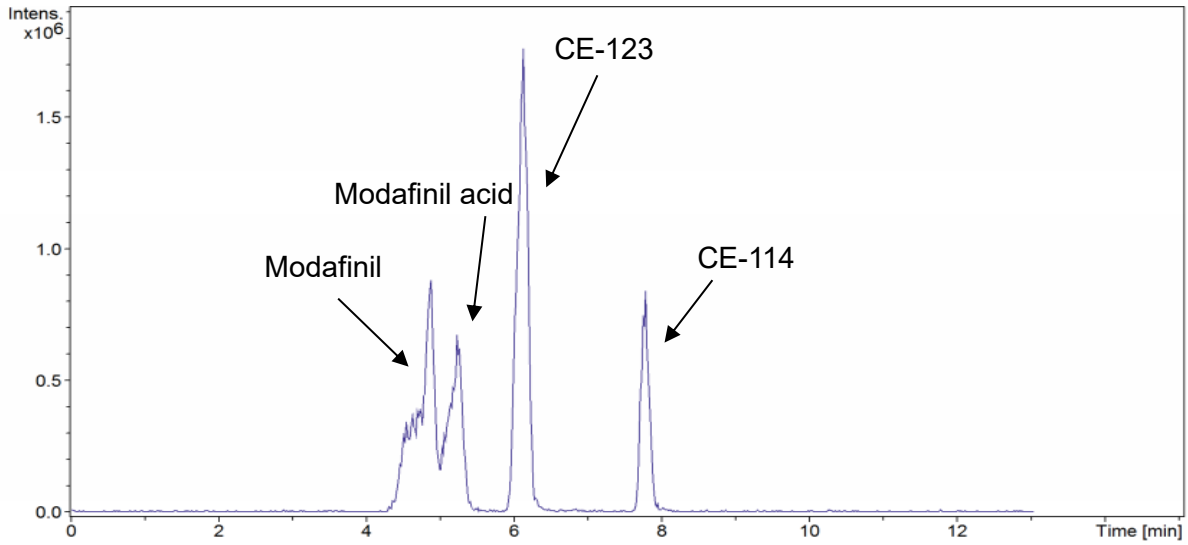

Figure 18: EIC chromatogram of *R*-modafinil (*m/z*=167.0848) and modafinil acid (*m/z*=167.0848) in the mixture of *R*-modafinil (100 µg/mL), modafinil acid (100 µg/mL), *S*-CE-123 (100 µg/mL) and CE-114 (100 µg/mL) with gradient 3. Baseline separation was not achieved.

The timetable of gradient 4, which was extended by two minutes, is depicted in **Table 10**. The following multi-step gradient was applied: 5 % B from 0.01 to 1.50 min; 23 % B from 1.51 to 3.50 min; 3.50-7.50 min, linear from 23 to 35% B; 7.50-11.50 min, increasing 70% B; a washing phase at 95% B from 11.51 to 13.50 min and column re-equilibration with 5 % B from 13.51 to 15.00 min.

Table 10: HPLC gradient 4. The following multi-step gradient was applied: 5 % solvent B at 0.01 min, 23 % solvent B from 1.51 min to 3.50 min, 35 % solvent B from 3.51 min to 7.50 min, 70 % solvent B at 11.50 min, a washing phase at 97 % solvent B from 11.51 to 13.50 min and column re-equilibration with 5 % solvent B from 13.51 to 15.00 min.

Time, min	Mobile phase A (%)	Mobile phase B (%)
0.01	95	5
1.50	95	5
1.51	77	23
3.50	77	23
3.51	65	35
7.50	65	35
11.50	30	70
11.51	5	95
13.50	5	95
13.51	95	5
15.00	95	5

The EIC at *m/z*=167.0848 of the mixture *R*-modafinil (100 µg/mL), modafinil acid (100 µg/mL), *S*-CE-123 (100 µg/mL) and CE-114 (100 µg/mL) are shown **in Figure 19.**

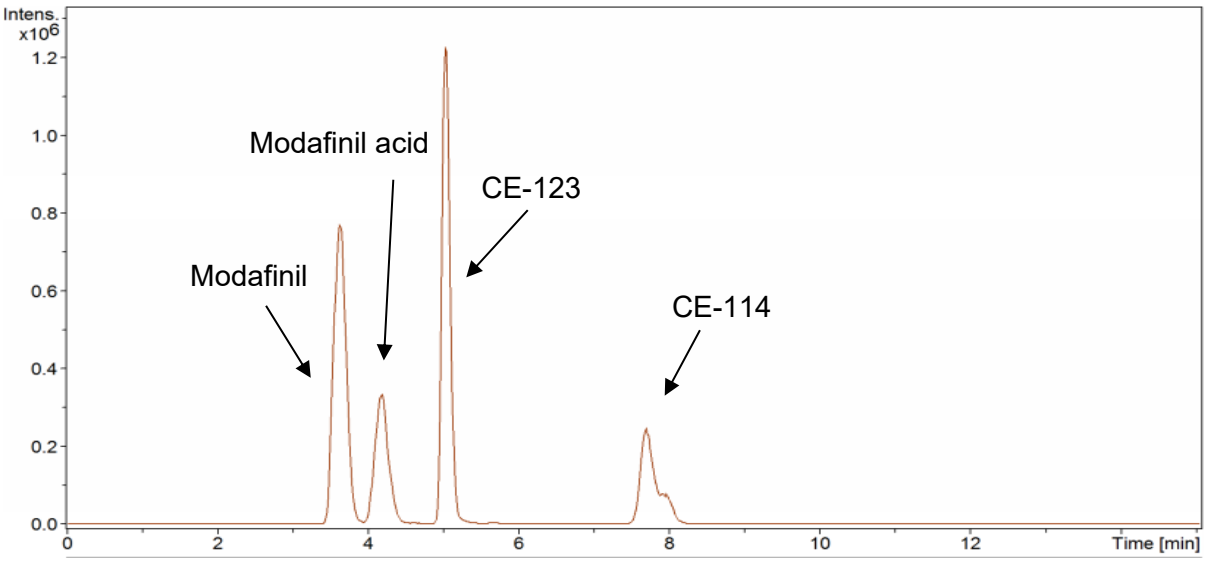

Figure 19: EIC chromatogram at *m/z*=167.0848 of *R*-modafinil and modafinil acid in the mixture of *R*-modafinil (100 µg/mL), modafinil acid (100 µg/mL), *S*-CE-123 (100 µg/mL) and CE-114 (100 µg/mL) with gradient 4. The baseline separation between *R*-modafinil and modafinil acid was not accomplished.

It was necessary to further extend the gradient, because *R*-modafinil and modafinil acid both eluted as the DPM cation at similar timepoints. The aim was to achieve baseline separation between the peaks. *R*-Modafinil had a retention time of 3.6 minutes and modafinil acid had a retention time of 4.2 minutes, but complete separation was not accomplished. The next gradient was shortened for 4 minutes, because all substances had already eluted after 10 minutes using the gradient 4.

The gradient elution (**Table 11**) was: min 0 with 5% solvent B to min 1.50; min 1.51 with 23% B to min 3.50; min 3.51 with 35% B to min 6.00; 6.00-8.80 min, increasing 95% B; a washing phase at 95% B from 8.00 to 10.00 min and column re-equilibration with 5 % B from 10.01 to 11.00 min.

Table 11: HPLC gradient 5. The following multi-step gradient was applied: 5 % solvent B at 0.01 min, 23 % solvent B from 1.51 min to 3.50 min, 35 % solvent B from 3.51 min to 6.00 min, 95 % solvent B at 8.00 min and column re-equilibration with 5 % solvent B from 10.01 to 11.00 min.

Time, min	Mobile phase A (%)	Mobile phase B (%)
0.01	95	5
1.50	95	5
1.51	77	23
3.50	77	23
3.51	65	35
6.00	65	35
8.00	5	95
10.00	5	95
10.01	95	5
11.00	95	5

The EIC at *m/z*=167.0848 of a mixture of *R*-modafinil, modafinil acid, *S*-CE-123 and CE-114 are illustrated in **Figure 20**. The concentration of each drug was 100 µg/mL. *R*-Modafinil had a retention time of 3.6 minutes and modafinil acid had a retention time of 4.2 minutes.

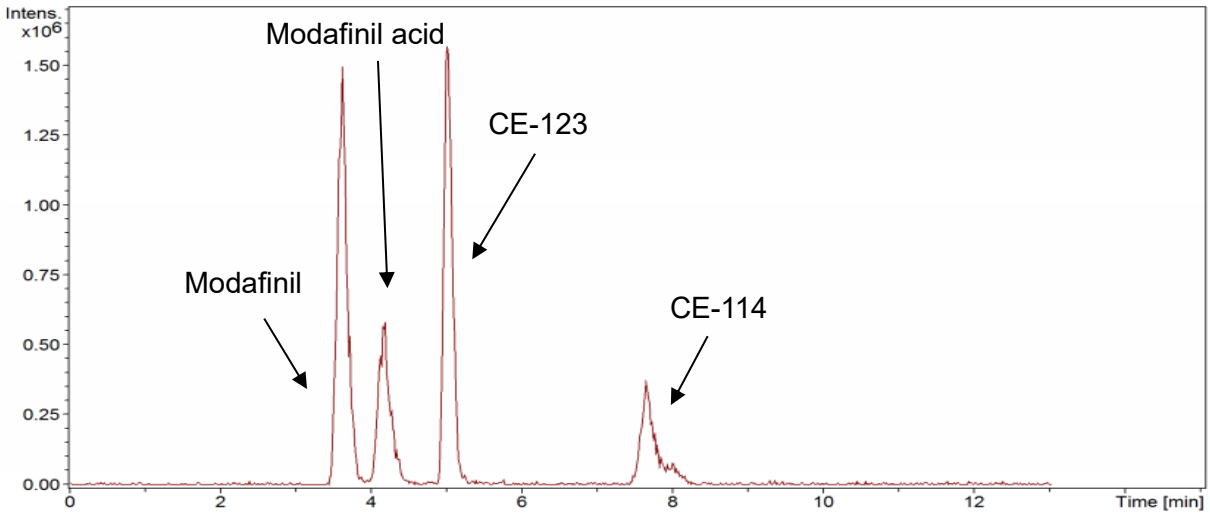

Figure 20: EIC chromatogram at *m/z*=167.0848 of *R*-modafinil and modafinil acid in the mixture of *R*-modafinil (100 µg/mL), modafinil acid (100 µg/mL), *S*-CE-123 (100 µg/mL) and CE-114 (100 µg/mL) with gradient 4. The retention time of *R*-modafinil was 3.6 minutes and the retention time of modafinil acid was 4.2 minutes.

This gradient shows the best baseline separation between all compounds and was chosen to be suitable for the project.

3.5.4. Plasma stability test of *R*-modafinil

During measurement of standard solutions prepared from the Uppsala University, the used assay procedure was unable to determine concentrations of *R*-modafinil and its circulating metabolite, modafinil acid, in rat plasma.

Modafinil is primarily hydrolyzed by an esterase and/or amidase into modafinil acid, which is the major metabolite [34]. According to P. Robertson and E. Hellriegel [11] the major circulating metabolite does not appear to exert any significant activity in the brain or periphery.

One method of inhibition of drug metabolism has previously been reported for analysis of *R*-modafinil in human plasma. In this method developed by Gorman [51], stock solutions (500 mg/mL) containing *R*-modafinil and modafinil acid were prepared in *N',N'*- DMF. Working solutions were made by serial dilution of these stock solutions with DMF and then an aliquot of a test sample was mixed with human plasma (10:90, v/v).

Short time stability of *R*-modafinil in drug free plasma and in plasma with 10% DMF which is supposed to inhibit metabolization of *R*-modafinil to modafinil acid was analyzed. For that purpose, samples containing 1 µg/mL *R*-modafinil were incubated at 37°C (water bath) and measured at time point 0, 1, 2 and 4 hours by LC-HRMS. The procedures of samples and spiking solutions preparation are described in sections **3.3** and **3.5.1.**

3.5.5. Calibration curve of *R*-modafinil and modafinil acid in plasma

After measurement of standard solutions prepared from the Uppsala University, the major metabolite of *R*-modafinil was detected. The aim was to create a statistically significant calibration curves by making the standard solutions of *R*-modafinil and correlation.

For sample preparation stock solutions with concentrations of approx. 1 mg/mL and the working solutions (100 µg/mL) of *R*-modafinil and CE-114 in ACN were prepared following the procedure described in section **3.3.**

The intermediate standard with a concentration of 5 µg/mL (IM 5000) was prepared. 20 µL of *R*-modafinil working solution (100 µg/mL) were added to 200 µL phosphate buffered saline (PBS) buffer and 180 µL of rat blood plasma. The intermediate standard was used to prepare some of the standards. The dilution series for all substances was generated, which is listed in **Table 12** below.

Table 12: Standard preparation for calibration curve in plasma.

Standards (ng/mL)	Standard (µg/mL) Volume	Standard (µL) Volume	Volume plasma (µL)	Total volume (µL)	Volume left (µL) + Spiking solution (µL) used
2500	IM 5000	50	50	100	=50+100
1000	IM 5000	30	120	150	=50+100
600	IM 5000	12	88	100	=50+100
500	IM 5000	13	119	132	=50+100
300	STD 1000	30	70	100	=50+100
200	STD 1000	20	80	100	=50+100
100	STD 500	20	80	100	=50+100
50	STD 500	12	108	120	=50+100
10	STD 50	20	80	100	=50+100

The spiking solution, containing CE-114 (500 ng/mL) as IS, was prepared following the procedure described in section **3.5.1**. This concentration was found to be a suitable IS

concentration for the expected analyte, because of the similar peak height of analytes and IS, and used for all plasma samples that were prepared for this pharmacokinetic study.

A stock solution of modafinil acid in ACN with a concentration of 1 mg/mL was prepared using 10.03 mg of drug. The powder was weighed on an analytical balance, transferred into a 10 mL volumetric flask and filled with ACN. A 1:100 dilution of modafinil acid was prepared in ACN. This working solution of modafinil acid (10 µg/mL) has been prepared and further diluted to nominal concentrations of 25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL. All samples were analyzed three times.

Table 13: Standard preparation of modafinil acid calibration curve (in plasma).

Standards (ng/mL)	Standard (µg/mL)	Volume (µL)	Volume (plasma) (µL)	Total volume (µL)
200	IM1000	30	120	150
100	IM1000	15	135	150
50	ST200	25	75	100
25	ST100	25	75	100

In this procedure of making standard solutions of *R*-modafinil and modafinil acid, in contrast to procedure described in section **3.3**, DMF was added to rat plasma (10:90, v:v). After each of the standards has been spiked with an ice-cold spiking solution (ACN + 0.2 % FA) in a ratio of plasma/ACN + 0.2 % FA 1:3 (v:v), they were vortexed for one minute and centrifuged at 4500 x *g* at 4°C for 10 min. All samples should be vortexed for one minute to assure proper homogeneity of solutions before LC-HRMS measurements. The experiment was implemented in triplicate.

After centrifugation, certain amounts of supernatant were taken and mixed with water for LC- MS in ratio supernatant/water 1:2 (v:v). All further information is listed in the appendix (**Appendix Table 1** and **Appendix Table 2**). Before the samples were analyzed with LC-HRMS each HPLC vial was vortexed for around one minute.

3.5.6. Rat samples preparation

The rat plasma samples with unknown concentrations of modafinil and CE-123 had to be vortexed for one minute before using the LC-HRMS for the detailed analysis. 50 µL rat plasma was spiked with ice-cold 100 µL spiking solutions containing CE-114 (500 ng/mL) as IS. The preparing of the spiking solution is described in section **3.5.1**.

After each of the standards was spiked with an ice-cold spiking solution (ACN + 0.2 % FA) in a ratio of standard/ACN + 0.2 % FA 1:3 (v:v), they were vortexed for one min and centrifuged at 4500 x *g* at 4°C for 10 min. Then, 50 µL of supernatant were transferred into HPLC vials and mixed 1:2 (v:v) with water. The experiment was implemented in triplicate.

4. Results

4.1. Plasma stability test of *R*-modafinil

The relative amount of *R*-modafinil during incubation at 37°C decreases over time to about 70% in the first two hours and down to approximately 50% during 4 hours (see **Table 14** and **Figure 21**). At time point 0 and 1 h modafinil acid was not detected. After 2 and 4 h modafinil acid was detected and results are depicted in **Figure 21**. The complete list of values is shown in the appendix (**Appendix Table 3** and **Appendix Table 4**).

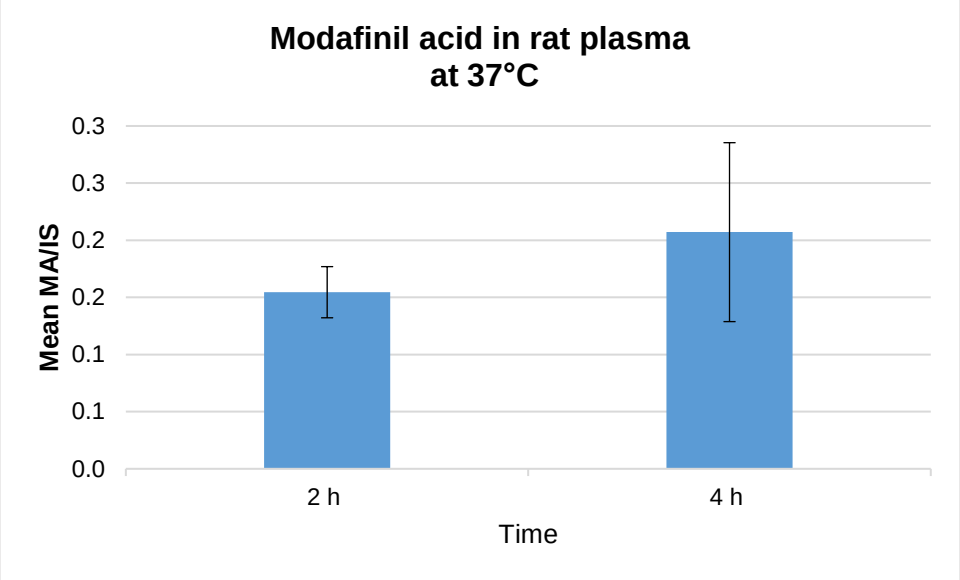

Figure 21: Formation of modafinil acid during incubation of rat plasma containing *R*-modafinil at 37°C after 2 and 4 hours (n=3, mean ± SD).

Stability of *R*-modafinil in rat plasma containing 10% DMF was tested in duplicate and measured in triplicate. The relative amount of drug drops down to a minimal value of 93.6% observed after 2 hours in one of the independent preparations. In addition, more information is listed in the appendix (**Appendix Table 5** and **Appendix Table 6**). However, there is no consistent decrease of *R*-modafinil in plasma containing 10% DMF depending on time when incubated at 37°C (see **Table 14** and **Figure 22**).

Table 14: Amount of R-modafinil (MO) in rat blood plasma and plasma containing 10% DMF after incubation at 37°C (water bath) for 0, 1, 2 and 4 hours (n=3, mean). Values are presented as AUC (area under the curve) of R-modafinil divided by AUC of IS and as percentage assuming 100.0% at time point 0 h.

	MO in plasma		MO in plasma with10% DMF, sample 1		MO in plasma with10% DMF, sample 2	
Time	**Mean area (MO)/area(IS)**	**% of MO**	**Mean area (MO)/area(IS)**	**% of MO**	**Mean area (MO)/area(IS)**	**% of MO**
0 h	4.2101	100.0	3.5762	100.0	3.9176	100.0
1 h	3.0953	73.5	3.3786	94.5	3.8978	99.5
2 h	3.0423	72.3	3.3466	93.6	3.8961	99.5
4 h	2.0357	48.4	3.2360	95.8	3.8650	99.2

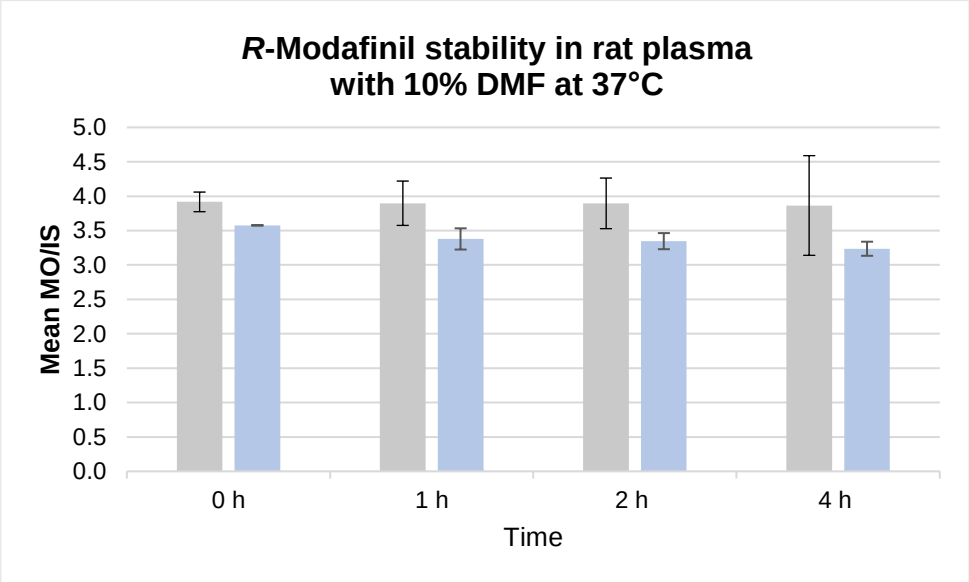

Figure 22: Stability of *R*-modafinil in rat blood plasma containing 10% DMF at 37°C, measured at four different time points of two independent preparations (gray and blue) (n=3, mean ± SD).

4.2. Calibration curves

Calibration curve in bioanalytical method is a linear relationship between concentration (independent variable) and response (dependent variable) using a least squares method. This relationship is built to predict the unknown concentrations of the analyte in a complicated matrix [53].

A calibration curves of *R*-modafinil and *S*-CE-123 consist of a blank sample (ACN), a zero sample (plasma sample processed with IS), and nine non-zero samples covering the expected range (10 – 2500 ng/mL). The data of the ratio of peak area for drug to IS versus drug concentration were treated by linear least square regression analysis. The samples were analyzed in triplicate.

In the **Appendix Table 7** are shown the results of LC-HRMS measuring *R*-modafinil (*m/z* 167.0848) and *CE-114* (*m/z* 357.1065) and calibration curve of modafinil is show in the **Figure 23**. The results showed good linearity with a correlation coefficient of 0.997. According to Prasenjit *et al*. [54] the correlation quotient must exceed 0.980 for the values to be statistically significant, which was achieved. The regression equation derived from the calibration curve was y = 0.4531x+0.0119 where y is the peak area of *R*-modafinil obtained from LC-HRMS analysis and x is the concentration of the drug in ng/mL.

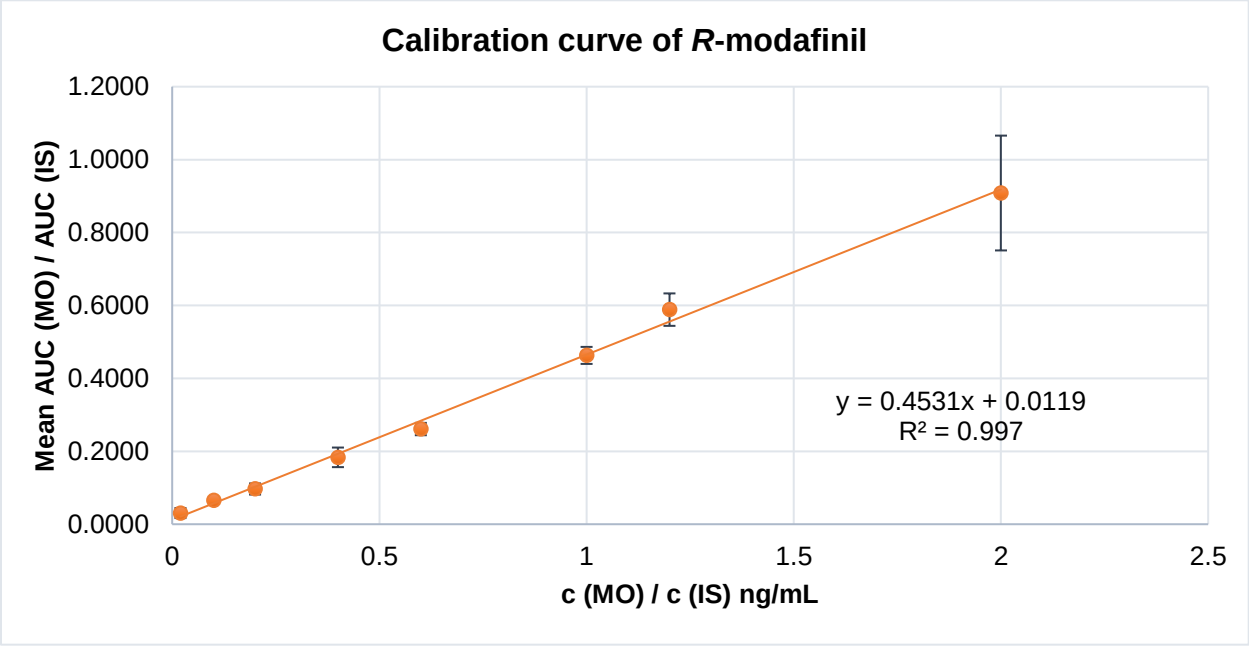

Figure 23: Distribution of values of *R*-modafinil (MO) applying the concentrations of the dilution series. The dilutions series were prepared as following: A mixture of analyte *R*-modafinil at 9 different initial concentrations (10 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 500

ng/mL, 600 ng/mL, 1000 ng/mL and 2500ng/mL) and CE-114 as IS at a constant amount (500 ng/mL). The procedure of preparing dilution series is described in section 3.5.5. *R*-Modafinil was detected at all points of calibration curve. The linear equation revealed a slope of 0.4531 and an intercept of 0.0119 (n=3, mean ± SD).

The calibration curve of *S*-CE-123 is reported in **Figure 24** The calibration curve resulted with correlation quotient (R^2) of 0.9953 and in the linear range from 50 – 2500 ng/mL. This standard curve was used for further quantification of *S*-CE-123 by LC-HRMS. The regression equation derived from the calibration curve was y = 0.3394x+0.0083. All further information is listed in the appendix (**Appendix Table 8**).

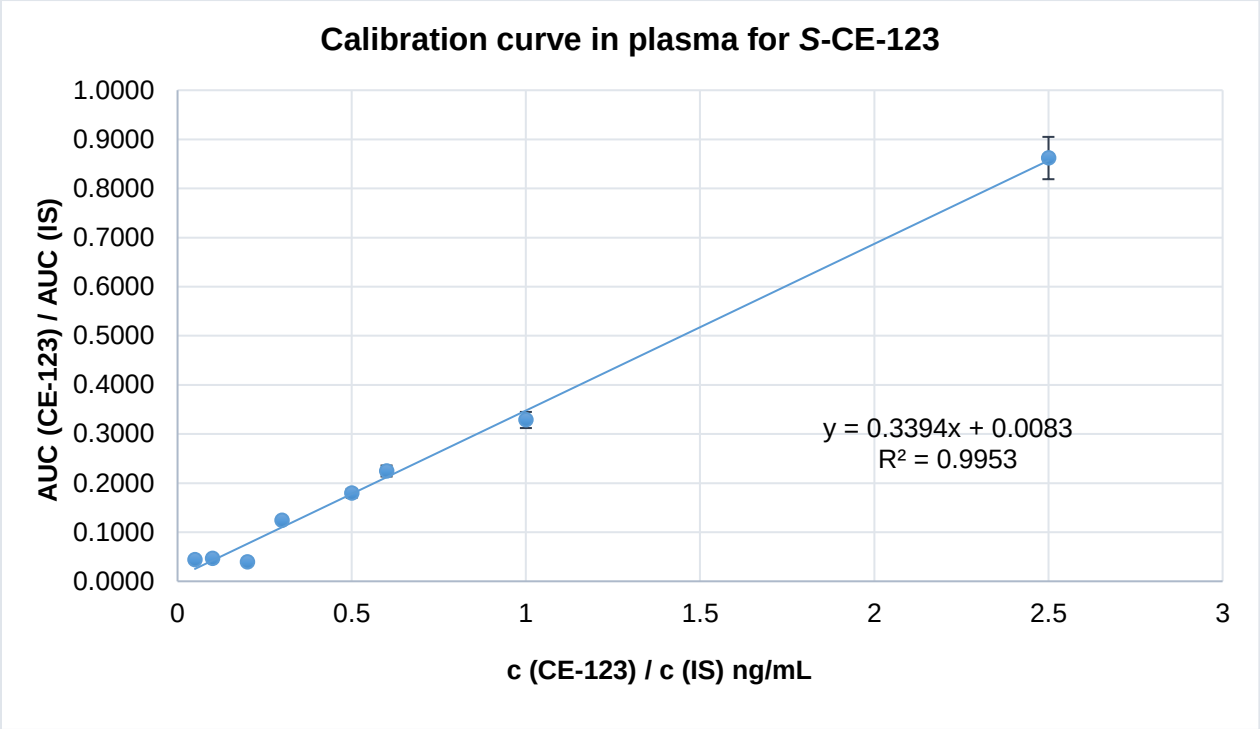

Figure 24: Distribution of values of *S*-CE-123 applying the concentrations of the dilution series. The dilutions series were prepared as following: A mixture of analyte *S*-CE-123 at 9 different initial concentrations (10 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 500 ng/mL, 600 ng/mL, 1000 ng/mL and 2500ng/mL) and CE-114 as IS at a constant amount (1000 ng/mL). The procedure of preparing dilution series is described in section 3.5.1. *S*-CE-123 was detected at 8 points of calibration curve. At point with 10 ng/mL *S*-CE-123 was not detected. The linear equation revealed a slope of 0.3394 and an intercept of 0.0083 (n=3, mean ± SD).

Therefore, additional standards of modafinil acid were prepared at following initial concentration 25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL. The resulting calibration curve for modafinil acid (**Figure 25**) was also linear (R^2 is 0.9951). All further information is listed in the appendix (**Appendix Table 9**).

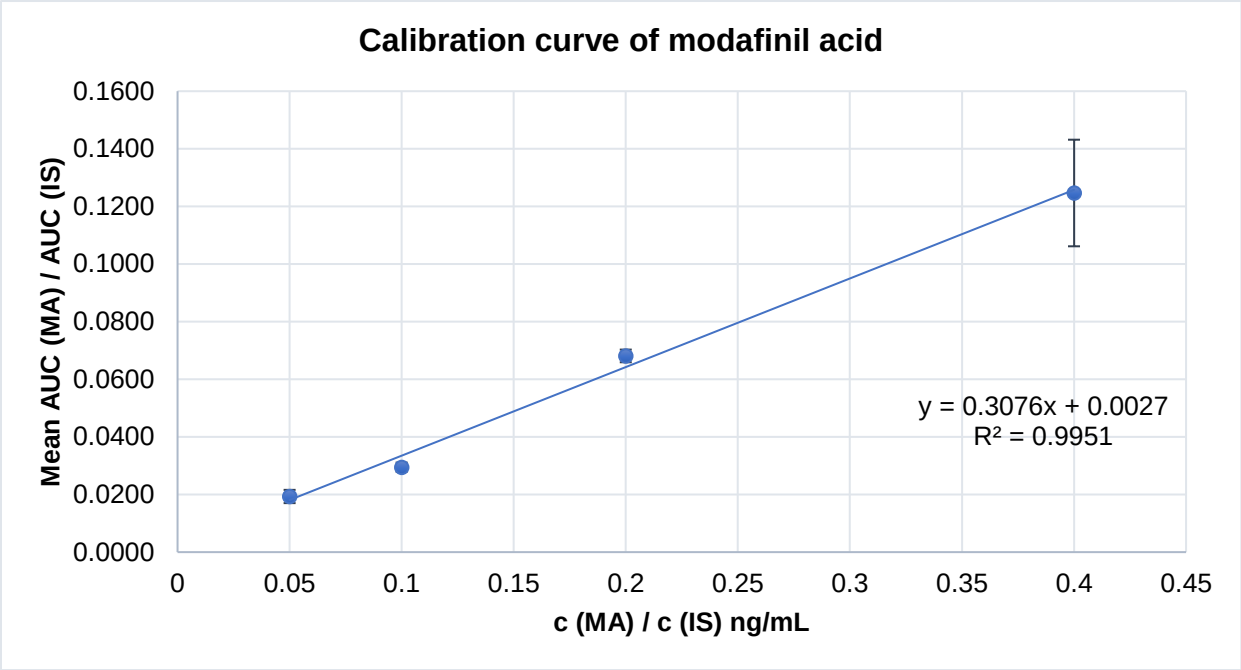

Figure 25: Distribution of values of modafinil acid (MA) applying the concentrations of the dilution series. The dilutions series were prepared as following: A mixture of modafinil acid at 4 different concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) and CE-114 as IS at a constant amount (500 ng/mL). The procedure of preparing dilution series is described in section 3.5.5. Modafinil acid was detected at all points of calibration curve. The linear equation revealed a slope of 0.3076 and an intercept of 0.0027 (n=3, mean ± SD).

4.3. Extracted ion chromatogram (EIC)

The samples from the LC-HRMS measurements were analyzed using software Compass DataAnalysis 4.3 with the aid of ″Extracted ion chromatogram″. The calculated *m/z* values of *R*-modafinil, modafinil acid, *S*-CE-123 and CE-114 positively charged with H^+ and Na^+ were extracted according to their masses with a deviation of ± 0.01 g/mol. For the integration of the peaks the *m/z* 167.0848 for *R*-modafinil and modafinil acid are chosen since they possess a high resolution. The compression of the two adducts shows the significant difference concerning the AUCs. The DPM cations of *R*-modafinil and modafinil acid have the areas of 4.0×10^6 and 4.5×10^5, whereas the hydrogen adducts are 1.1×10^5 and 1.8×10^4, respectively. Regarding *S*-CE-123 and CE-114 the hydrogen adducts have a higher AUCs, in contrast to DPM cation. For the integration of peaks *m/z* 314.0646 for *S*-CE-123 and the *m/z* 357.1065 for CE-114 were applied. **Table 15** demonstrates the respective areas of all substances charged with H^+ and DPM cations in plasma. For all evaluations conducted later in the study, only the more prominent adduct of the drugs was used, which was DPM cation for *R*-Modafinil and modafinil acid, and hydrogen for *S*-CE-123 and CE-114.- The analyzed samples are in lower concentration range, in which less strong adducts cannot be correctly integrated due to decreased resolution and noise issues.

Table 15: Overview of the areas of modafinil, modafinil acid, *S*-CE-123 and CE-114 positively charged with H^+ and DPM cations in plasma. The concentration of modafinil, CE-123 and CE-114 was 1000 ng/mL. The concentration of modafinil acid was 200 ng ng/mL. The *m/z* values used in this study are highlighted in bold.

Test item	*m/z* $[DPM]^+$	*m/z* $[M+H]^+$
***R*-modafinil**	**1146007**	400323
Modafinil acid	**186547**	45517
***S*-CE-123**	1294038	**1317248**
CE-114	985326	**4009841**

4.4. Plasma rat samples

19 rat plasma samples were measured by LC-HRMC and the results were evaluated. The concentration of modafinil was determined for five (MO 500 Tissue 6h, MO 1000 Buffer 6h, MO 1000 Tissue 6h, MO 1000 Thermo Bef, MO 1000 Thermo Aft 6 h) of eleven samples with the calibration curve depicted in **Figure 21**. The remaining six samples were not quantifiable, due to a signal-to-noise ratio below the limit of quantification (LOQ). The limit of detection (LOD) is the lowest concentration of analyte which can be detected and not quantitated as an accurate value. The LOQ is the lowest concentration of analyte which can be quantified [55]. For *R*-modafinil a LOD of 2 ng/mL was determined and for the analyte *S*-CE-123 a LOD of 3 ng/mL. Furthermore, for *R*-modafinil and *S*-CE-123 a LOQ of 10 ng/mL was determined. The results measurements of *R*-Modafinil are reported in **Table 16**. All further information is listed in the appendix (**Appendix Table 10**).

The concentrations were calculated applying the equation of the calibration curve in **Figure 23**. An example of the calculation process of a "**MO 1000 Thermo Bef (Replicate 1)**" sample is demonstrated below:

y=0.4531x+ 0.0119
y=Area (Analyte) / area (IS) =0.186001279
0.186001279=0.4531x+0.0119
x = Concentration (Analyte)/Concentration (IS) = 0.3842
Concentration IS = 500 ng/mL
Concentration (Analyte) = 0.3842 *500 ng/mL ≈ 191 ng/mL

Table 16: Calculated concentrations of *R*-modafinil in rat plasma samples (n=3 for each sample, mean ± CV) with calibration curve for *R*-modafinil.

Sample	Concentration of *R*-modafinil [ng/mL]	CV [%]
MO_500 Buffer_PPB_6 h	-	-
MO_500 Tissue_PPB_6 h	24	9.2
MO_500 Buffer_PPB_24 h	-	-
MO_500 Tissue_PPB_24 h	-	-
MO_1000 Buffer_PPB_6 h	22	6.1
MO_1000 Tissue_PPB_6 h	90	2.9
MO_1000 Thermo_Bef	185	4.4
MO_1000 Thermo_Aft_6 h	28	5.2
MO_1000 Buffer_PPB_24h	-	-
MO_1000 Tissue_PPB_24h	-	-
MO_1000 Thermo_Aft_ 24 h	-	-

As it can be seen in **Table 16**, after the incubation at 37°C for 6 and 24 h six samples out of eleven were not quantifiable. The metabolization of modafinil to modafinil acid in plasma has previously been reported by Wong [12]. We can confirm that *R*-modafinil was metabolized to its inactive metabolite.

In the frame of this work, we developed the HPLC method for the separation of *R*-modafinil from its main metabolite. In nine samples treated with *R*-modafinil, modafinil acid was detected (**Table 17**). The results were evaluated with the calibration curve depicted in **Figure 25**. The other two samples (MO 500 Buffer 6h, MO 500 Buffer 24h) were not quantifiable. The complete list of values is shown in the appendix (**Appendix Table 11**).

The concentrations were calculated applying the equation of the calibration curve in **Figure 25**. An example of the calculation process of a sample "**MO 500 Tissue PPB 6h (Replicate 1)**" is demonstrated below:

y = 0.3076x+ 0.0027

y = Area (Analyte) / area (IS) = 0.076821714

0.076821714 = 0.3076x+ 0.0027

x = Concentration (Analyte)/Concentration (IS) = 0.2409

Concentration IS = 500 ng/mL

Concentration (Analyte) = 0.2409 *500 ng/mL ≈ 120 ng/mL

Table 17: Calculated concentrations of modafinil acid in rat plasma samples (n=3 for each sample, mean ± CV) with calibration curve for modafinil acid.

Sample	Concentration of modafinil acid [ng/mL]	CV [%]
MO_500 Buffer_PPB_6 h	-	-
MO_500 Tissue_PPB_6 h	119	2.7
MO_500 Buffer_PPB_24 h	-	-
MO_500 Tissue_PPB_24 h	146	1.5
MO_1000 Buffer_PPB_6 h	116	2.3
MO_1000 Tissue_PPB_6 h	763	1.4
MO_1000 Thermo_Bef	301	1.9
MO_1000 Thermo_Aft_6 h	411	2.4
MO_1000 Buffer_PPB_24h	98	3.7
MO_1000 Tissue_PPB_24h	392	1.3
MO_1000 Thermo_Aft_ 24 h	636	1.4

The concentration of *S*-CE-123 was determined in all rat samples with the calibration curve depicted in **Figure 24**. The results of calculated concentrations are listed in **Table 18**. All further information are listed in the appendix (**Appendix Table 12**). An example of the calculation of a sample "**CE 500 Buffer PPB 6h (Replicate 1)**" is shown below:

y = 0.3394x+ 0.0083

y = Area (Analyte) / area (IS) = 0.0552311761

0.0552311761= 0.3394x+ 0.0083

x = Concentration (Analyte)/Concentration (IS) = 0.1383

Concentration IS = 500 ng/mL

Concentration (Analyte) = 0.1383 *500 ng/mL ≈ 69 ng/mL

Table 18: Calculated concentrations of *S*-CE-123 in rat plasma samples (n=3 for each sample, mean ± CV) with calibration curve for *S*-CE-123.

Sample	Concentration of *S*-CE-123 [ng/mL]	CV [%]
CE_500 Buffer_PPB_6 h	68	5.23
CE_500 Tissue_PPB_6 h	257	2.6
CE_500 Buffer_PPB _24 h	83	2.7
CE_500 Tissue_PPB _24 h	234	3.2
CE_1000 Buffer_PPB _6 h	160	1.3
CE_1000 Tissue_PPB _6 h	646	1.1
CE_1000 Buffer_PPB _24h	158	3.3
CE_1000 Tissue_PPB _24h	501	0.7

4.5. Discussion

The LC-HRMS assay for simultaneous quantification of modafinil, its main metabolite modafinil acid and the novel analog CE-123 in rat plasma was developed. This method is based on PPT and LC-HRMS analysis with a total run time of less than 11 min.

During the LC-HRMS run a new compound was detected as DPM cation (*m/z*=167.0848) at a retention time of 3.2 minutes (**Figure 16**). Using *m/z* values and EIC we can confirm that new detected compound is modafinil acid (**Figure 17**). Modafinil has been reported to be further metabolized to modafinil acid, which has no pharmacological effect [10]. It was necessary to develop a method to inhibit the metabolization of *R*-modafinil to its metabolite in rat blood plasma. In this work we investigated the method described by Gorman [51] using plasma with 10% DMF. After 2 and 4 h incubation at 37°C the relative amount of modafinil drops down to a minimal value of 93.6% observed after 2 hours in one of the independent preparations. For future research, it is recommended to use DMF (10% by volume) by sample preparation in order to avoid the metabolization of modafinil.

The standard solutions contain *R*-modafinil at nine different initial concentrations (10 – 2500 ng/mL) prepared from the Uppsala University could not be used for the quantification due to containing its metabolite. As there is no baseline separation between *R*-modafinil and modafinil acid (both are detected as DPM cation) we were not able to quantify both compounds in rat plasma samples. The new statistically significant calibration curves of *R*-modafinil (10 – 2500 ng/mL) and modafinil acid (25 – 200 ng/mL) in rat blood plasma with 10 % DMF show good linearity with a correlation coefficient of 0.997 for modafinil and 0.995 for modafinil acid. This process required additional time and resources, however it enabled us to create a statistically significant calibration curve for *R*-modafinil and modafinil acid.

In comparison to modafinil, *S*-CE-123 does not metabolize *in vitro* and as such is more stable. This phenomena could be explained by looking at CE-123 on a molecular level. It has on position 5 an isoproyl-thiadiazole substituent instead of carboxyl-amide group in modafinil (**Figure 3**).

In the preceded study of *S*-CE-123 IS method was applied [47], which used diazepam as IS to quantificate CE-123 in plasma samples. In this study, we investigated diazepam as a potential IS and concluded that diazepam (**Figure 14)** is not suitable as

IS because there is no baseline separation between modafinil and CE-123. An modafinil analog, CE-114 was tested as the IS and shows a retention time of 8.9 minutes. Thus, performing LC-HRMS measurements, is better to utilize CE-114 as IS owing to its higher intensity and not overlapping with the peaks of the plasma.

In this study rat plasma samples were prepared with the use of a commonly applied procedure including protein precipitation with ACN and centrifugation. This technique is rapid and widely used in preparing LC/MS samples for bioanalysis [38], but it has disadvantages. During LC-HRMS analysis of rat plasma samples, a low S/N ratio of samples containing low analyte concentrations was noticed. A possible reason can be a presence of particles (salts, proteins, lipids) in samples [56].

A probable reason of high matrix effect can be reduced by improving the strategy of the sample pretreatment. Using solid phase extraction instead of protein precipitation, for example, can reduce ionization suppression by phospholipids [56].

5. Conclusion

The aim of this diploma thesis was to develop an LC-HRMS assay for simultaneous quantification of modafinil, its main metabolite modafinil acid and the novel analog CE-123 in rat plasma. To reach optimal analytical performance for this assay, chromatographic conditions and new IS were investigated and optimized for our needs. The finally chosen chromatographic parameters and IS are summarized in **3.4.2** and **3.4.3** sections. During the chromatographic analysis of standard solutions containing *R*-modafinil and *S*-CE-123 prepared by the Uppsala University, the main metabolite of *R*-modafinil, modafinil acid, was detected. Thus it was required to develop a method to inhibit a metabolization of *R*-modafinil in rat blood plasma. The literature research revealed that addition of DMF (10% by volume) to the rat plasma inhibited the degradation of modafinil (<5.5% loss after 3 h at room temperature) [51]. The next step was to create a statistically significant calibration curves of *R*-modafinil and modafinil acid in rat blood plasma with 10 % DMF.

The method described has been successfully applied for the simultaneous quantification of CE-123 in rat plasma samples. *S*-CE-123 was previously spiked with the rat plasma to a final drug concentration of 500 ng/mL and 1000 ng/mL respectively and then left to equilibrate between the buffer and plasma compartments in the RED device. After the 6 h and 24 h incubation, the *S*-CE-123 plasma and buffer samples are collected and stored at —80 °C until LC-HRMS analysis. The concentrations of all eight samples were calculated (**Appendix Table 12**). Applying the S/N approach for *S*-CE-123 a LOD of 3 ng/mL and a LOQ of 10 ng/mL were determined.

After LC-HRMS analysis of rat plasma samples containing *R*-modafinil, it was found, that modafinil was metabolized to its main metabolite in all samples. The concentrations of modafinil from five out of eleven samples were calculated (**Appendix Table 10**). The signals of the other rat plasma samples in the present study were below detection limits. Modafinil acid was detected and quantified in nine out of eleven samples containing *R*-modafinil (**Appendix Table 11**). The intensity of modafinil acid in two samples was lower that the LOQ, hence a quantification was not possible. Applying the S/N ratio approach for *R*-modafinil and modafinil acid a LOD of 2 ng/mL and a LOQ of 10 ng/mL were determined.

6. References

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7.Appendix

Appendix Table 1: Applying the stock solution, the dilution series for *R*-modafinil was generated. The table depicts the mixing of standard solutions with water in the ratio supernatant/water 1:2 (v:v).

Standards solutions of *R*-modafinil (ng/mL)	Volume of supernatant taken (µL)	Volume of water added to supernatant (µL)
2500	50	50
1000	50	50
600	50	50
500	50	50
300	50	50
200	50	50
100	50	50
50	50	50
10	50	50

Appendix Table 2: Mixing standards of modafinil acid after the centrifugation with water in the ratio supernatant/water 1:1 (v:v).

Standards solutions of modafinil acid (ng/mL)	Volume of supernatant taken (µL)	Volume of water added to supernatant (µL)
200	50	50
100	50	50
50	50	50
25	50	50

Appendix Table 3: Relative amount of *R*-modafinil (MO) in rat plasma samples (n=3, mean ± SD) during incubation at 37°C (water bath) for 0, 1, 2 and 4 hours.

Time	Repl.	Area (MO)	Area (IS)	Area (MO)/ area (IS)	Mean area (MO)/ area (IS)	SD [ng/mL]	CV (%)
0 h	1	1901849	446263	4.2617	4.2101	0.33	7.75
	2	2344360	520126	4.5073			
	3	2020795	523368	3.8611			
1 h	1	1391510	453632	3.0675	3.0423	0.13	4.14
	2	1888293	598750	3.1537			
	3	1948094	670434	2.9057			
2 h	1	1443850	471152	3.0645	3.0953	0.05	1.56
	2	1784231	581096	3.0705			
	3	1844170	585299	3.1508			
4 h	1	1245550	626730	1.9874	2.0357	0.21	10.41
	2	1420469	766962	1.8521			
	3	1455058	641691	2.2675			

Appendix Table 4: Relative amount of modafinil acid (MA) in rat plasma samples (n=3, mean ± SD) during incubation at 37°C (water bath) for 0, 1, 2 and 4 hours.

Time	Repl.	Area (MA)	Area (IS)	Area(MA)/ area (IS)	Mean area (MA)/ area (IS)	SD [ng/mL]	CV (%)
0 h	1	s.n.d	446.263	-	-	-	-
	2	s.n.d	520126	-			
	3	s.n.d	523368	-			
1 h	1	s.n.d	453632	-	-	-	-
	2	s.n.d	598750	-			
	3	s.n.d	670434	-			
2 h	1	82956	471152	0.1761	0.1545	0.02	14.48
	2	90722	581096	0.1561			
	3	76905	585299	0.1314			
3 **h**	1	186241	626730	0.2972	0.2071	0.08	37.81
	2	129321	766962	0.1686			
	3	99764	641691	0.1555			

*s.n.d.: signal not detected

Appendix Table 5: Relative amount of *R*-modafinil (MO) in rat plasma sample №1 after incubation at 37°C (water bath) for 0, 1, 2 and 4 hours (n=3, mean ± SD).

Time	Repl.	Area (MO)	Area (IS)	Area (MO)/ area (IS)	Mean area (MO)/ area (IS)	SD [ng/mL]	CV (%)
0 h	1	1699847	551218	3.0838	3.5762	0.51	14.38
	2	1967241	556458	3.5353			
	3	1964516	478036	4.1096			
1 h	1	1131576	327603	3.4541	3.3786	0.15	4.56
	2	1952426	609861	3.2014			
	3	2194990	630674	3.4804			
2 h	1	1853840	532489	3.4815	3.3466	0.12	3.50
	2	1898801	580328	3.2719			
	3	1930736	587471	3.2865			
4 h	1	1504581	448810	3.3524	3.2360	0.10	3.17
	2	1771782	554343	3.1962			
	3	1983847	627908	3.1595			

Appendix Table 6: Relative amount of *R*-modafinil (MO) in plasma sample №2 after incubation at 37°C (water bath) for 0, 1, 2 and 4 hours (n=3, mean ± SD).

Time	Repl.	Area (MO)	Area (IS)	Area (MO)/ area (IS)	Mean area (MO)/ area (IS)	SD [ng/mL]	CV (%)
0 h	1	1839273	451976	4.0694	3.9176	0.14	3.65
	2	1968461	505036	3.8977			
	3	1940512	512600	3.7856			
1 h	1	1704852	472725	3.6064	3.8978	0.32	8.27
	2	2294292	540556	4.2443			
	3	2448048	637083	3.8426			
2 h	1	1953021	538263	3.6284	3.8961	0.37	9.45
	2	2230738	595807	3.7441			
	3	2207724	511534	4.3159			
4 h	1	1423287	304784	4.6698	3.8650	0.72	18.75
	2	1754443	537585	3.2636			
	3	2054180	561005	3.6616			

Appendix Table 7: Calibration curve of *R*-modafinil (MO) (*m/z* 167.0848) in plasma with CE-114 (*m/z* 357.1065) as IS.

c (MO) [ng/mL]	Repli.	Area (MO)	Area (IS)	Area (MO)/area (IS)	Mean area (MO) /area (IS)	SD [ng/mL]	CV (%)
2500	1	3392612	1467699	2.311517552	2.1304	0.1575	7.39
	2	4911920	2424367	2.026062886			
	3	5556641	2705794	2.053608294			
1000	1	1146007	1233370	0.929167241	0.9083	0.0445	4.89
	2	2165568	2526096	0.857278583			
	3	2581463	2750474	0.938552046			
600	1	1006042	1638931	0.613840363	0.5885	0.0233	3.96
	2	1393584	2387065	0.583806474			
	3	1645319	2897048	0.567929492			
500	1	851192	1764340	0.48244216	0.4630	0.0169	3.65
	2	1242328	2747974	0.452088702			
	3	1206182	2654588	0.454376348			
300	1	482631	1654796	0.29165589	0.2610	0.0268	10.28
	2	678957	2721368	0.249491065			
	3	678234	2804759	0.241815429			
200	1	277156	1617624	0.171335242	0.1834	0.0152	8.31
	2	526373	2625276	0.200501966			
	3	469689	2635283	0.178230953			
100	1	161814	1615215	0.10018109	0.0966	0.0031	3.19
	2	260484	2754361	0.094571481			
	3	265726	2792315	0.095163332			
50	1	160290	2001423	0.080088017	0.0651	0.0138	21.19
	2	156869	2518115	0.062296202			
	3	134617	2543350	0.052929011			
10	1	60286	1912553	0.031521218	0.0307	0.0012	3.76
	2	75826	2536906	0.029889164			
	3	<LOD	2488950	-			

* LOD: limit of detection

Appendix Table 8: Calibration curve of *S*-CE-123 (*m/z* 314.0646) in plasma with CE-114 (*m/z* 357.1065) as IS.

c (CE-123) [ng/mL]	Repli.	Area (CE-123)	Area (IS)	Area (CE-123)/ area (IS)	Mean area (CE-123)/ area (IS)	SD [ng/mL]	CV (%)
5000	1	7036156	4508678	1.560580729	1.5701	0.0111	0.71
	2	7034672	4445631	1.582378744			
	3	7208578	4598941	1.567443027			
2500	1	3821623	4528945	0.843821906	0.8619	0.0171	1.98
	2	3990052	4545808	0.877743187			
	3	4004805	4634125	0.864198743			
1000	1	1404562	4399621	0.319246135	0.3285	0.0098	2.98
	2	1491053	4552689	0.327510401			
	3	1503245	4437906	0.338728445			
600	1	1102673	4695962	0.234813016	0.2246	0.0089	3.95
	2	1026493	4697890	0.218500859			
	3	1013045	4591967	0.220612430			
500	1	779357	4524577	0.172249693	0.1793	0.0066	3.66
	2	827567	4467890	0.185225464			
	3	816032	4523571	0.180395533			
300	1	570218	4399031	0.129623547	0.1243	0.0059	4.76
	2	541280	4589893	0.117928675			
	3	563465	4498575	0.125254108			
200	1	181279	4694124	0.038618281	0.0392	0.0025	6.39
	2	173790	4687533	0.037074939			
	3	192684	4590641	0.041973223			
100	1	212642	4558451	0.046647863	0.0463	0.0046	9.84
	2	184997	4445743	0.041612167			
	3	226481	4465941	0.050712940			
50	1	199267	4492313	0.044357328	0.0442	0.0021	4.69
	2	189466	4504941	0.042057377			
	3	209611	4537312	0.046197176			
10	1	s.n.d	s.n.d	-	-	-	-
	2	s.n.d	s.n.d	**-**			
	3	s.n.d	s.n.d	**-**			

*s.n.d.: signal not detected

Appendix Table 9: Calibration curve of modafinil acid (MA) *(m/z* 167.0848) in plasma with CE-114 (*m/z* 357.1065) as IS.

c (MA) [ng/mL]	Repli.	Area (MA)	Area (IS)	Area (MA)/ area (IS)	Mean area (MA)/area (IS)	SD [ng/mL]	CV (%)
200	1	186547	1350599	0.138121678	0.1247	0.0185	14.81
	2	308657	2334105	0.132237838			
	3	312282	3013767	0.103618495			
100	1	99248	1429757	0.069415992	0.0681	0.0022	3.16
	2	148950	2268895	0.065648697			
	3	180158	2597909	0.06934731			
50	1	67001	2218808	0.030196844	0.0295	0.0016	5.48
	2	60464	2187900	0.027635632			
	3	68571	2239045	0.03062511			
25	1	32284	1472233	0.021928594	0.0193	0.0023	11.92
	2	36530	1983325	0.018418565			
	3	45546	2588700	0.017594159			

Appendix Table 10: Calculated concentrations of *R*-modafinil (MO) in rat plasma samples (n=3, mean ± SD) calculated with the calibration curve for *R*-modafinil. *R*-Modafinil was determined for five of eleven samples. The other samples were not quantifiable.

Sample	Repli.	Area (MO)	Area (IS)	Area (MO)/ area (IS)	c (MO) [ng/mL]	Mean [ng/mL]	SD [ng/mL]	CV [%]
MO_500 Buffer_PPB_ 6 h	1	s.n.d	2774687					
	2	s.n.d	2835623					
	3	s.n.d	3038830					
MO_500 Tissue_PPB_ 6 h	1	91671	2870100	0.031940002	22.11			
	2	96258	2862315	0.033629422	23.98	**24**	2.2187	9.16
	3	104884	2917976	0.035944093	26.53			
MO_500 Buffer_PPB_ 24 h	1	s.n.d	2821589					
	2	s.n.d	2774607					
	3	s.n.d	2897904					
MO_500 Tissue_PPB_ 24 h	1	s.n.d	2458379					
	2	s.n.d	2487250					
	3	s.n.d	2517238					
MO_1000 Buffer_PPB_ 6 h	1	72909	2192787	0.033249467	23.56			
	2	69391	2225889	0.031174510	21.27	**22**	1.3458	6.11
	3	69202	2225188	0.031099395	21.19			
MO_1000 Tissue_PPB_ 6 h	1	100522	1057544	0.095052310	91.76			
	2	98104	1084096	0.090493831	86.73	**90**	2.5785	2.88
	3	97380	1039626	0.093668300	90.23			
MO_1000 Thermo_Bef	1	406139	2183528	0.186001279	192.12			
	2	373027	2178562	0.171226249	175.82	**185**	8.2075	4.45
	3	388058	2154262	0.180135007	185.65			
MO_1000 Thermo_Aft_ 6 h	1	101067	2644481	0.038218085	29.04			
	2	98760	2474687	0.035593204	26.15	**28**	1.4455	5.23
	3	104822	2774687	0.036966127	27.66			
MO_1000 Buffer_PPB_ 24h	1	s.n.d	2835623					
	2	s.n.d	3038830					
	3	s.n.d.	2934325					
MO_1000 Tissue_PPB_ 24h	1	s.n.d	2183390					
	2	s.n.d	2148133					
	3	s.n.d	2185970					
MO_1000 Thermo_Aft_ 24 h	1	s.n.d	2063746					
	2	s.n.d	2089005					
	3	s.n.d	2052270					

*s.n.d.: signal not detected

Appendix Table 11: Calculated concentrations of modafinil acid (MA) in rat plasma samples (n=3, mean ± SD) calculated with the calibration curve for modafinil acid. As you can see, *R*-modafinil was metabolized to its metabolite. Modafinil acid was determined for nine of eleven samples. The other two samples were not quantifiable.

Sample	Repli.	Area (MA)	Area (IS)	Area (MA)/ area (IS)	c (MA) [ng/mL]	Mean [ng/mL]	SD [ng/mL]	CV [%]
MO_500 Buffer_PPB_6 h	1	s.n.d.	2774687					
	2	s.n.d.	2835623					
	3	s.n.d.	3038830					
MO_500 Tissue_PPB_6 h	1	220486	2870100	0.076821714	120.45	**119**	3.1968	2.69
	2	210421	2862315	0.073514271	115.08			
	3	224750	2917976	0.077022566	120.77			
MO_500 Buffer_PPB_24 h	1	s.n.d.	2821589					
	2	90370	2774607	0.032570378	48.60			
	3	s.n.d.	2897904					
MO_500 Tissue_PPB_24 h	1	231156	2458379	0.094027813	148.41	**146**	2.2142	1.52
	2	227272	2487250	0.091374812	144.18			
	3	231550	2517238	0.091985740	145.16			
MO_1000 Buffer_PPB_6 h	1	166013	2192787	0.075708676	118.66	**116**	2.7129	2.33
	2	161191	2225889	0.072416459	113.30			
	3	165712	2225188	0.074471011	116.71			
MO_1000 Tissue_PPB_6 h	1	498908	1057544	0.471760986	762.52	**763**	10.9820	1.44
	2	504795	1084096	0.465636807	752.44			
	3	498113	1039626	0.479127109	774.38			
MO_1000 Thermo_Bef	1	418664	2183528	0.191737408	307.22	**301**	5.5905	1.86
	2	407382	2178562	0.186995826	299.58			
	3	398604	2154262	0.185030419	296.33			
MO_1000 Thermo_Aft _6 h	1	692752	2644481	0.261961421	421.49	**411**	9.7655	2.38
	2	693984	2774687	0.250112535	402.15			
	3	721917	2835623	0.254588498	409.46			
MO_1000 Buffer_PPB_24h	1	182697	2995269	0.060995189	94.77	**98**	3.5853	3.67
	2	189192	3038830	0.062258172	96.88			
	3	191714	2934325	0.065334958	101.76			
MO_1000 Tissue_PPB_24h	1	548556	2283390	0.240237542	386.05	**392**	5.1959	1.33
	2	526521	2148133	0.245106332	394.02			
	3	540563	2195970	0.246161377	395.81			
MO_1000 Thermo_Aft _24 h	1	761551	1913746	0.397937344	642.39	**636**	8.8508	1.39
	2	809821	2089005	0.387658718	625.81			
	3	720630	1819270	0.396109429	639.47			

*s.n.d.: signal not detected

Appendix Table 12: Calculated concentrations of *S*-CE-123 in rat samples (n=3, mean ± SD) calculated with the calibration curve for *S*-CE-123. *S*-CE-123 was determined for eight samples.

Sample	Repli.	AUC (CE-123)	AUC (IS)	AUC (CE-123) / AUC (IS)	c (CE-123) [ng/mL]	Mean [ng/mL]	SD [ng/mL]	CV [%]
CE_500 Buffer_6 h	1	169851	3075274	0.0552311761	69.14	**69**	3.5864	5.25
	2	148786	2859421	0.0520336110	64.43			
	3	167442	2947191	0.0568140986	71.47			
CE_500 Tissue_6 h	1	370793	2070345	0.1790972036	251.62	**257**	6.7026	2.60
	2	376754	2068950	0.1820991324	256.04			
	3	388856	2067964	0.1880380896	264.79			
CE_500 Buffer_24 h	1	197426	3048872	0.0647537844	83.17	**83**	2.2857	2.74
	2	189360	2847743	0.0664947645	85.73			
	3	194290	3064620	0.0633977459	81.17			
CE_500 Tissue_24 h	1	440168	2698445	0.456155362	228.08	**234**	7.4630	3.19
	2	424162	2563214	0.463112922	231.56			
	3	475110	2748975	0.484772221	242.39			
CE_1000 Buffer_6 h	1	343504	2981772	0.1152012964	157.49	**160**	2.0651	1.29
	2	336110	2848191	0.1180082375	161.62			
	3	344280	2951906	0.1166297301	159.59			
CE_1000 Tissue_6 h	1	965532	2184827	0.4419260655	638.81	**646**	6.9452	1.08
	2	994491	2203366	0.4513507969	652.70			
	3	981627	2197501	0.4467015032	645.85			
CE_1000 Buffer_ 24h	1	348894	2923234	0.1193520601	163.60	**158**	5.1444	3.26
	2	323956	2844322	0.1138956841	155.56			
	3	345474	3061395	0.1128485543	154.02			
CE_1000 Tissue_24h	1	550554	1579866	0.3484814535	499.56	**501**	3.4738	0.69
	2	570488	1628097	0.3504017267	522.71			
	3	527880	1526938	0.3457114827	515.26			