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„Quantification of the Modafinil derivative CE-123 in rat plasma *via* LC-HRMS for pharmacokinetic evaluation“

verfasst von / submitted by

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1. Zusammenfassung/Abstract

1.1 Zusammenfassung

Schlafstörungen und deren Auswirkungen auf unsere Gesundheit sind Themen zunehmenden Interesses. Die Zahl der zugelassenen Arzneimittel für Krankheiten, wie Narkolepsie, ist sehr gering. Die Entwicklung neuer Wirkstoffe ist daher angebracht. Ein neuer und vielversprechender Arzneistoff ist das Derivat des Dopamin-Wiederaufnahmehemmers Modafinil, namens CE-123. Verglichen mit der Muttersubstanz Modafinil, zeigt CE-123 eine selektive Hemmung des Dopamin-Transporters (DAT). Diese Hemmung könnte der Grund für die Abwesenheit von neurologischen Nebenwirkungen sein, die in Studien beobachtet wurde. Außerdem wurden, im Vergleich zu Modafinil, höhere Konzentrationen an CE-123 im Gehirn festgestellt. Studien belegen außerdem, dass CE-123, im Gegensatz zu Modafinil, zu kognitiver Verbesserung führt, ohne ein impulsives Ansprechen zu verursachen. CE-123 wurde deswegen für weitere Tests ausgewählt. Das Ziel unserer Studie war es, eine LC-HRMS Methode zu entwickeln, um die Konzentration von *S*-CE-123 im Plasma älterer Ratten nachzuweisen, welches 2 Stunden nach Arzneistoffverabreichung (10 mg/kg *S*-CE-123) *via* intraperitoneale Injektion entnommen wurde. Als interner Standard wurde eine konstante Menge an *R*-Modafinil (10600 ng/mL) verwendet. Eine Kalibriergerade wurde erstellt und die Geradengleichung zeigte eine Steigung von 2,7942 und einen Achsenabschnitt von 0,365 (R^2= 0,9965). Die Methode wurde für einen linearen Bereich von 650-8700 ng/mL, mit einer akzeptablen Richtigkeit, Präzision und Autosampler Stabilität validiert. Die Nachweisgrenze (LLOD) für *R*-Modafinil lag bei 5 ng/mL. Für *S*-CE-123 wurde eine Nachweisgrenze (LLOD) von 2 ng/mL und eine Quantifizierungsgrenze (LLOQ) von 6 ng/mL bestimmt. Die mit der validierten Methode ausgewerteten

Konzentrationen des Analyten in Rattenplasma, umfassen einen Bereich von 1220-4080 ng/mL mit einem Mittelwert von 3098 ng/mL (%CV = 3,20).

1.2 Abstract

Sleep disorders and their impact on our health are topics of growing interest. The number of drugs authorized for diseases like narcolepsy is very small, therefore the development of new active substances is required. A very promising substance is CE-123, which is a derivative of the dopamine reuptake inhibitor Modafinil. Compared to the parent compound Modafinil, CE-123 apparently is a selective inhibitor of the dopamine transporter (DAT), which could be a reason for the absence of neurological adverse reactions observed in studies. Furthermore, higher brain levels of CE-123 compared to *R*-Modafinil were determined. Studies also show that unlike Modafinil, CE-123 demonstrates cognitive enhancement without impulsive responding and was therefore suitable for further testing. The aim of the present study was to develop a LC-HRMS method for measuring the concentration of *S*-CE-123 in rat plasma. Samples of aged rats were taken 2 hours after administration (10 mg/kg *S*-CE-123) *via* intraperitoneal injection. In this study a constant amount of *R*-Modafinil was applied as the internal standard (10600 ng/mL). A calibration curve was generated and the linear equation revealed a slope of 2.7942 and an intercept of 0.365 (R^2= 0.9965). The method was validated in a linear range of 650-8700 ng/mL, achieving the acceptance criteria for accuracy, precision and autosampler stability. For *R*-Modafinil a LLOD of 5 ng/mL was defined. For analyte *S*-CE-123 the LLOQ was 6 ng/mL and the LLOD was 2 ng/mL. The analyte concentrations in treated rat plasma using the validated method were in a range of 1220-4080 ng/mL with a mean of 3098 ng/mL (%CV = 3.20).

2. Introduction

2.1 Hypersomnia and Psychostimulants

2.1.1 Hypersomnia

Hypersomnia is the generic term for diseases that manifest themselves in excessive daytime sleepiness and include narcolepsy type 1 and 2, idiopathic hypersomnia, Kleine-Levin-Syndrome, sleep deficiency syndrome, hypersomnia caused by organic disease, substance induced hypersomnia and hypersomnia caused by a psychiatric disorder (Rodenbeck *et al*., 2014).

2.1.1.1 Narcolepsy

Narcolepsy is a sleeping sickness with symptoms of recurring periods of hypersomnia or spontaneous falling asleep over at least 3 months. The disease can affect adults, but also children, who then have an excessively long night's sleep or a recurring after-lunch sleep (Rodenbeck *et al*., 2014). The reason for the occurrence of narcolepsy is unexplained. They may be associated with infections or autoimmune diseases. Narcolepsy has a prevalence of 0.05 % and an annual incidence of 0.00074 %, though a high number of unreported cases is assumed (Deutsche Gesellschaft für Neurologie, 2012). Due to the ambiguity of the causes and the low number of authorized drugs, further examination of the disease is indicated. There are two types of narcolepsy: narcolepsy type 1 and 2. The diagnosis criteria for narcolepsy type 1 include a hypocretin deficit in the cerebrospinal fluid, with a maximal hypocretin value of 110 pg/mL or the occurrence of cataplexy with an average sleep latency of maximally 8 minutes. Furthermore, the performance of a multiple sleep latency test (MSLT) or a polysomnography is necessary, whereby two or more sleep-onset rapid eye movement (SOREM) periods must be displayed by the MSLT (Rodenbeck *et al*.,

2014). SOREM is a rapid and abnormal shift from wakefulness to rapid eye movement sleep. The beginning of a sleep period is normally characterized by a time of non-REM sleep, which is skipped when experiencing SOREM (Binder *et al*., 2009). Cataplexy is a syndrome of short periods of muscle weakness caused by intense excitement. The atony can include all skeletal muscles, but commonly involves slurred speech and transient face and neck weakness. The disease can also be accompanied by hypnagogic hallucinations (Scammell *et al*., 2009).

The diagnosis criteria for narcolepsy type 2 contain the absence of cataplexy, the same MSLT criteria as type 1, no hypocretin deficit in the cerebrospinal fluid and no explanation for the symptoms by another disease. Although cataplexy is absent, short periods of muscle weakness caused by stress and anger can occur (Rodenbeck *et al*., 2014).

2.1.1.2 Idiopathic hypersomnia

Patients with idiopathic hypersomnia also suffer from recurring periods of hypersomnia or spontaneous falling asleep over at least 3 months. In contrast to narcolepsy type 1, cataplexy is absent. The criteria request an average sleep latency of maximally 8 minutes or a total sleep time longer than 660 minutes within 24 hours. Furthermore, the polysomnography and a subsequent MSLT demonstrate less than two SOREM periods (Rodenbeck *et al*., 2014).

2.1.1.3 Kleine-Levin-Syndrome

Patients suffering from Kleine-Levin-Syndrome have at least two recurrent episodes of hypersomnia more than once a year or at least every 18 months. The episodes can last from two days to 5 weeks, with symptoms like cognitive dysfunction, changed perception, eating disorder or uninhibited behaviour of

which at least one must occur. Between episodes, patients regain all cognitive functions, as well as normal mood and behaviour (Rodenbeck *et al*., 2014).

2.1.1.4 Sleep deficiency syndrome

This disease also manifests itself in recurring periods of hypersomnia or spontaneous falling asleep over at least 3 months. Furthermore, the illness expresses itself for prepubertal children in behavioural problems if they experience a short sleeping time, caused by matutinal awakening by alarm for at least 3 months. Freedom of symptoms is achieved by the extension of sleeping time (Rodenbeck *et al*., 2014).

2.1.1.5 Hypersomnia caused by organic disease/ substances/ psychiatric disorder

Symptoms for hypersomnia caused by an organic disease, drugs or psychiatric disorder are equal to other forms of hypersomnia and occur for at least 3 months. The diagnosis criteria request an average sleep latency of maximally 8 minutes and an MSLT demonstrates maximally two SOREM periods. All symptoms must be due to the diseases of this group. Examples of typical organic diseases leading to hypersomnia at times are Parkinson’s disease, endocrine disruptions, tumors, metabolic encephalopathies, chronic renal failure, pancreatic insufficiency, toxins and genetic disorders. Substances sometimes causing hypersomnia are intoxicating drugs, alcohol and sedative drugs. Symptoms can also occur when the application of amphetamine or caffeine is stopped. When hypersomnia is caused by a psychiatric disorder, the symptoms of sleepiness occur simultaneously with the psychiatric disease (Rodenbeck *et al*., 2014).

2.1.2 Authorized Drugs for narcolepsy in Austria

- Natrium oxybate: authorized in Austria for narcolepsy and cataplexy for adults (Fachinformation Xyrem®, 2015)
- Modafinil (racemate): authorized in Austria for daytime sleepiness with narcolepsy with or without cataplexy (Deutsche Gesellschaft für Neurologie, 2012)
- Methylphenidate: authorized in Austria for cataplexy (Fachinformation Ritalin®, 2018)
- Antidepressants (SSRI i.e. fluoxetine, reboxetine, venlafaxine; TCA; TeCA; MAOI): authorized in Austria for cataplexy (Deutsche Gesellschaft für Neurologie, 2012)
- Pitolisant hydrochloride (histamine H3 receptor antagonist): authorized in Austria for narcolepsy with or without cataplexy (Fachinformation Waxis®, 2018)

2.1.3 Modafinil

Modafinil is a wake-promoting drug authorized for daytime sleepiness with narcolepsy with (Type 1) or without cataplexy (Type 2), with the registered name Modasomil® (100 mg tablet, Teva Pharma BV, GA Haarlem, Netherlands) (Deutsche Gesellschaft für Neurologie, 2012) (Fachinformation, 2017). The drug was registered in Austria in 1998. The recommended initial daily dose for Modasomil® is 200 mg. The 200 mg dose can be prescribed once daily or 100 mg can be administered twice, in the morning and at midday (Fachinformation, 2017). The first studies regarding Modafinil were conducted in the eighties and nineties, when the substance was registered. A double-blinded,

randomized, placebo controlled, parallel-group study was implemented in 1998 and evaluated the efficacy and safety of Modafinil, making it a new drug for the treatment of sleepiness in narcolepsy. Patients, who were diagnosed with narcolepsy, were treated for 9 weeks with a daily dose of Modafinil of either 200 mg, 400 mg or placebo. Afterwards, an open-label treatment period was conducted. The drug significantly reduced sleepiness and improved the illness significantly. During this study only few adverse events were observed, mostly mild to moderate and dose-dependent. The exact mechanism of Modafinil was unidentified and the dopaminergic interaction of Modafinil was mainly unknown in 1998 (The U.S. Modafinil in Narcolepsy Multicenter Study Group authors., 1998). Three years later a study in narcoleptic dogs was conducted, where the role of the neurotransmitter dopamine in sleep regulation was examined. The outcome of the study was that the antinarcoleptic drugs Modafinil and amphetamine increase extracellular dopamine. Furthermore, in mice the dopamine transporter (DAT) was deleted, leading to increased wakefulness and reducing the time of non-rapid eye movement sleep. Furthermore, these mice did not respond to the wake-promoting effects of Modafinil or methylphenidate. Therefore, the importance of dopamine transporters in sleep regulation were proven (Wisor *et al.*, 2001). Further studies also reported that dopaminergic mechanisms control sleep, therefore the loss of wake-active dopamine cells promotes sleep. Drugs applied in the treatment of narcolepsy have a positive effect on dopamine transmission like reuptake inhibition, increase receptor expression and dopamine release (Burgess *et al*, 2010).

Modafinil shows evidence-based interactions with different receptors, like adenosine, benzodiazepine, dopamine, GABA, histamine, melatonin, noradrenaline, orexin and serotonin, which are all regulators of the sleep-wake

rhythm. *In vitro* and *in vivo* studies showed that Modafinil binds to the dopamine transporter (DAT), leading to a dopamine reuptake inhibition and therefore increased dopamine concentrations in the prefrontal cortex and hippocampus (Fachinformation, 2017) (Kristofova *et al.*, 2018). Furthermore, the binding of Modafinil to the dopamine transporter is different in comparison to cocaine and amphetamine as there was no report of any release of dopamine regarding these drugs (Schmitt Kyle C., Reith Maarten E. A., 2011). DAT inhibitors like cocaine bind to the conformation of DAT which is outward-facing. The binding of the enantiomers *R*- and *S*-Modafinil is distinct from the conformation which is cocaine induced (Loland *et al.*, 2012). Modafinil also binds to the noradrenaline transporter, leading to a noradrenaline reuptake inhibition, which was found to be weaker compared to the dopamine reuptake inhibition. The drug only affects the brain areas responsible for sleep, awakening and vigilance, unlike classical psychostimulants (Methylphenidate, Amphetamine) which affect the neuronal activity of the whole brain (Fachinformation, 2017).

Common side effects of Modafinil are mental problems like nervousness, anxiety, depression and disorientation. Furthermore headaches, a blurred vision, but also tachycardia and palpitations were reported (Fachinformation, 2017). In addition, studies reported that Modafinil can produce impulsive responding, while other analogues like CE-123 demonstrate cognitive enhancement without impulsive responding (Nikiforuk *et al*., 2017). Moreover, other analogues of Modafinil were synthesized, which had higher activities and a higher selectivity towards the DAT than Modafinil (Kalaba *et al*., 2017)

2.1.3.1 Structural formula

The IUPAC name of Modafinil is 2-[(Diphenylmethyl)sulfinyl]acetamide (National Center for Biotechnology Information, 2019). The structural formula of Modafinil is demonstrated in **Figure 1.** Modafinil is one of about 40% of drugs, containing of one or multiple asymmetrical (chiral) centres (Wong *et al*., 1999). Regarding Modafinil, the sulfoxide function possesses the asymmetric centre. The drug was usually administrated as a racemate, because both enantiomers (*S*- and *R*-enantiomer) have a significant pharmacological effect. However, studies showed pharmacological differences between the two enantiomers: The half-life of the *R*-enantiomer is longer than the *S*-enantiomer and the concentration of circulating *R*-Modafinil is three times higher than of *S*-Modafinil (Wang *et al*., 2012). Because of the longer half-life of *R*-Modafinil, the interval between doses can be enhanced and therefore the compliance increases. Racemic Modafinil (Modasomil®) has an effective elimination half-life after multiple dose administration of 100 mg of 15 hours, a plasma protein binding of 60 % and a t_{max} of 2-4 hours. It is metabolized in the liver and its main metabolite is modafinil acid, which has no pharmacological effect (Fachinformation, 2017).

Due to the pharmacological differences between the enantiomers, a drug containing only *R*-Modafinil, called Nuvigil® (formerly called Armodafinil®) (Teva Pharmaceuticals USA, Inc.) was produced. The substance was authorized by the FDA and registered in the USA in 2007 for treatment of narcolepsy, shift work disorder and obstructive sleep apnoea (Wang *et al*., 2012) (Teva Pharmaceuticals USA, 2017).

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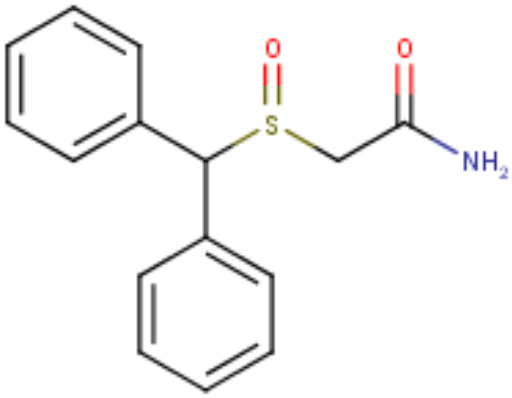

Figure 1: Structural formula of Modafinil, which contains one asymmetrical (chiral) centre (sulfoxide function) (Wong *et al*., 1999) (Wang *et al*., 2012). The IUPAC name of Modafinil is 2-[(Diphenylmethyl)sulfinyl]acetamide (National Center for Biotechnology Information, 2019).

2.1.4 CE-123

The Modafinil derivative CE-123 is a novel compound for enhancing cognitive function which is intensively tested as potential drug. CE-123 showed a blockage of the reuptake of dopamine, while it had only negligible effects on the serotonin and norepinephrine transporter. Compared to the parent compound Modafinil, CE-123 apparently is a selective inhibitor of DAT, which may have been the reason for the absence of neurological adverse reactions was observed in a study. Furthermore, in contrast to cocaine, the drug showed no ionic interaction with ASP79 with a negative charge. In addition, higher brain levels of CE-123 compared to *R*-Modafinil were observed. The evaluation of the memory-enhancing potential of CE-123 in male Sprague-Dawley rats in a spatial-hole-board task showed a unique memory acquisition enhancing effect on day 1 after administration of a single dose (10 mg/kg). Furthermore, Pharmacelsus (Saarbrücken, Germany) performed a pharmacokinetic analysis, where the plasma, brain and CSF levels of CE-123 were examined. Rats were injected with

10 mg/kg CE-123 or *R*-Modafinil and samples of blood, CSF and brain were taken from all rats and measured *via* HPLC–MS (Thermo TSQ Quantum Discovery Max, Thermo Fisher Scientific, USA) 15, 60 and 420 minutes after administration. The plasma levels after 15 minutes was found to be 7.7 ± 1.1 µg/mL for CE-123 and 2.3 ± 1.0 µg/mL for *R*-Modafinil. After 1 hour the respective values were 3.0 ± 0.9 µg/mL for CE-123 and 0.2 ± 0.1 µg/mL for *R*-Modafinil. Brain levels were 4.4 ± 0.5 µg/g for CE-123 and 1.1 ± 0.3 µg/g for *R*-Modafinil after 15 minutes and 2.0 ± 0.4 µg/g for CE-123 and 0.1 ± 0.0 µg/g for *R*-Modafinil after 1 hour. The values for CSF were 0.3 ± 0.1 µg/mL for CE-123 and 0.5 ± 0.2 µg/ mL for *R*-Modafinil after 15 minutes and 0.3 ± 0.04 µg/mL for CE-123 and 0.1 ± 0.03 µg/mL for *R*-Modafinil after 1 hour. Seven hours after administration CE-123 was still detectable in plasma, brain and CSF. As a result, the different plasma, brain and CSF levels of *R*-Modafinil and CE-123 15 minutes and 1 hour after administration demonstrated that the elimination rate constant is higher for CE-123 than for *R*-Modafinil (Kristofova *et al.*, 2018).

Following the pharmacokinetic analysis performed in this study, a modified method was applied to the presented study. 14 rats (Dr. István Gyertyán - Semmelweis University, Institute of Pharmacology and Pharmacotherapy, Budapest, Hungary) at an age of 2 years were injected with 10 mg/kg *S*-CE-123 and 2 hours after the administration, blood samples were taken from all rats to finally quantify the drug content *via* LC-HRMS.

Furthermore, other studies reported that the enantiomeric *S*-form enhances cognitive flexibility without causing impulsive responding at low and high dosages (Nikiforuk *et al*., 2017). Therefore, this derivative is attractive for further testing and was chosen as the analyte for this project. Modafinil was applied as the internal standard, because of its similarity to the analyte.

2.1.4.1 Structural formula

5-[(Diphenylmethylsulfinyl)methyl]thiazole (IUPAC) is a racemate as well as Modafinil. As can be seen in the structural formula shown in **Figure 2**, CE-123 possesses a thiazole instead of the acetamide group present in Modafinil. The substitution of the thiazole instead of the carboxyl-amide group could lead to an increased metabolic stability of CE-123 compared to Modafinil (Kristofova *et al.*, 2018).

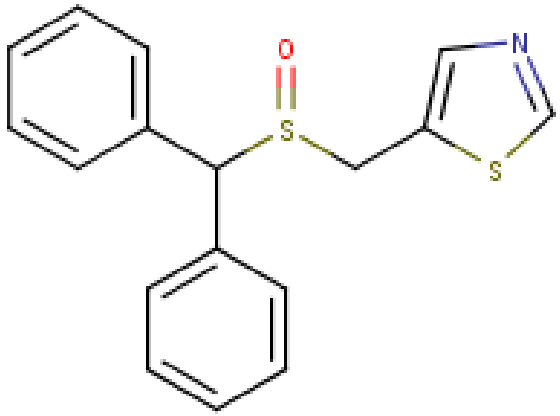

Figure 2: This figure depicts the structural formula of CE-123, which has an IUPAC name of 5-[(Diphenylmethylsulfinyl)methyl]thiazole.

2.2 High-performance liquid chromatography

2.2.1 Chromatography – Introduction

Chromatographic methods are used for separation, detection and quantification of substances of all sorts. In a chromatographic instrument, a sample is inserted at the inlet of a column or other material containing the stationary phase and is transported by the mobile phase throughout the column. The stationary phase can be solid or liquid, while for the mobile phase a gas or liquid is used. Based

on various interactions by the components of the sample with the stationary phase, the components elute from the chromatographic column at different times. The times are called retention times and are defined as the time between sample application and elution. At the end of the chromatographic system, a suitable detector provides signals for the components, which are then analysed (Lundanes *et al*., 2014).

2.2.2 High-performance liquid chromatography – General Concept

High-performance liquid chromatography (HPLC) was a development from the classical column liquid chromatography. The advantage of the HPLC is the higher separation efficiency given by smaller particle sizes of the stationary phase compared to classical methods. The use of smaller particles generated higher backpressure and for this reason, high-pressure delivery units for the mobile phase were required. HPLC can be performed in gradient mode or using isocratic elution. With isocratic elution the composition of the mobile phase is constant, in contrast to a gradient, where the composition changes during the time-dependent gradient (Lundanes *et al*., 2014).

2.2.3 Separation principles

2.2.3.1 Normal-Phase HPLC

Normal-Phase HPLC uses a nonpolar mobile phase and a polar stationary phase for separation. The most common material used as stationary phase is silica, with pore sizes from 6 to 30 nm. The advantage is the rigidness of the silica particles, which is the reason for the frequent use as HPLC packing materials. Silica is used for normal-phase chromatography with organic/nonpolar solvents as mobile phase, such as hexane. Hexane is often combined with more polar solvents, like

ethyl acetate or dichloromethane. With this separation technique analytes containing polar groups, like OH, NH, CO, SO, SH, NO, etc. are stronger retained than ones without polar groups (Lundanes *et al*., 2014).

2.2.3.2 Reversed-Phase HPLC

Reversed-phase HPLC is performed having a reversed elution order compared to normal-phase HPLC. For this reason, based on hydrophobic interactions, the retention of nonpolar analytes is stronger than polar analytes. Aqueous solvents, such as a mixture of acetonitrile/water or methanol/water, often used with additives such as acids or buffers, are common mobile phases for RP-HPLC. Furthermore, nonaqueous solvents are used for hydrophobic molecules, such as lipids. If the HPLC is performed in gradient mode, general starting conditions are 5 % acetonitrile or methanol in water, with a gradually increasing percentage of organic solvent. Common stationary phases in RP-HPLC are based on silica with covalently bonded phenyl-, C2-, C8- and C18-groups. (Lundanes *et al*., 2014).

2.2.4 HPLC instrumentation

The HPLC instrument consists of one or multiple pumps, an injector with automatic or manual injection, one or multiple HPLC columns, at least one detector and a device for data evaluation. Furthermore, the analytical instrument consists of stainless steel or PEEK tubes, through which the solvent is transported between the units. The chromatographic run starts at the inlet of a suitable column, where the sample is introduced often by an autoinjector, followed by the separation in the column and detection. Afterwards, signals are transferred to a PC, where the data is evaluated. The addition of another column is possible, if a single one is not sufficient. Furthermore, a guard column can be

added for protection of the analytical column (Lundanes *et al*., 2014). The schematic illustration of a HPLC instrument is depicted in **Figure 3**.

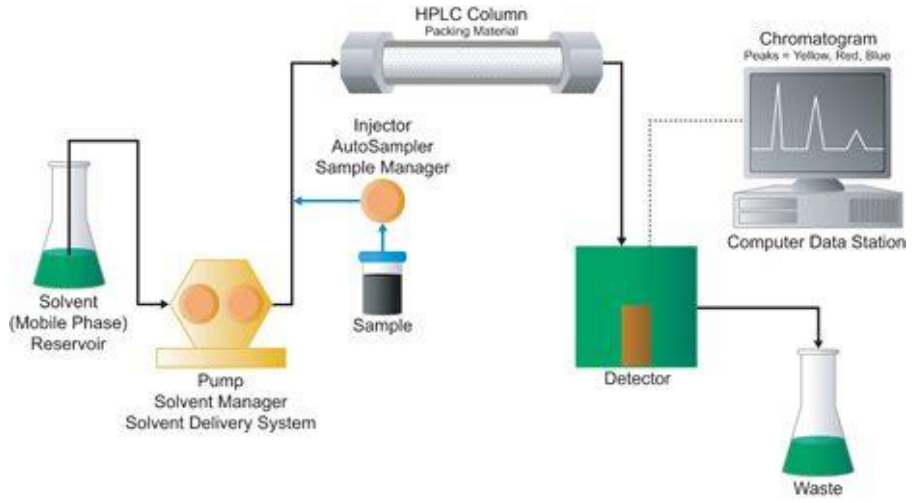

Figure 3: This figure depicts a schematic illustration of a HPLC instrument (Waters Ges.m.b.H, High-Performance Liquid Chromatography [HPLC] System), which consists of one or multiple pumps, an injector with automatic or manual injection, one or multiple HPLC columns, at least one detector and a device for data evaluation (Lundanes *et al*., 2014).

2.2.4.1 Pumps

The mobile phase is delivered by one or two HPLC piston pumps, with a usual flow rate from 0.5-5 mL/min depending on the column size (Lundanes *et al*., 2014) (Lough W. J., Wainer I. W., 1996). The HPLC pump has a pump head, containing check valves and a moving piston. Furthermore, there are miniaturized systems, operating with a flow rate of 1-10 nL/min. Solvents are delivered from a solvent container (minimum of two solvent containers for gradient mode) and a mixing unit. The analytical procedure can be performed using low- or high-pressure mixing. The process is called low-pressure mixing, when the mixture of solvents is generated, before the entrance in the high-pressure system. In contrast, using high-pressure mixing, the solvents are delivered at increased

pressure to the mixing unit. Conventional pump pressures are up to 400 bar, whereas there are also ultrahigh pressure systems operating up to 1000-1200 bar. These systems are applied for columns with small particle sizes below about 3 µm and require connecting tubes to tolerate the high pressures. To guarantee long-term maintenance, it is of great importance to remove all acidic or basic mobile phases or salt solutions from the column. Even if the column is only stored overnight in these conditions, it needs to be washed with aqueous solution and then with 70-100 % acetonitrile or methanol to prevent salt precipitation, corrosion and growth of microbes (Lundanes *et al*., 2014).

2.2.4.2 Sample injection

For manual injection an injection valve is applied. Using a syringe, the sample is transferred into a loop of polyetheretherketone- (PEEK) or stainless-steel tubing or into a rotor groove, by partial- or overfilling. The rotor is located in the load position. When switching to inject position, the loaded contents contact with the mobile phase, which pushes them into the column. The switch can be conducted by an electromotor, manually or by pneumatic activation (Lundanes *et al*., 2014). Automatic injection can be achieved using an autosampler, where the injection step is controlled by a software. Therefore, reproducibility is increased (Markowski Wojciech, 2002.).

2.2.4.3 Columns

Most columns are made from stainless steel, except for capillary and nanoflow columns, which contain fused silica tubing. Furthermore, steel columns are more inert when using a glass inner wall. At the ends of the column, frits are located to hold the stationary phase particles in place. Conventional columns have an inner

diameter of 2-5 mm, a length of 3-25 cm and contain particles of 3-5 µm in diameter. Columns used for UHPLC have an inner diameter of 1-2 mm, a length of 3-15 cm, containing particles of 1.7-1.9 µm in diameter and using a typical flow rate of 100-1000 µL/min. Furthermore, there are also microbore-, capillary- and nanoflow columns, which have an inner diameter between 0.01-1 mm, at a length and particle size similar to conventional columns but using a flow rate between 0.02-100 µL/min (Lundanes *et al*., 2014).

2.2.4.4 Detectors

Traditionally, UV detection are applied in HPLC, but nowadays benchtop mass spectrometers have grown in popularity and are often used in combination with UV detection. Nevertheless, UV detectors are cheaper and more easily accessible and thus preferred if a more sensitive or selective detection is not needed. Other detectors used in HPLC are light scattering, fluorescence, refractive index, electrochemical, corona discharge and conductivity detectors (Lundanes *et al*., 2014).

2.3 Mass spectrometric detection

Detecting with a mass spectrometer is nowadays a very common method, because of its increased robustness, automation, performance and the decreasing cost of instruments (Lundanes *et al*., 2014). The base of mass spectrometry is the ion production from the analyte, their analysation respecting their mass to charge ratio (m/z) and their detection (Lavagnini *et al*., 2006). Essential components of a mass spectrometer are interface, mass analyser and detector (Lundanes *et al*., 2014).

2.3.1 Function and structure

2.3.1.1 Interface

The difference concerning the conditions between HPLC and mass spectrometry is the application of liquids and high pressure in HPLC, while the mass spectrometer operates under high vacuum. For this reason, when coupling the two instruments, a device in between, called an interface, is necessary. In this unit, the ionization and transfer from liquid to gas phase of the samples occur. Most common interfaces in LC-MS are atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), inductively coupled plasma ionization (ICP) and atmospheric pressure photoionization (APPI). ESI, APCI and APPI have a wide range of applications, while ICP is commonly used for the determination of metals. The choice of interface depends on the polarity and size of the analytes (Lundanes *et al*., 2014). In this study, an ESI interface (maXis HD ESI—Qq-TOF mass spectrometer - Bruker Corporation, Bremen, Germany) was applied which can be used for flow rates below 50 mL/min down to nL/min. A reduced flow increases the ionization rate (Lundanes *et al*., 2014). Furthermore, ESI is a suitable interface for our study, because it allows the ionization molecules and even macromolecules with no or only little fragmentation, by making the unfragmented molecule accessible to mass separation and allowing accurate molar mass determination (Altuntas *et al*., 2011).

2.3.1.1.1 Electrospray ionization

In this study the Bruker Apollo II Electrospray Source (Bruker Corporation, Bremen, Germany) was applied. In ESI high voltage (typically + 5 or – 5 kV) is utilized on a liquid steam, which often is the effluent from the HPLC (Lundanes *et al*., 2014) (Bruker Daltonics Training Document). Applying pure electrospray in

mass spectrometry, a nebulizing gas is not necessary. When higher flow rates are applied in LC, a sheat gas is used for nebulization. Larger droplets explode and produce smaller droplets in ESI, when the force of Coulomb repulsion exceeds surface tension. The smaller droplets are exposed to further evaporation. Ion evaporation occurs, when the surface tension is exceeded by the electrical field strength on the droplet surface. The ions are directly emitted into gas phase. In positive mode protonated species and adducts with e.g. sodium, potassium, ammonium etc. are produced. In contrast to this, deprotonation occurs in negative ion mode. Basic analytes, like compounds containing amino groups, are ionized easily by protonation in positive ion mode. Compounds containing acidic groups, like phenols, carboxylic or sulfonic acids, are ionized in negative mode. The molecule loses a proton and receives a negative charge. Ionization *via* adduct formation occurs for polar neutral species which are difficult to protonate or deprotonate. Most important adducts in positive ion mode are Na^+, K^+ and NH_4^+ and $CHOO^-$, CH_3COO^- and Cl^- in negative ion mode. Which adducts are formed depends on the solvent composition (e.g. buffers) and chemical character of the analyte. ESI requires polar solvents, like for instance water, acetonitrile, methanol and iso-propanol. The ionization efficiency depends on the pH of the solvent. Positive ions request acidic pH (<7) and negative ions demand basic pH (>7). Acids, like formic acid 0.1-1 %, acetic acid 0.1-1 % and trifluoroacetic acid ≤0.05 % can be applied as additives in positive ion mode. Typical buffers for ESI are ammonium acetate and ammonium formate (Bruker Daltonics Training Document).

2.3.1.2 Mass analyser

After ionization, the analytes enter the spectrometer through a number of lenses and skimmers and are then analysed by a mass analyser. Common analysers are the quadrupole mass analyser, ion trap-, time-of-flight- (TOF) and fourier transform analysers (FT) (Lundanes *et al*., 2014). In this study a (maXis HD ESI—Qq-TOF mass spectrometer - Bruker Corporation, Bremen, Germany) was applied, because the combination of ESI-MS and high-resolution mass analysers, like the quadrupole-time-of-flight (Q-TOF) analysers provides correct molar masses for samples analysed with accuracies in the ppm area. Valuable information on chemical structures even of macromolecules can be derived (Altuntas *et al*., 2011).

2.3.1.2.1 Quadrupole mass analyser

A quadrupole mass analyser consists of four parallel rods, which create an oscillating electric field by applying direct current (DC) and radio frequency (RF). The entrance of ions in the z-direction causes them to oscillate to the x- and y-direction. The ions are detected by the quadrupole, when they have a steady trajectory and therefore do not collide with the quadrupole rods. The application of specific direct current and RF values causes that only certain *m/z* values pass the quadrupole. By varying DC and RF, whole mass spectra are produced (Lundanes *et al*., 2014).

2.3.1.2.2 Time-of-flight analyser

The analyser measures the time of flight of ions, when entering a field-free drift tube. The TOF consists of two plates and a pulsed source, which has a large potential difference. The source accelerates the ions and when receiving similar

kinetic energy, the ions move toward the drift tube. The flight time of heavy ions is longer than that of light ones, because of the inverse proportionality of velocity and *m/z* value. For this reason, separation with high resolution can occur (Lundanes *et al*., 2014).

2.4 Quantification

Quantification is the determination of concentration of one or multiple analytes in a sample. There are various methods for quantification. In column chromatography, it is based upon an established relationship between the concentration of analyte, which passes the detector and the response of the detector. The response is characterized as the peak area or the peak height, with a signal-to-noise ratio over 10:1 (Lundanes *et al*., 2014).

2.4.1 Calibration methods

There are four different calibration methods, which are used for quantification: normalizing peak areas, external standard, internal standard and standard addition. **Normalizing peak areas** is the simplest method, whereby the sum of the area of all peaks is calculated. Then the quantity of each component is calculated as area%, supposing equal detector response. This method is applied to determine the relative concentration of components in samples, when all compounds elute at given conditions. The other 3 methods give more accurate quantification.

The second method is using **external standards** and a calibration curve is produced by examining calibration solutions with known concentrations of the analyte standard. The response of the detector is plotted as a function of the analyte concentration. The analyte concentration of the samples is calculated by

substituting the value y (peak area or height) in the equation of the calibration curve.

The third method is the **internal standard** (IS) method, where a known concentration of internal standard is added to the samples and all calibration samples. It is necessary for the IS to be separated from the analyte in the chromatogram, unless the detection is conducted with a mass spectrometer. When using this method, the analyte concentration varies, whereas the concentration of the internal standard is kept constant. Furthermore, it is necessary for the IS to have a retention time near the analyte. The IS also needs to have similar behaviour to the analyte regarding sample preparation, derivatization, extraction etc. Moreover, it is important for the IS to have a similar peak height to the expected concentration of analyte, as well as being stable and available with high purity. The calibration curve plots the ratio of the analyte and IS signal intensity as a function of the ratio of the analyte (A) and the IS concentration (Lundanes *et al*., 2014). This was the method applied to the study *R*-Modafinil as internal standard and *S*-CE-123 as analyte. For method evaluation Diazepam was also tested as internal standard. The method was utilized because it is the best option concerning sample preparation and error prevention when the internal standard has similar behaviour to analyte and is available with high purity, which was the case for *R*-Modafinil and Diazepam. Various analytical errors affect an analytical procedure like manipulations of sample and standard solutions or instrumental problems. Therefore, the addition of an internal standard is implied, which compensates the analytical errors. Furthermore, the internal standard is especially effective for highly complex substrates (Lavagnini *et al*., 2006).

The fourth method is **standard addition**, which is used relatively little, because of the need of several analyses per sample, which is very time consuming

(Lundanes *et al*., 2014). The method can be applied for determining an unknown concentration of a known analyte when it situated in an unreproducible matrix (Lavagnini *et al*., 2006). In this method, the sample is split into aliquots, with an addition of various amounts of standard analyte to each aliquot. Then the samples are measured and evaluated, plotting the response as a function of the standard analyte concentration. Extrapolation of the calibration curve leads to the concentration of the analyte in the samples (Lundanes *et al*., 2014).

2.4.2 Method Validation

The method applied in this study was validated for Accuracy, Precision, Linearity, Range, Stability, Detection Limit and Quantification Limit.

2.4.2.1 Accuracy and Precision

Accuracy is the closeness between the experimentally found value and the true value. Precision is the closeness between a number of measurements of the same sample under equal conditions. When examining precision, three levels can be considered: repeatability (intra-day precision: short interval), intermediate precision (inter-day precision) and reproducibility (precision between laboratories). Precision is commonly expressed as variance, standard deviation or %CV (coefficient of variation) (ICH Expert Working Group, 1994).

2.4.2.2 Linearity and Range

Linearity is the ability to obtain results directly proportional to the analyte concentration in the sample. Range is the interval between upper and lower analyte concentrations for which a suitable level of accuracy, precision and linearity was demonstrated (ICH Expert Working Group, 1994).

2.4.2.3 Stability

Method validation also includes measuring the chemical stability of an analyte in a matrix. This includes the effect of collection, handling and storage of the samples. Examples of stability studies are benchtop (stability during expected laboratory conditions for study samples), autosampler (stability of samples stored in the autosampler), stock solution (stability of samples in stock solution), freeze-thaw (stability of samples when undergoing freeze-thaw cycles) and long-term stability (stability of samples over a period of time) (Food and Drug Administration Office, 2018).

2.4.2.4 Detection Limit and Quantification Limit

The limit of detection is the lowest concentration of analyte which can be detected and not quantitated as an accurate value. The limit of quantification is the lowest concentration of analyte which can be quantified (ICH Expert Working Group, 1994).

3. Materials and methods

3.1 Chemicals and materials

R-Modafinil and *S*-CE-123 were synthesised by Dr. Predrag Kalaba, MSc and Vladimir Dragačević, MSc (Jung *et al.*, 2012) (Kristofova *et al.*, 2018). The chemicals used were Diazepam (Batch No 1563, Vitrum AB, Stockholm), water (LC-MS grade, Merck KGaA, Darmstadt, Germany), acetonitrile (ACN) (LC-MS grade, Merck KGaA, Darmstadt, Germany) and formic acid (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 Dr. István Gyertyán (Semmelweis University, Institute of Pharmacology and Pharmacotherapy, Budapest, Hungary).

3.2 Method evaluation

3.2.1 High-performance liquid chromatography/ UV detection

The Shimadzu Nexera XR UHPLC system (DGU-20A5R Degassing unit, LC-20ADXR Liquid Chromatograph Pump, SIL-20AXR Autosampler, SPD-M20A Prominence Diode Array Detector, CTO-20AC Prominence Column Oven) was used for method evaluation. Separation was conducted by applying a Kinetex Phenyl-Hexyl 2.6 µm 100 Å LC column (50x2.1 mm I.D, Phenomenex, Inc., Torrance, CA, United States), preceded by a suitable guard column. The UHPLC run was performed using a gradient with LC-MS grade acetonitrile and LC-MS grade water containing 0.1 % formic acid. The flow rate was set to 600 µL/min.

3.2.1.1 Sample preparation

Stock solutions of the drugs Diazepam, *R*-Modafinil and *S*-CE-123 in ACN with a concentration of 1 mg/mL was prepared, respectively. The powder of each drug

was weighed on an analytical balance (MC210P, Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany), transferred into a 10 mL volumetric flask and filled with acetonitrile. 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 ACN of each drug were then diluted. For the separate measurement of each drug, a 1:10 dilution of Diazepam, *R*-Modafinil and *S*-CE-123 was prepared in ACN. An aliquot of each solution was then filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing) and transferred into 1.5 mL HPLC vials. A mixture of the three 1:10 dilutions was prepared as well. An aliquot of the combination of the three drugs, containing each drug with a concentration of 30 µg/mL in ACN, was filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing) and transferred into a 1.5 mL HPLC vial. Furthermore, a blank sample, containing 1 mL ACN, was prepared. The measurement of a blank sample is necessary to differentiate between the signal of the drug and the background. A blank sample should be included in every analytical measurement.

3.2.1.2 HPLC gradient

Gradient optimization was necessary to achieve baseline separation of analyte and internal standard. Baseline separation is accomplished when the peaks of the compounds do not overlap and the response of the detector returns to baseline between the peaks (Fox M. A., Whitesell J. K., 1994).

Table 1: Gradient 1 is depicted. The following solvent gradient was applied: 5 % solvent B at 0 min, a washing phase at 97 % solvent B from 0.6 to 1.7 min and column reequilibration with 5 % solvent B from 1.8 to 2.5 min.

Mobile phase	Time [min]					
	0.0	0.1	0.6	1.7	1.8	2.5
B [%]	5	5	97	97	5	5
A [%]	95	95	3	3	95	95

The first UHPLC measurement of the 5 prepared samples was performed using the gradient shown in **Table 1**. The following solvent gradient was applied: 5 % solvent B at 0 min, a washing phase at 97 % solvent B from 0.6 to 1.7 min and column reequilibration with 5 % solvent B from 1.8 to 2.5 min. The oven temperature was set to 40 ºC and the sample injection volume was 20 µL, which were chosen as settings for every measurement of this study. The measurements were conducted with an average pump pressure of 285 bar.

The signals were recorded with a diode array detector, observing wavelengths 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.

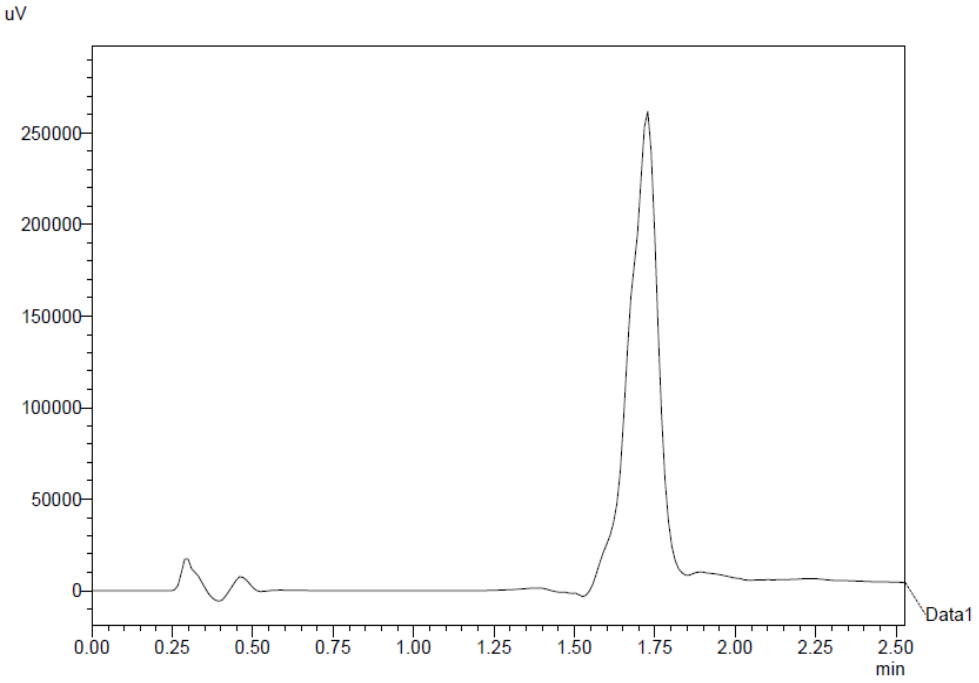

Figure 4: UV chromatogram (254 nm) of the mixture of Diazepam (30 µg/mL), *R*-Modafinil (30 µg/mL) and *S*-CE-123 (30 µg/mL) with gradient 1 showing one peak between 1.5 and 2.0 minutes. Baseline separation was not achieved with this gradient.

As shown in **Figure 4**, baseline separation was not achieved and only one peak was detected (between 1.5 and 2.0 minutes), because the time for separation was not sufficient. Consequently, the gradient was optimized (see **Table 5**).

The timetable of gradient 2, which was extended by 1 minute is depicted in **Table 2**. The following solvent gradient was applied: 5 % solvent B at 0 min, a washing phase at 97 % solvent B from 2.7 to 2.8 min and column reequilibration with 5 % solvent B from 2.8 to 3.5 min. The UV chromatogram of a mixture of Diazepam (10 µg/mL), *R*-Modafinil (10 µg/mL) and *S*-CE-123 (10 µg/mL) with gradient 2 is shown in **Figure 5**. Furthermore, the drugs were measured separately. The comparison of the UV chromatograms (254 nm) of Diazepam (10 µg/mL), *R*-Modafinil (10 µg/mL), *S*-CE-123 (10 µg/mL) and a blank sample (ACN - black) measured with gradient 2 is given in **Figure 6**.

Table 2: Gradient 2 is depicted. The following solvent gradient was applied: 5 % solvent B at 0 min, a washing phase at 97 % solvent B from 2.7 to 2.8 min and column reequilibration with 5 % solvent B from 2.8 to 3.5 min.

Mobile phase	Time [min]				
	0.0	0.1	2.7	2.8	3.5
B [%]	5	5	97	5	5
A [%]	95	95	3	95	95

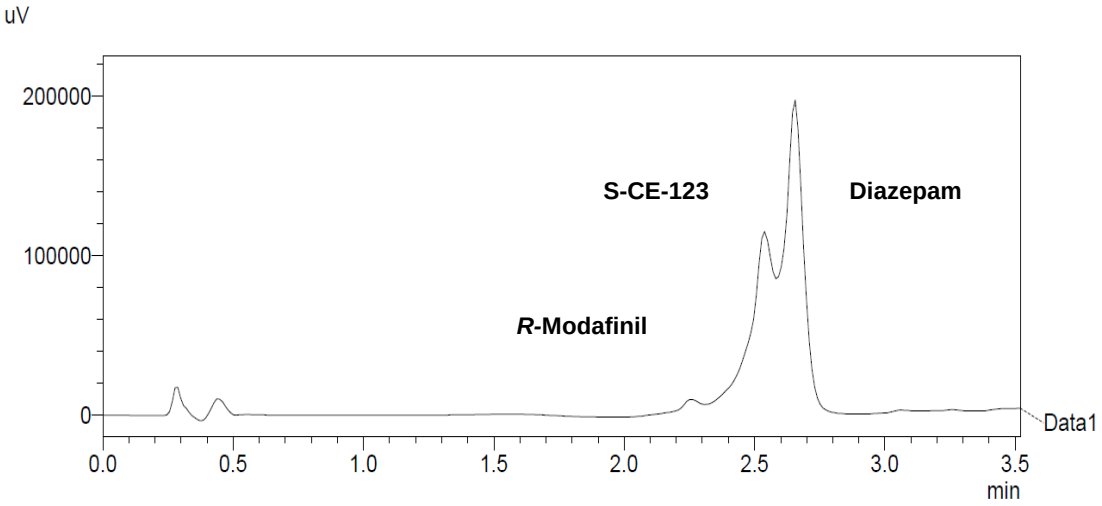

Figure 5: UV chromatogram (254 nm) of the mixture of Diazepam (10 µg/mL), *R*-Modafinil (10 µg/mL), *S*-CE-123 (10 µg/mL) with gradient 2.

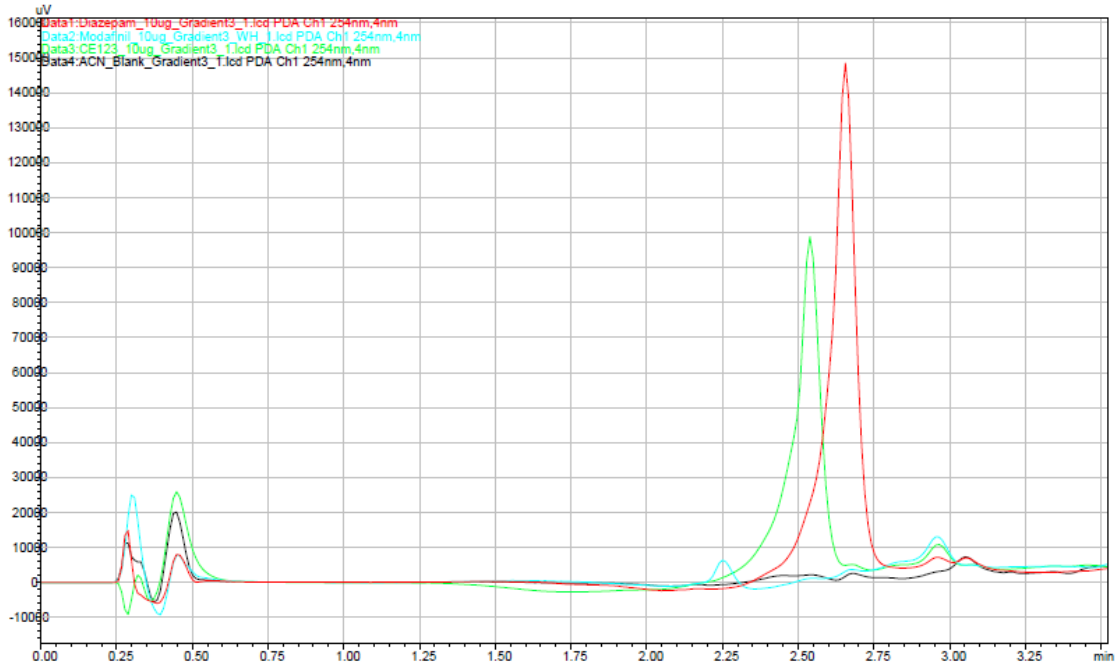

Figure 6: Comparison of the UV chromatograms (254 nm) of Diazepam (10 µg/mL - red), *R*-Modafinil (10 µg/mL - turquoise), *S*-CE-123 (10 µg/mL - green) and a blank (ACN - black) with gradient 2. Baseline separation was not achieved. Gradient adaptation was necessary.

Baseline separation of the three drugs was not achieved with gradient 2. Therefore, gradient 3 was generated, which was further extended to 8 minutes and had a slower increase of concentration of solvent B (ACN) (70% at minute 3.2). The timetable of gradient 3 is depicted in **Table 3** and the UV chromatogram (254 nm) of the mixture of Diazepam (5 µg/mL), *R*-Modafinil (5 µg/mL) and *S*-CE-123 (5 µg/mL) is shown in **Figure 7**. The following solvent gradient was applied: 5 % solvent B at 0 min, 70 % solvent B at 3.2 min, a washing phase at 97 % solvent B from 4.0 to 6.0 min and column reequilibration with 5 % solvent B from 7.0 to 8.0 min.

Table 3: Gradient 3 is depicted. The following solvent gradient was applied: 5 % solvent B at 0 min, 70 % solvent B at 3.2 min, a washing phase at 97 % solvent B from 4.0 to 6.0 min and column reequilibration with 5 % solvent B from 7.0 to 8.0 min.

Mobile phase	Time [min]						
	0.0	0.1	3.2	4.0	6.0	7.0	8.0
B [%]	5	5	70	97	70	5	5
A [%]	95	95	30	3	30	95	95

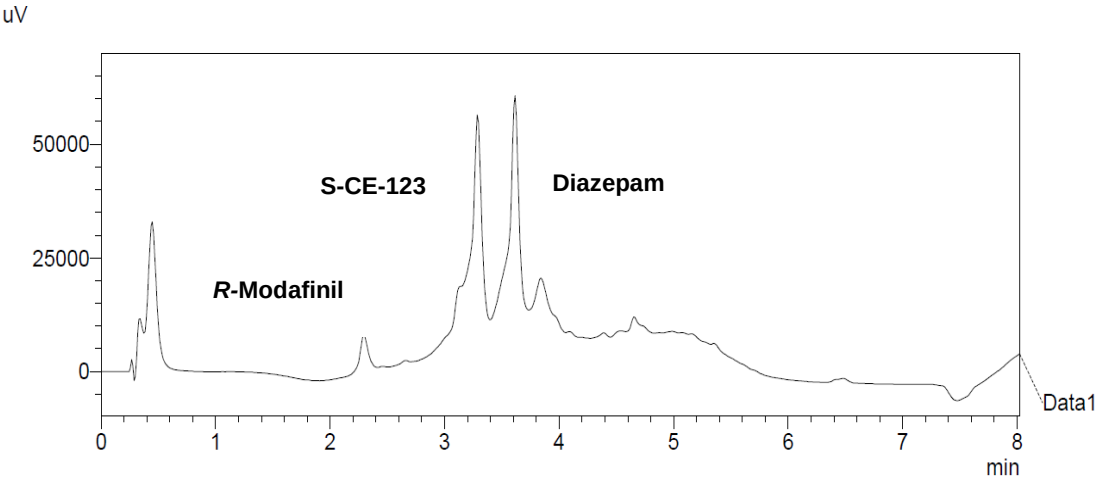

Figure 7: UV chromatogram (254 nm) of the mixture of Diazepam (5 µg/mL), *R*-Modafinil (5 µg/mL), *S*-CE-123 (5 µg/mL) with gradient 3. Baseline separation was not achieved. Further gradient adaptation was necessary.

It was necessary to further extend the gradient, because *S*-CE-123 and Diazepam both eluted at similar timepoints. Therefore, gradient 4 was created, which had a slower increase of concentration of solvent B (ACN) from 30% at minute 5 to 35% at minute 20, which is depicted in **Table 4**. The following solvent gradient was applied: 5 % solvent B at 0 min, 30 % solvent B at 5.0 min, 35 % solvent B at 20 min. The aim was to achieve baseline separation between the peaks of *S*-CE-123 and Diazepam, which was not accomplished. The UV chromatogram (254 nm) of the mixture of Diazepam (5 µg/mL), *R*-Modafinil (5 µg/mL) and *S*-CE-123 (5 µg/mL) with gradient 4 is illustrated in **Figure 8**. The gradient was terminated after 10 minutes, because all substances had already

eluted. Although it would have been possible to separate Diazepam and *S*-CE-123 by the mass spectrometer with Extracted-Ion Chromatography, the disadvantage would have been a reduced accuracy. Consequently, the decision was made to continue the study with Modafinil as internal standard.

Table 4: Gradient 4 is depicted. The following solvent gradient was applied: 5 % solvent B at 0 min, 30 % solvent B at 5.0 min, 35 % solvent B at 20 min.

Mobile phase	Time [min]			
	0.0	0.1	5.0	20.0
B [%]	5	5	30	35
A [%]	95	95	70	65

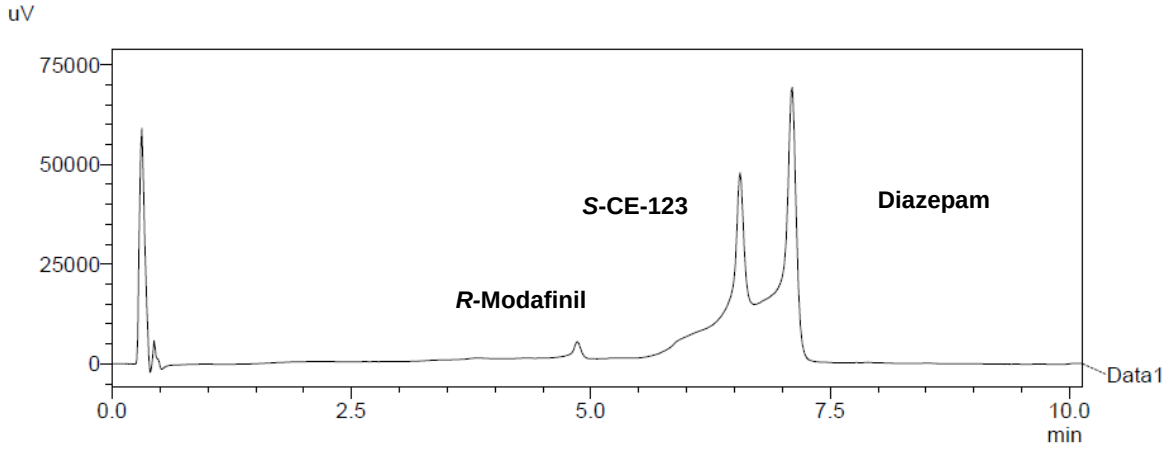

Figure 8: UV chromatogram (254 nm) of the mixture of Diazepam (5 µg/mL), *R*-Modafinil (5 µg/mL), *S*-CE-123 (5 µg/mL) with gradient 4.

The optimized gradient applied for further studies is shown in **Table 5**. The gradient started with 5 % ACN to eluate the hydrophilic components, followed by a washing phase (95 % ACN) to elute the hydrophobic elements and ended with column reequilibration (5 % ACN). A long washing step is necessary to remove all interfering components from the column.

Table 5: Optimized gradient applied for LC-MS studies: 5 % solvent B at 0 min, 30 % solvent B at 5 min, 45 % solvent B at 10 min, 70 % solvent B at 12 min, a washing phase at 97 % solvent B from 13 to 14.5 min and column reequilibration with 5 % solvent B from 14.5 to 15.5 min.

Mobile phase	Time [min]								
	0.0	0.1	5.0	10.0	12.0	13.0	14.0	14.5	15.5
B [%]	5	5	30	45	70	97	97	5	5
A [%]	95	95	70	55	30	3	3	95	95

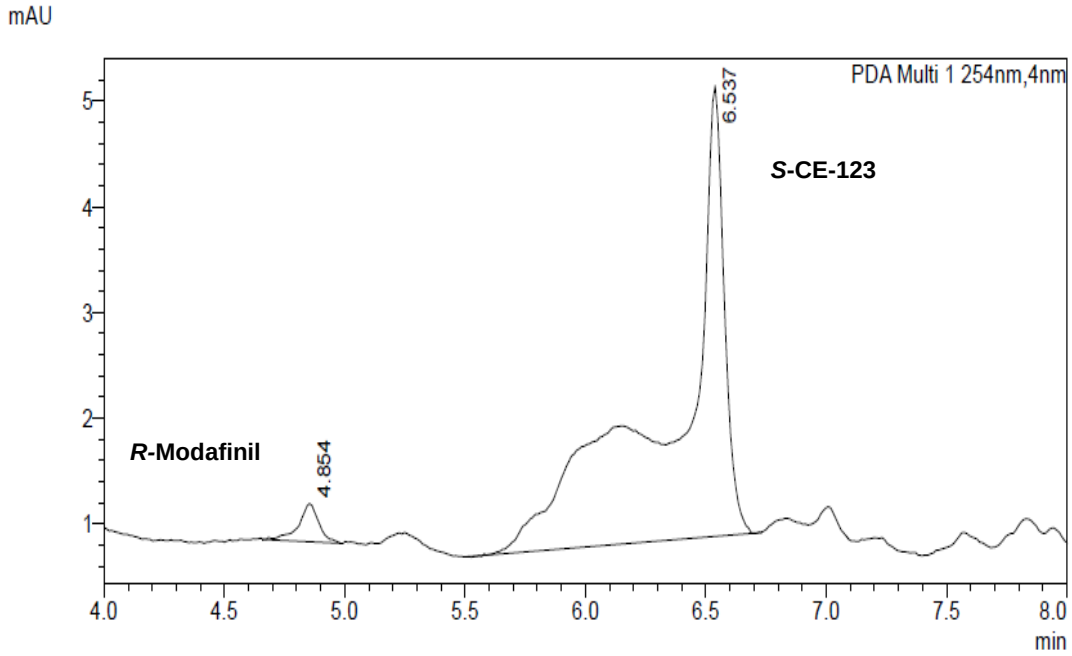

Figure 9: UV chromatogram (254 nm) of the mixture of *R*-Modafinil (10 µg/mL) and *S*-CE-123 (10 µg/mL) with gradient 5. The retention time of *R*-Modafinil was 4.9 minutes at an ACN content of 27 %. *S*-CE-123 had a retention time of 6.5 minutes at an ACN content of 33 %.

The chromatogram of a mixture of *R*-Modafinil and *S*-CE-123 is illustrated in **Figure 9**. The concentration of each drug was 10 µg/mL. *R*-Modafinil had a retention time of 4.9 minutes at an ACN content of 27 %. The retention time for *S*-CE-123 was 6.5 minutes at an ACN content of 33 %. This gradient was chosen to be suitable for the project. The work was proceeded with LC-HRMS, because

mass spectrometry was selected as the best detection method for this study. The sensitivity of the mass spectrometer is higher than the UV detector, therefore low concentrations of the analyte can be quantified.

3.2.2 LC-HRMS

3.2.2.1 Settings

For the liquid chromatography an UltiMate 3000 RSLC-series system (Dionex; Thermo Fisher Scientific, Inc., Gemering, Germany) paired with a maXis HD ESI—Qq-TOF mass spectrometer (Electrospray Ionisation – Double Quadrupole mass analyser – Time-of-flight mass analyser) (Bruker Corporation, Bremen, Germany) was used. The settings of the ESI ion source were: 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 ºC. Mass spectra were recorded in full-scan positive mode in the range of *m/z* 50-2500. Data were analysed using Compass DataAnalysis 4.2 (Bruker Corporation). For quantification, Extracted Ion Chromatograms (EIC) were generated for *S*-CE-123, as analyte, and *R*-Modafinil, as internal standard, which allowed the display of chromatograms of specific *m/z* values. The measurements were performed with an average pump pressure of 289 bar and with an oven temperature of 40 ºC. The flow rate was set to 600 µL/min and the injection volume for every sample was 20 µL. The calculated m/z values of Modafinil and CE-123 positively charged with H^+ and Na^+ and their respective retention times are shown in **Table 6** below. All UPLC-HRMS measurements were achieved with an error $\Delta < 10$ ppm.

Table 6: UPLC-HRMS: Overview of calculated m/z values of *R*-Modafinil and *S*-CE-123 positively charged with H^+ and Na^+ and their respective retention times.

Test item	*m/z* $[M+H]^+$	*m/z* $[M+Na]^+$	RT [min]	Sum Formula
***R*-Modafinil**	274.0878	296.0716	4.5	$C_{15}H_{15}NO_2S$
***S*-CE-123**	314.0644	336.0487	6.2	$C_{17}H_{15}NOS_2$

3.2.2.2 Measurements

A mixture of *R*-Modafinil (5200 ng/1005 µL) and *S*-CE-123 (2600 ng/1005 µL) was measured with gradient 5 *via* LC-HRMS. The comparison of the base peak chromatograms (BPC) of a mixture of *R*-Modafinil (5200 ng/1005 µL), *S*-CE-123 (2600 ng/1005 µL) and a blank sample (ACN) is illustrated in **Figure 10**. The chromatogram of the ACN blank sample shows that the peaks that occur after 9 minutes are caused by the solvents of the gradient (ACN, H_2O, formic acid) and are not associated with the analyte or internal standard. The EICs of *R*-Modafinil (5200 ng/1005 µL) positively charged with H^+ and Na^+ are illustrated in **Figure 11**. The comparison of the two adducts shows the significant difference concerning the intensities. The sodium adduct has an approximate intensity of 1.5×10^6, whereas the intensity of the hydrogen adduct is only about 0.6×10^5. The EICs of *S*-CE-123 (2600 ng/1005 µL) positively charged with H^+ and Na^+ are depicted in **Figure 14**. The comparison shows the significant difference concerning the intensities of the two adducts. The hydrogen adduct has an approximate intensity of 4×10^6, whereas the intensity of the sodium adduct is only about 3×10^5.The identification *via* spectrum identifier is depicted for the sodium adduct of *R*-Modafinil in **Figure 12** and for the hydrogen adduct in **Figure 13**. The molecules were identified with SmartFormula which calculates the possible elemental compositions from a selected mass (Bruker Corporation, Bremen, Germany). For the sodium adduct of *R*-Modafinil the sum formula is

$C_{15}H_{15}NNaO_2S$ with a *m/z* of 296.0716. The sum formula of the hydrogen adduct of *R*-Modafinil is $C_{15}H_{16}NO_2S$ with a *m/z* of 274.0878. The hydrogen adduct of *S*-CE-123 has a sum formula of $C_{17}H_{16}NOS_2$ with a *m/z* of 314.0644, whereas the sum formula of the sodium adduct is $C_{17}H_{15}NNaOS_2$ with a *m/z* of 336.0487. The identification *via* spectrum identifier is illustrated for the hydrogen adduct of *S*-CE-123 in **Figure 15** and for the sodium adduct in **Figure 16**. The measured mass of all molecules corresponded with the calculated mass with an error (Δ) of < 10 ppm.

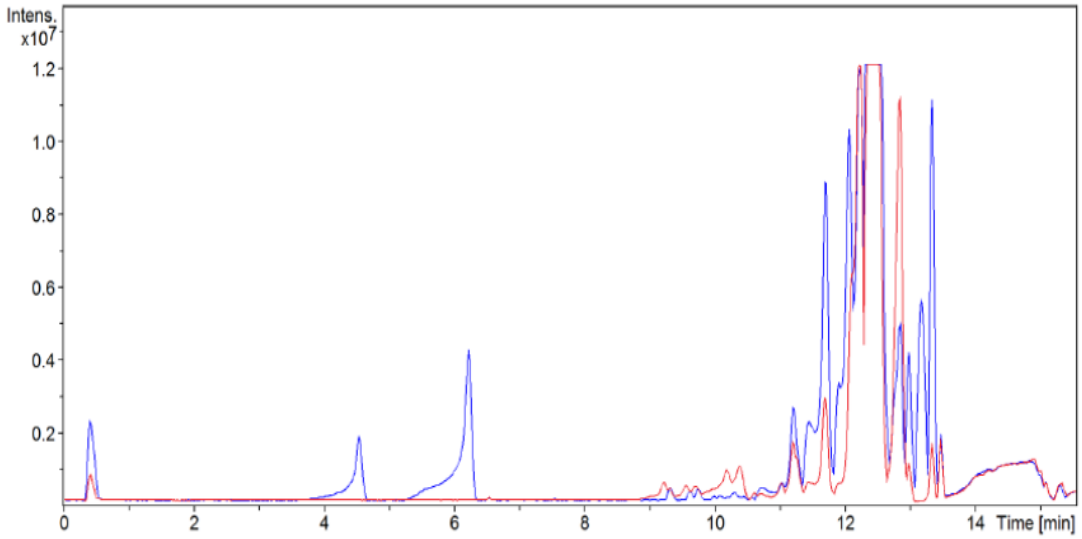

Figure 10: Comparison of the base peak chromatograms (BPC) of *R*-Modafinil (5200 ng/1005 µL - blue), *S*-CE-123 (2600 ng/1005 µL - blue) and a blank sample (ACN - red) measured with gradient 5 *via* LC-HRMS.

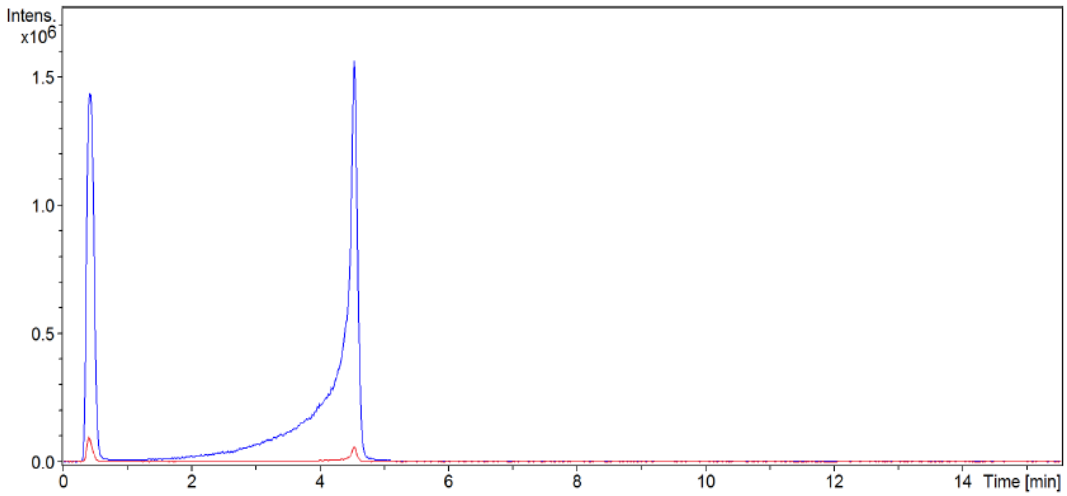

Figure 11: The EICs of *R*-Modafinil (5200 ng/1005 µL) positively charged with H^+ (red) and Na^+ (blue) are depicted in this figure. The comparison shows the significant difference concerning the intensities of the two adducts. The sodium adduct has an approximate intensity of 1.5 x 10^6, whereas the intensity of the hydrogen adduct is only about 0.6 x 10^5.

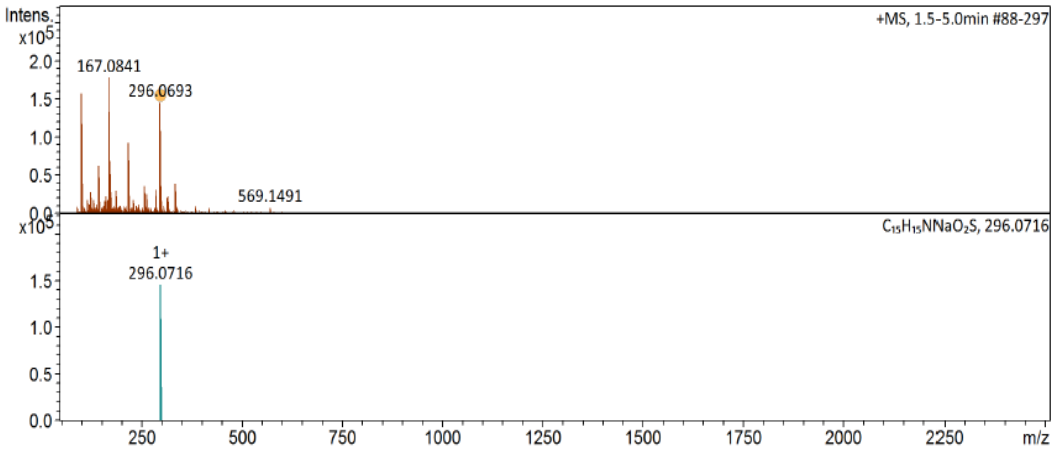

Figure 12: Identification of the sodium adduct of *R*-Modafinil *via* SmartFormula (Bruker Corporation, Bremen, Germany). The measured mass spectrum of *R*-Modafinil with a mark on the sodium adduct (*m/z* = 296.0693) is depicted in the upper chromatogram. The lower chromatogram shows the calculated *m/z* (*m/z* = 296.0716) for the sodium adduct of *R*-Modafinil with a sum formula of $C_{15}H_{15}NNaO_2S$ (Δ <10 ppm).

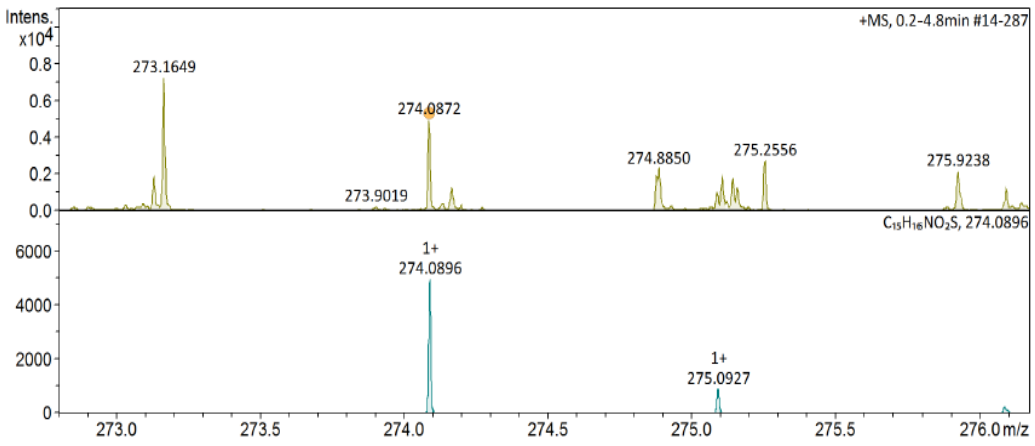

Figure 13: : Identification of the hydrogen adduct of *R*-Modafinil *via* SmartFormula (Bruker Corporation, Bremen, Germany). The measured mass spectrum of *R*-Modafinil with a mark on the hydrogen adduct (*m/z* = 274.0872) is depicted in the upper chromatogram. The lower chromatogram shows the calculated *m/z* (*m/z* = 274.0896) for the hydrogen adduct of *R*-Modafinil with a sum formula of $C_{15}H_{16}NO_2S$ (Δ <10 ppm).

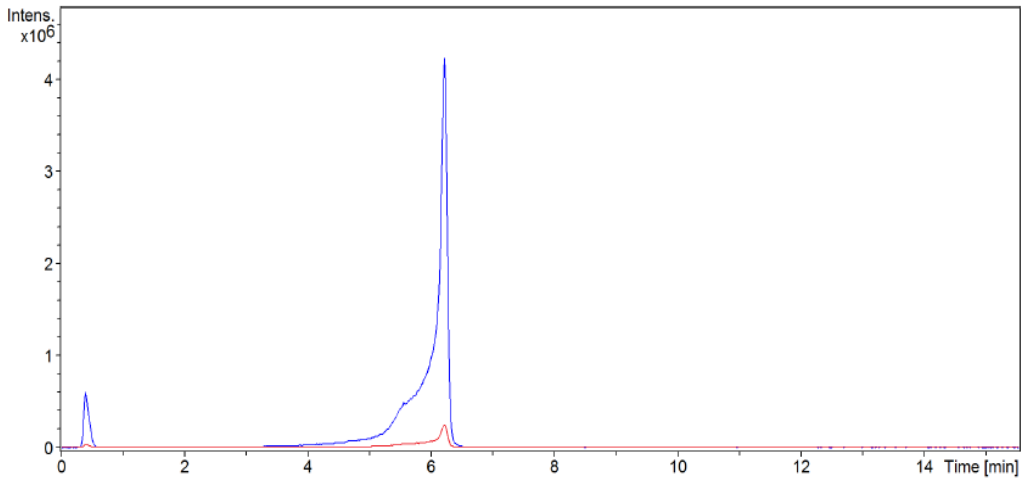

Figure 14: The EICs of *S*-CE-123 (2600 ng/1005 µL) positively charged with H^+ (blue) and Na^+ (red) are depicted in this figure. The comparison shows the significant difference concerning the intensities of the two adducts. The hydrogen adduct has an approximate intensity of 4 x 10^6, whereas the intensity of the sodium adduct is only about 3 x 10^5.

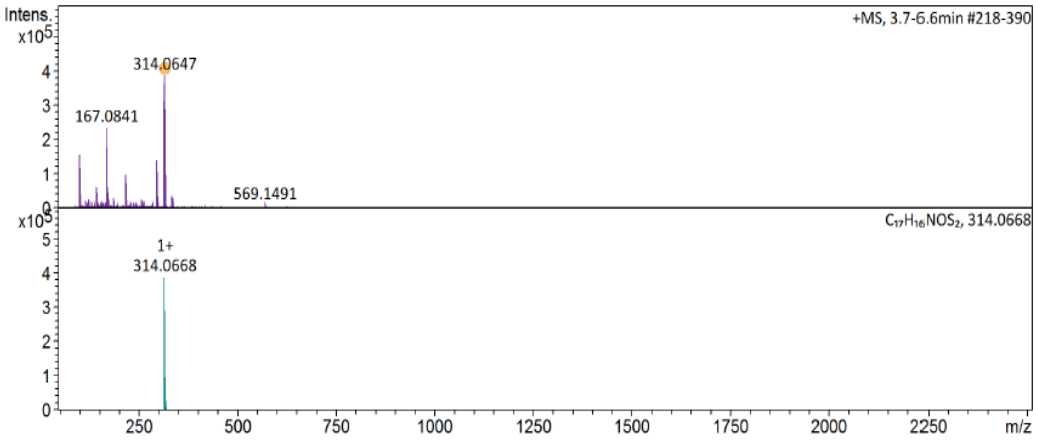

Figure 15 Identification of the hydrogen adduct of *S*-CE-123 *via* SmartFormula (Bruker Corporation, Bremen, Germany). The measured mass spectrum of *S*-CE-123 with a mark on the hydrogen adduct (*m/z* = 314.0647) is depicted in the upper chromatogram. The lower chromatogram shows the calculated *m/z* (*m/z* = 314.0668) for the hydrogen adduct of *S*-CE-123 with a sum formula of $C_{17}H_{16}NOS_2$ (Δ <10 ppm).

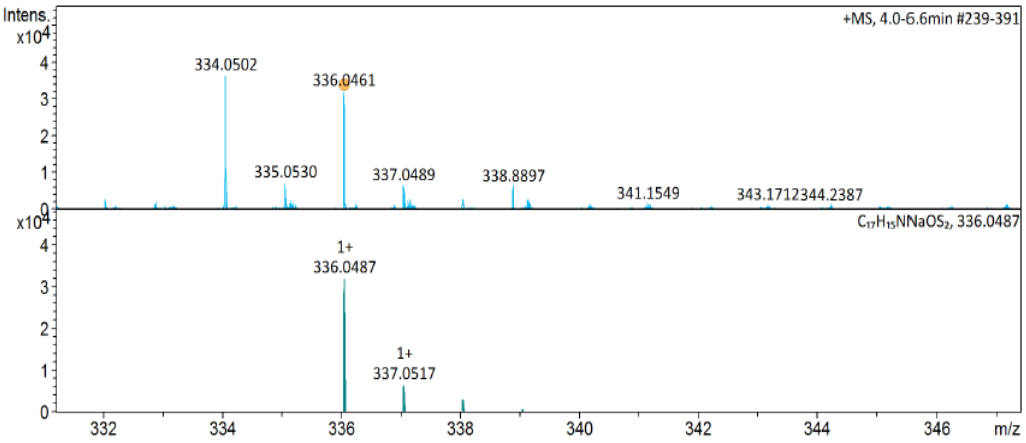

Figure 16 Identification of the sodium adduct of *S*-CE-123 *via* SmartFormula (Bruker Corporation, Bremen, Germany). The measured mass spectrum of *S*-CE-123 with a mark on the sodium adduct (*m/z* = 336.0461) is depicted in the upper chromatogram. The lower chromatogram shows the calculated *m/z* (*m/z* = 336.0487) for the sodium adduct of *S*-CE-123 with a sum formula of $C_{17}H_{15}NNaOS_2$ (Δ <10 ppm).

Although as solvent A acidified water was applied in order to increase the intensity of hydrogen adducts of the compounds (Markgraf B., 2018), the sodium adduct of *R*-Modafinil still shows a significant higher intensity (see 4.3). Regarding *S*-CE-123 the hydrogen adduct has a higher intensity, in contrast to the sodium adduct. For all evaluations conducted later in the study, only the more prominent adduct of the two drugs was used, which was sodium for *R*-Modafinil and hydrogen for *S*-CE-123. The reason for this is that for lower concentrations the less strong adducts were not quantifiable and unevenness would have resolved.

The comparison of the intensities of the two drugs shows that the intensity of *S*-CE-123 is 3 times higher at the same concentration (10000 ng/mL *S*-CE-123 and *R*-Modafinil) than the one of *R*-Modafinil. This characteristic was important to consider, because for an internal standard it is necessary to have a similar peak height to the expected concentration of analyte (Lundanes *et al*., 2014). Therefore, it was necessary to test rat samples with certain amounts of the internal standard *R*-Modafinil, to select a concentration with a similar peak height to *S*-CE-123 of the tested rat sample (see 3.2.2.5).

3.2.2.3 Calibration curve in ACN

A calibration curve of analyte *S*-CE-123 and internal standard *R*-Modafinil was created. The aim was to test the range of linear correlation and to use a similar range for the calibration curve in rat plasma further in the study.

For sample preparation stock solutions of *R*-Modafinil and *S*-CE-123 were prepared following the procedure described in section 3.2.1.1. Using the stock solution, the dilution series for *S*-CE-123 was generated, which is listed in **Table 7** below. A mixture of the two drugs, containing 1000 µL of the respective dilution

of *S*-CE-123 and 5 µL *R*-Modafinil stock solution, was prepared. The concentration of *R*-Modafinil (5000 ng/1005 µL) was kept constant in every mixture. The samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing) and measured *via* LC-HRMS. The measurements were conducted with an average pump pressure of 289 bar. The experiment was implemented in triplicate.

Table 7: Applying the stock solution, the dilution series for *S*-CE-123 was generated. This table depicts the dilution series of analyte *S*-CE-123 and the resulting concentrations [ng/1005 µL].

Concentration of *S*-CE-123 [ng/1005 µL]	Dilution
260	1:1 mixture with solution [530 ng/mL]
530	1:1 mixture with solution [1100 ng/mL]
1100	1:10 dilution of solution [10600 ng/mL]
2600	1:1 mixture with solution [5300 ng/mL]
5300	1:1 mixture with solution [10600 ng/mL]
6600	1:1 mixture with solution [13300 ng/mL]
10600	1:100 dilution of stock solution [1 mg/mL]
13200	1:1 mixture with solution [26600 ng/mL]
26400	1:1 mixture with solution [53100 ng/mL]
52800	1:20 dilution of stock solution [1 mg/mL]

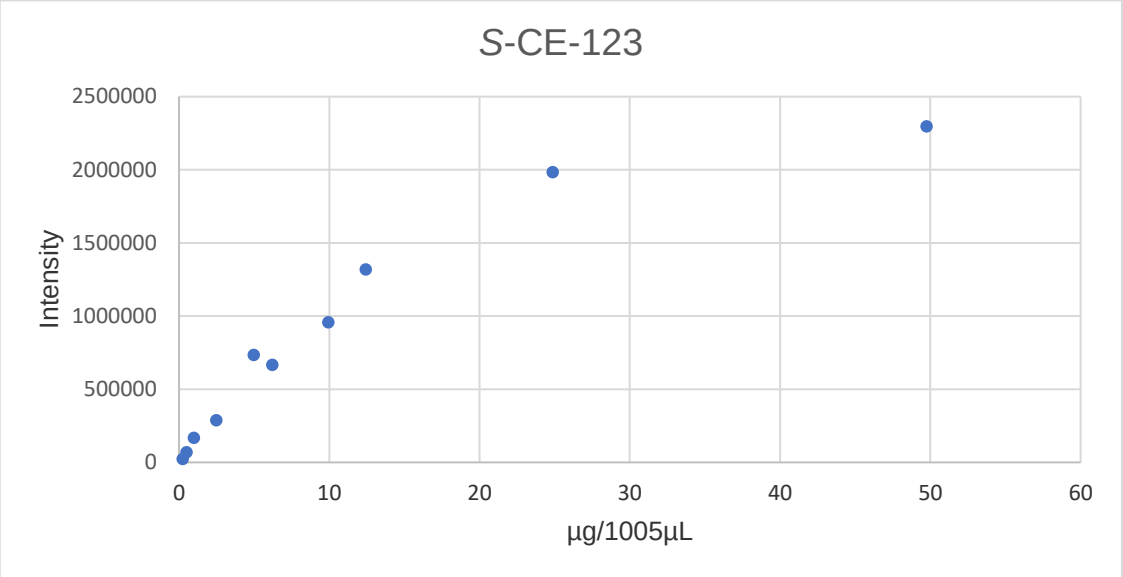

Figure 17: Distribution of values of analyte *S*-CE-123 applying the concentrations of the dilution series. The intensity of S-CE-123 as a function of the concentration is shown. Without considering the outlier at 5300 ng/1005 µL, linear correlation is given up to a concentration of 10600 ng/1005 µL.

The distribution of values of *S*-CE-123 is demonstrated in **Figure 17**. The figure shows the intensity of *S*-CE-123 as a function of the concentration. Without considering the outlier at 5300 ng/1005 µL, linear correlation is given up to a concentration of 10600 ng/1005 µL. At higher concentrations the curve flattens off. For this reason, only these values were included in the calibration curve and are shown in **Table 8**.

Table 8: This table depicts the dilution series and concentrations [ng/1005 µL] included in the calibration curve of analyte *S*-CE-123.

Concentration of *S*-CE-123 [ng/1005 µL]	Dilution
260	1:1 mixture with solution [530 ng/mL]
530	1:1 mixture with solution [1100 ng/mL]
1100	1:10 dilution of solution [10600 ng/mL]
2600	1:1 mixture with solution [5300 ng/mL]
6600	1:1 mixture with solution [13300 ng/mL]
10600	1:100 dilution of stock solution [1 mg/mL]

The calibration curve was generated by plotting the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=3 for each concentration, mean ± SD). The linear equation revealed a slope of 3.3081 and an intercept of 0.3722 (Lundanes *et al*., 2014).

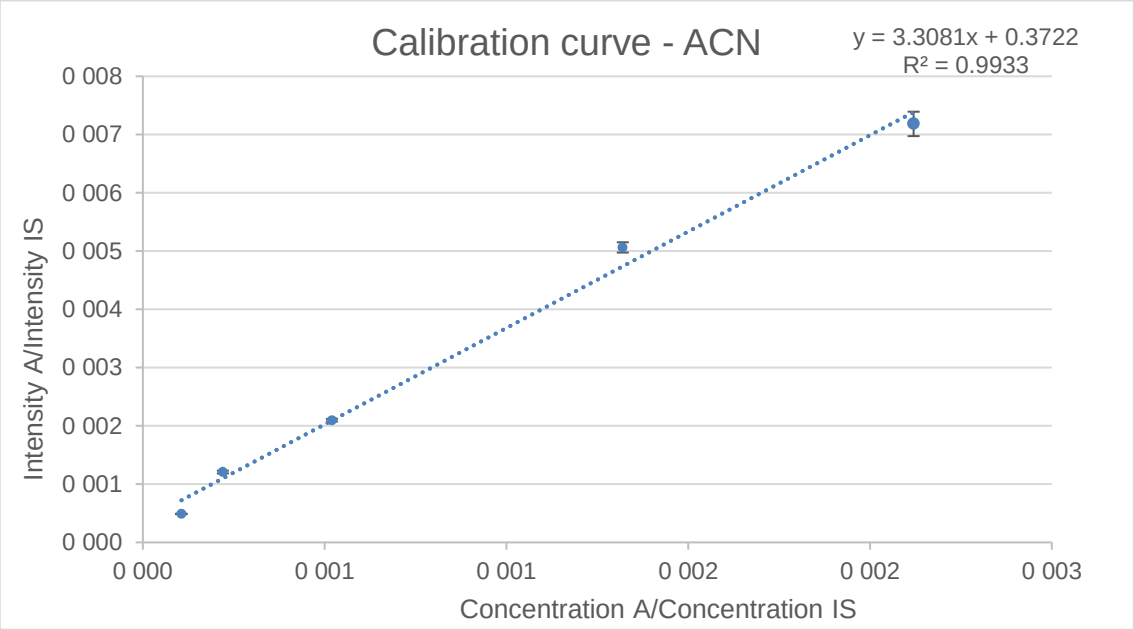

Figure 18: Samples were prepared as following: A mixture of analyte *S*-CE-123 at 6 different concentrations (260 ng/1005 µL, 530 ng/1005 µL, 1100 ng/1005 µL, 2600 ng/1005 µL, 6600 ng/1005 µL and 10600 ng/1005 µL, respectively) and IS *R*-Modafinil at a constant amount (5000 ng/1005 µL). The samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing, Altmann Analytik, München, Germany) and aliquoted into 1.5 mL vials. They were immediately measured via LC-HRMS (20 µL injection volume). The calibration curve plots the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=3 for each concentration, mean ± SD). The linear equation revealed a slope of 3.3081 and an intercept of 0.3722.

The calibration curve is demonstrated in **Figure 18**. Furthermore, the error bars and the correlation coefficient are shown. The correlation coefficient has to surpass R^2= 0.98 (Prasenjit *et al*., 2016), which was achieved with this calibration curve (R^2 = 0.9933). All further information is listed in the appendix (*Appendix Table 1*). An extract of chromatograms of the calibration curve is depicted in **Figures 10-16**.

3.2.2.4 Gradient adaptation – Washing phase

Before a calibration curve in rat plasma was produced, it was necessary to evaluate whether the washing phase at the end of the gradient was sufficient or has to be prolonged. For this reason, the gradient was extended to a 5 minute longer washing step.

For evaluation, plasma of untreated rats was used. For each sample 100 µL control plasma was mixed with 200 µL ACN. Then the samples were centrifuged for 10 minutes at 4500 g at 20 °C and the supernatant was mixed 1:1 with water. Afterwards, they were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing), aliquoted into 200 µL vials and then measured *via* LC/HRMS. The average pump pressure was 289 bar. The samples were prepared in duplicates to avoid systematic error.

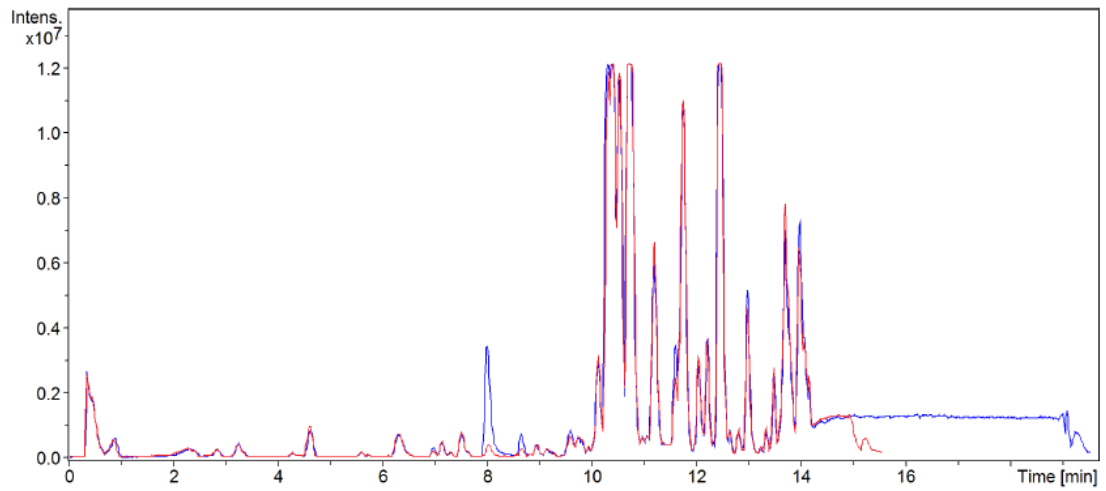

Figure 19: Comparison of the BPCs of a blank sample (ACN) measured with gradient 5 (red) and with the gradient containing an extended washing step (blue).

The comparison of the chromatograms of the two gradients is illustrated in **Figure 19** and shows that a longer washing phase was not necessary, because all interfering components were removed with gradient 5. For this reason, a change of gradient was not necessary.

3.2.2.5 Testing rat samples – Appropriate concentration of IS

Furthermore, it was important for the IS to have a similar peak height to the expected concentration of analyte (Lundanes *et al*., 2014). For this reason, a test sample from a rat treated with *S*-CE-123 was examined.

100 µL plasma of a treated rat was spiked with 100 µL ACN and 100 µL ACN, containing the internal standard in a test concentration. The further procedure was identical to 3.2.2.4. The EIC chromatogram of *R*-Modafinil and *S*-CE-123 is demonstrated in **Figure 20** below. After the examination, about 10000 ng/mL was found to be a suitable IS concentration for the expected analyte, because of the similar peak height of analyte and internal standard. Baseline separation was achieved as well with this IS concentration.

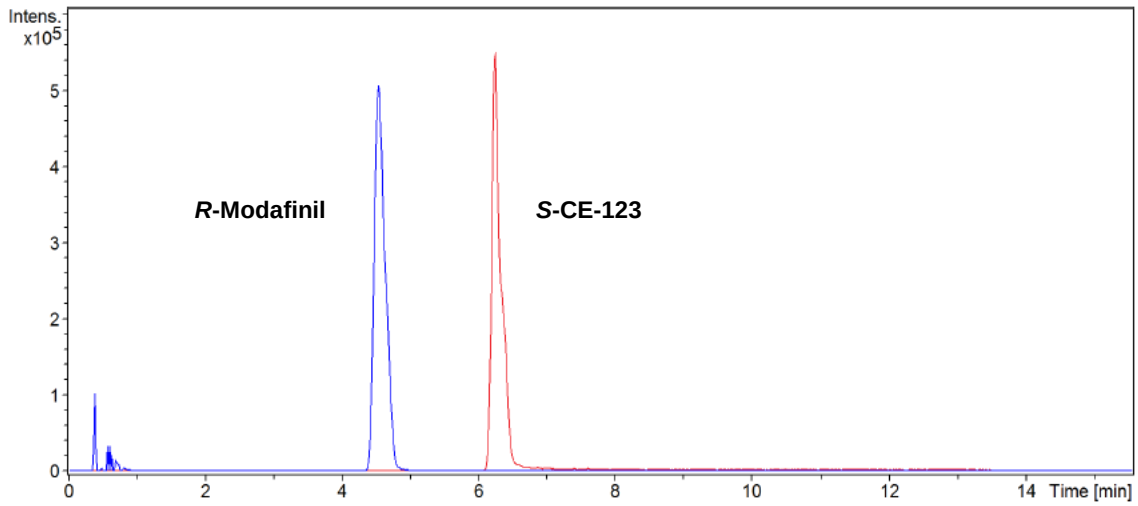

Figure 20: Rat 66: EIC of *R*-Modafinil (blue) (10000 ng/mL) as IS and *S*-CE-123 (red) as analyte. This IS concentration (10000 ng/mL) was found to be suitable for the expected analyte concentration, because of the similar peak height of analyte and internal standard.

3.2.2.6 Calibration curve – Plasma samples

For sample preparation, stock solutions of *R*-Modafinil and *S*-CE-123 were prepared following the protocol described in 3.2.1.1. Later, the dilution series for *S*-CE-123 was generated, which is listed in **Table 9** below.

Table 9 – Applying the stock solution, the dilution series for *S*-CE-123 was generated. This table depicts the dilution series of analyte *S*-CE-123 and the resulting concentrations [ng/mL].

Initial concentration of *S*-CE-123 [ng/mL]	Dilution
650	1:1 mixture with solution [1300 ng/mL]
1300	1:1 mixture with solution [2600 ng/mL]
2600	1:1 mixture with solution [5200 ng/mL]
4200	1:25 dilution of stock solution [1 mg/mL] - further diluted: 1:10
5200	1:20 dilution of stock solution [1 mg/mL] - further diluted: 1:10
8700	1:3 mixture with solution [104000 ng/mL] -further mixed: 1:2

After producing the dilution series, 100 µL control plasma was spiked with 100 µL ACN containing *S*-CE-123 at 6 different initial concentrations (650 ng/mL, 1300 ng/mL, 2600 ng/mL, 4200 ng/mL, 5200 ng/mL and 8700 ng/mL, respectively) and 100 µL ACN containing the IS *R*-Modafinil at a constant amount (10600 ng/mL). Then the samples were centrifuged for 10 minutes at 4500 g, 20 °C. The supernatant was mixed 1:1 with water. The samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing) and aliquoted into 200 µL vials. They were immediately measured *via* LC-HRMS. The average pump pressure was 290 bar. A blank sample, containing only plasma and ACN, was also measured.

An extract of chromatograms of the calibration curve (IS: *R*-Modafinil 10600 ng/mL, analyte: *S*-CE-123 2600 ng/mL) is illustrated in the figures below. The comparison of the BPCs of control plasma samples containing the internal standard (*R*-Modafinil 10600 ng/mL) and analyte (*S*-CE-123 2600 ng/mL) and of a blank plasma sample containing ACN is depicted in **Figure 21**. The addition of the blank plasma sample shows that the peaks that occur after 10 minutes are caused by plasma and the solvents of the gradient (ACN, H_2O, formic acid) and are not associated with the analyte or internal standard. The comparison of the EICs of IS *R*-Modafinil positively charged with Na^+ (10600 ng/mL) and analyte *S*-CE-123 positively charged with H^+ (2600 ng/mL) is demonstrated in **Figure 22**.The calibration curve, including error bars and a correlation coefficient of R^2= 0.9965, is depicted in **Figure 23**. The calibration curve plots the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=4 for each concentration, mean ± SD). The linear equation revealed a slope of 2.7942 and an intercept of 0.0365 applied for quantification. The complete list of values is shown in the appendix (*Appendix Table 2*).

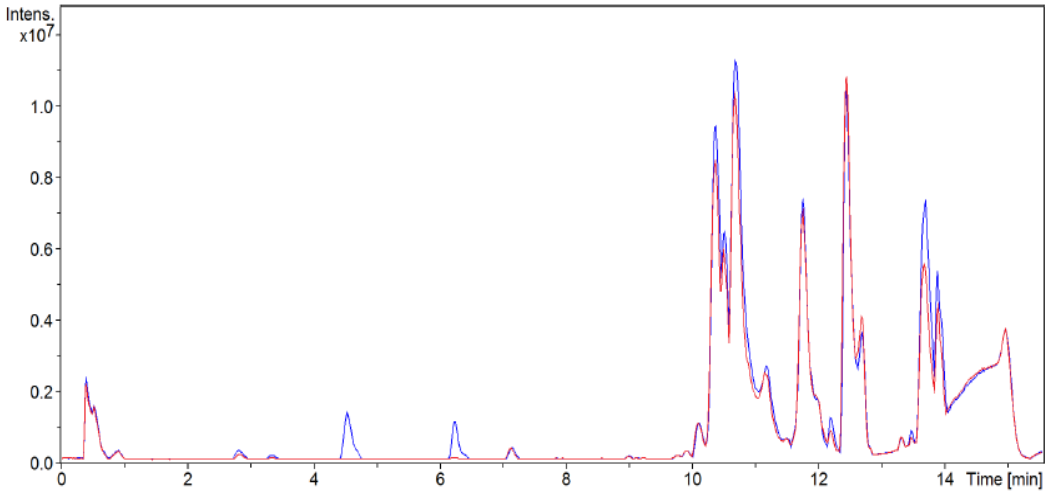

Figure 21: Comparison of the base peak chromatograms (BPC) of control plasma samples containing IS *R*-Modafinil (10600 ng/mL - blue) and analyte *S*-CE-123 (2600 ng/mL - blue) and of a blank plasma sample (ACN - red) measured with gradient 5 *via* LC-HRMS.

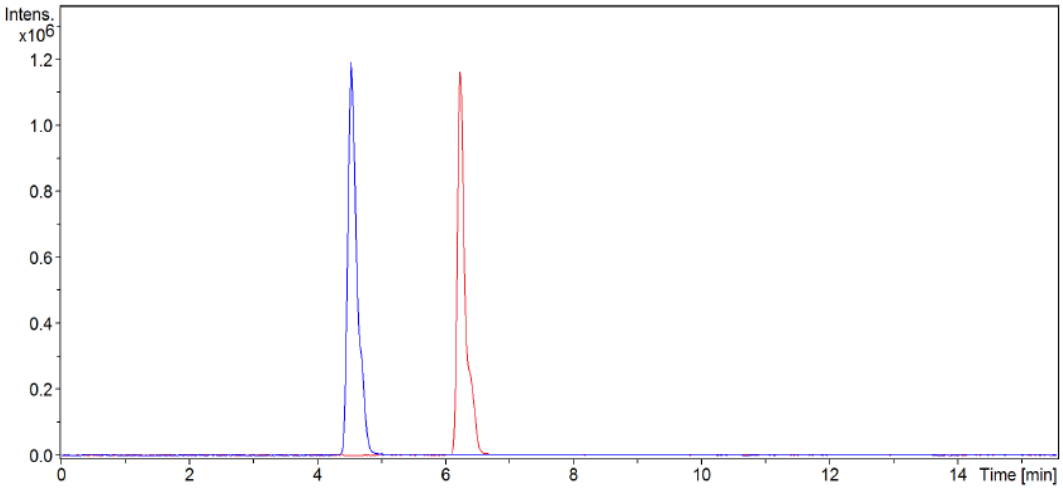

Figure 22: Comparison of the EICs of control plasma samples containing IS *R*-Modafinil positively charged with Na^+ (10600 ng/mL - blue) and analyte *S*-CE-123 positively charged with H^+ (2600 ng/mL - red) measured with gradient 5 *via* LC-HRMS.

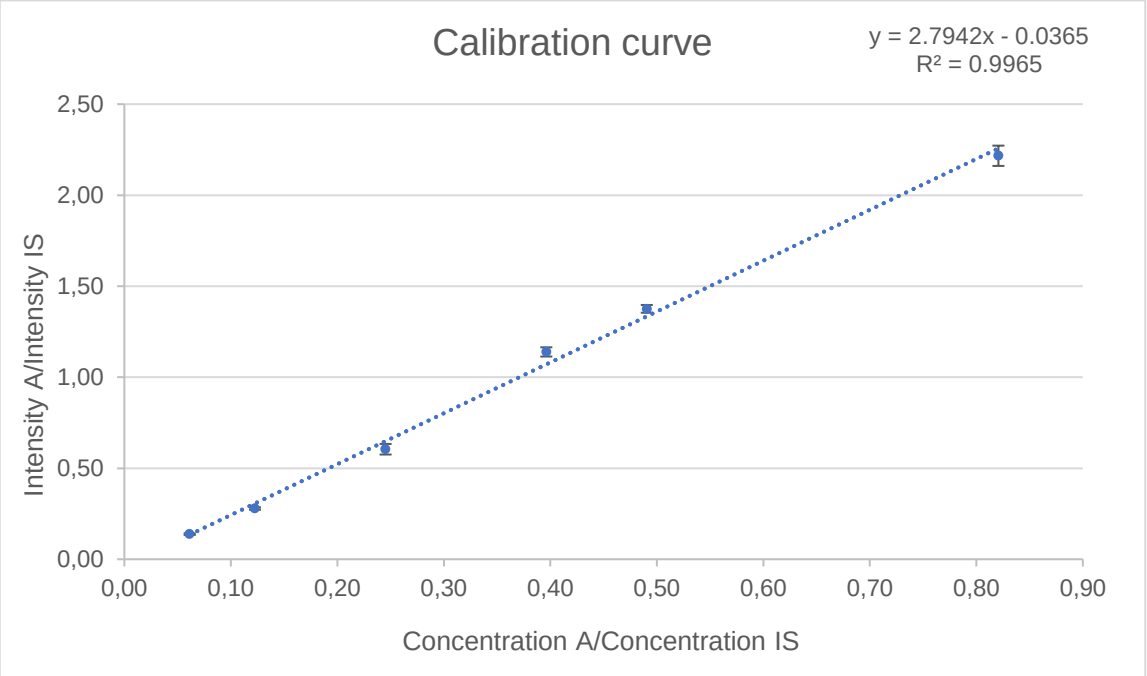

Figure 23: Samples were prepared as following: 100 µL control plasma was spiked with 100 µL ACN containing *S*-CE-123 at 6 different initial concentrations (650 ng/mL, 1300 ng/mL, 2600 ng/mL, 4200 ng/mL, 5200 ng/mL and 8700 ng/mL, respectively) and 100 µL ACN containing the IS *R*-Modafinil at a constant amount (10600 ng/mL). Samples were centrifuged for 10 minutes at 4500 g, 20 °C. The supernatant was mixed 1:1 with water. The samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing, Altmann Analytik, München, Germany) and aliquoted into 200 µL vials. They were immediately measured via LC-HRMS (20 µL injection volume). The calibration curve plots the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=4 for each concentration, mean ± SD). The linear equation revealed a slope of 2.7942 and an intercept of 0.0365 applied for quantification.

3.2.2.7 Calibration curve for lower concentrations

In addition, an alternative calibration curve for samples below 650 ng/mL of *S*-CE123 was generated.

The sample preparation was equal to the first calibration curve in plasma (see 3.2.2.6). The dilution series of *S*-CE-123 is listed in **Table 10** below.

Table 10: Applying the stock solution, the dilution series for *S*-CE-123 was generated. This table depicts the dilution series of analyte *S*-CE-123 and the resulting concentrations [ng/mL] for the calibration curve for low concentrations.

Initial concentration of *S*-CE-123 [ng/mL]	Dilution
100	1:10 dilution of stock solution [1 mg/mL] - further diluted: 1:1000
210	1:50 dilution of solution [10400 ng/mL]
310	1:2 mixture with solution [1000 ng/mL]
420	1:25 dilution of solution [10400 ng/mL]

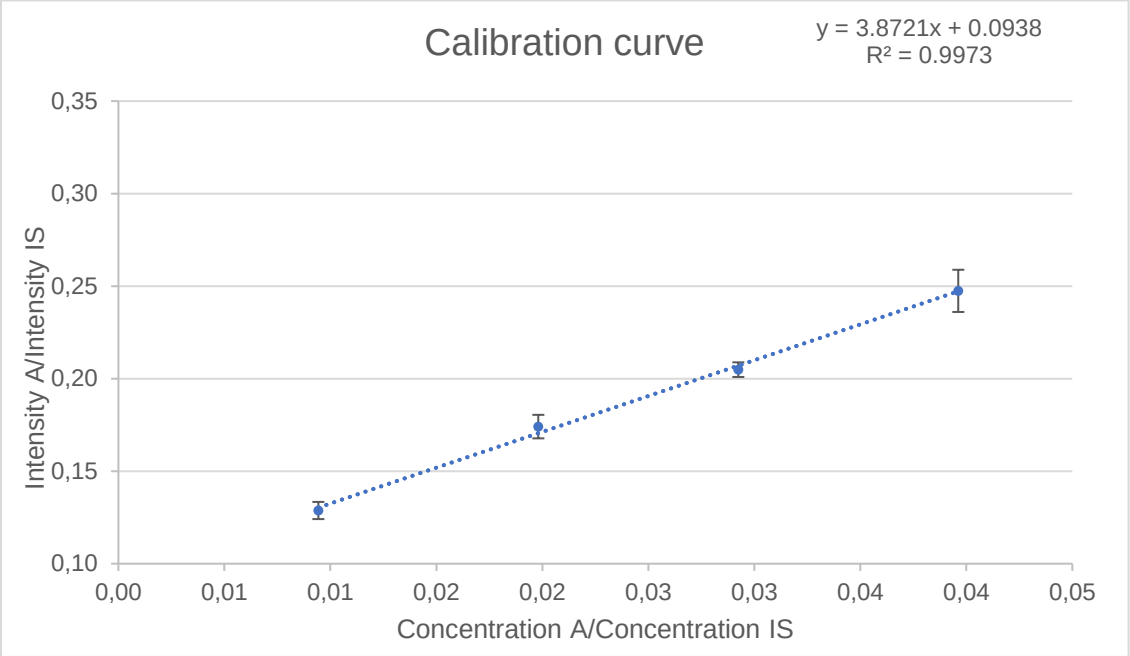

Figure 24: Samples were prepared as following: 100 µL control plasma was spiked with 100 µL ACN containing *S*-CE-123 at 4 different initial concentrations (100 ng/mL, 210 ng/mL, 310 ng/mL, 420 ng/mL, respectively) and 100 µL ACN containing the IS *R*-Modafinil at a constant amount (10600 ng/mL). Samples were centrifuged for 10 minutes at 4500 g, 20 °C. The supernatant was mixed 1:1 with water. The samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing, Altmann Analytik, München, Germany) and aliquoted into 200 µL vials. They were immediately measured via LC-HRMS (20 µL injection volume). The calibration curve plots the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=4 for each concentration, mean ± SD). The linear equation revealed a slope of 3.8721 and an intercept of 0.0938.

The calibration curve is demonstrated in **Figure 24**. Furthermore, a correlation coefficient of R^2= 0.9973 and error bars are included. The calibration curve plots the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=4 for each concentration, mean ± SD). The linear equation revealed a slope of 3.8721 and an intercept of 0.0938. In addition, more information is listed in the appendix (*Appendix Table 3*).

3.3 Rat samples

After the method was evaluated, the samples from rats treated with *S*-CE-123 were examined. 14 rats provided by Dr. István Gyertyán (Semmelweis University, Institute of Pharmacology and Pharmacotherapy, Budapest, Hungary), were injected with 10 mg/kg *S*-CE-123 *via* intraperitoneal injection. All animals were given a number for recognition – 5B, 6B, 50, 51, 52, 53, 54, 55, 57, 58, 59, 65, 66, 67. Two hours after the administration, blood samples were taken from all rats. Subsequently, the plasma was stored in a – 80 ºC freezer. The plasma of all 14 rats was examined during the presented pharmacokinetic study.

3.3.1 Sample preparation

100 µL treated rat plasma was spiked with 100 µL ACN and 100 µL ACN, containing *R*-Modafinil (10000 ng/mL). Afterwards, the samples were centrifuged for 10 minutes at 4500 g, 20 °C. Then the supernatant was mixed 1:1 with water. All samples were filtrated with a 0.2 µm filter (Micropur, PTFE, 15 mm, 0.20 µm, PP-casing) and aliquoted into 200 µL vials. All rat samples were prepared in triplicates and measured *via* LC-HRMS in triplicates. The average pump pressure was 289 bar.

3.4 Validation

The optimised method for rat plasma was validated *via* ICH harmonised tripartite guidelines for validation of analytical procedures, guidelines for bioanalytical method development and validation by USFDA and guidelines for the validation of analytical methods used in residue depletion studies (Food and Drug Administration Office, 2018) (ICH Expert Working Group, 1994) (VICH Steering

Committee, 2009). The validation included linearity, range, accuracy, precision, stability, LLOQ and LLOD.

3.4.1 Linearity

The developed method was evaluated for linearity in a concentration range of 650-8700 ng/mL. For the establishment of linearity at least 5 concentrations are necessary. The calibration curve was produced by plotting the ratio of the analyte (A) and IS signal intensity (I A/IS) as a function of the ratio of the A and the IS concentration (C A/IS) (n=4 for each concentration, mean ± SD) (Lundanes *et al*., 2014). The acceptance criteria for linearity was the correlation coefficient (R^2) and had to surpass 0.98 (Prasenjit *et al*., 2016).

3.4.2 Range

Range was established by achieving an acceptable level of linearity, precision and accuracy when deployed on samples containing the analyte. For the assay of a drug substance, a minimum calibration range from 80 to 120 % of test concentration is necessary (ICH Expert Working Group, 1994).

3.4.3 Accuracy

Accuracy was estimated using control plasma samples at initial concentrations within the validation range (650-8700 ng/mL), prepared in duplicates. Afterwards, the analyte concentration was calculated applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365 as shown in 4.1. The acceptance criteria for accuracy is ± 15 % % nominal values (Food and Drug Administration Office, 2018).

3.4.4 Precision

Precision was established by determining intra- and inter-day precision, using control plasma samples in triplicates at three different initial concentrations of *S*-CE123 within the validation range (2100 ng/mL, 4200 ng/mL and 8200 ng/mL), containing the IS at a constant concentration of 10400 ng/mL. For intra-day precision, the prepared samples were measured twice within the same batch. Inter-day precision was determined by remeasuring the same set of samples after three days. Concentrations were determined applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365 as shown in 4.1. The acceptance criteria for intra- and inter-day precision are ± 15 % relative standard deviation (%CV) of the average concentrations (Food and Drug Administration Office, 2018).

3.4.5 Stability

Stability studies were conducted by determining long-term- and autosampler stability.

Long-term stability was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration for three different storage temperatures (4 ºC, - 20 ºC, - 80 ºC) were prepared in order to measure the stability in each condition. The aim was to find the best storage temperature for the samples. Long-term stability was established for both samples containing solvent ACN and for samples in control rat plasma. The sample preparation for the samples only in ACN was the same as described in 3.2.2.3, as well as samples in plasma were produced as in 3.2.2.6. Afterwards, they were

transferred to the said storage facilities. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

Autosampler stability was established by measuring control plasma samples in triplicates at two different initial concentrations of S-CE123 within the validation range (4200 ng/mL and 8200 ng/mL), containing the IS at a constant initial concentration of 10400 ng/mL. After the first measurement, the samples were stored in the autosampler at 8 °C for 3 days and remeasured after this time period. Concentrations of S-CE123 were determined applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365 as shown in 4.1. They were considered stable if the accuracy (% nominal) at each level is within ± 15 % (Food and Drug Administration Office, 2018). The samples were prepared following the protocol described in 3.2.2.6.

3.4.6 LLOQ and LLOD

The lower limit of quantification was determined using the signal-to-noise approach. The acceptance criteria is a minimum signal-to-noise ratio of 10:1. The lower limit of detection was also defined, which has to exceed a signal-to-noise ratio of 3:1 (ICH Expert Working Group, 1994).

4. Results

4.1 Rat samples

The samples of 14 *S*-CE-123 treated rats were measured and the results were evaluated. The concentration of *S*-CE-123 was determined for 8 (Rats 51, 52, 53, 54, 57, 58, 65, 66) of 14 rats with the calibration curve depicted in **Figure 23**, due to a higher analyte signal. Two other rat samples (Rats 59, 67) were evaluated with the calibration curve for lower concentrations (see **Figure 24**). The remaining four test subjects (Rats 5B, 6B, 50, 55) were not quantifiable, due to a signal-to-noise ratios below 10:1.

The concentrations were calculated applying the equation of the calibration curve. An example of the calculation process of rat 51 is demonstrated below:

y = 2.7942x – 0.0365

y = Intensity A/ Intensity IS = 1.103

1.103 = 2.7942x – 0.0365

x = Concentration A/Concentration IS = 0.4078

Concentration IS = 10000 ng/mL

Concentration A = 0.4078 * 10000 ≈ **4080 ng/mL**

The mean of the calculated concentrations of 9 measurements of each rat was generated. The results for the samples evaluated with the first calibration curve are listed in **Table 11** below. The complete list of values is shown in the appendix (*Appendix Tables 4-11*). Furthermore, an example of an EIC chromatogram of rat number 66 is illustrated in 3.2.2.5 (**Figure 20**).

Table 11: Calculated concentrations of *S*-CE-123 in rat plasma samples (n=9 for each sample, mean ± SD; CV is shown) with calibration curve 1.

Rat Number	Concentration of *S*-CE-123 [ng/mL]	CV* [%]
51	4080	2.7
52	2240	3.6
53	2170	4,3
54	3630	3.7
57	3910	3.0
58	1220	3.4
65	3680	2.5
66	3850	2.4

Samples from rats' number 59 and 67 were analysed with the calibration curve for lower concentrations, due to their apparent lower *S*-CE-123 concentration. An example of the calculation of a sample from rat number 67 is shown below:

y = 3.8721x + 0.0938

y = Intensity A/ Intensity IS = 0.135

0.135= 3.8721x + 0.0938

x = Concentration A/Concentration IS = 0.011

Concentration IS = 10000 ng/mL

Concentration A = 0.011* 10000 = **110 ng/mL**

The results for samples evaluated with the alternative calibration curve are listed in **Table 12** below. Further information is shown in the appendix (*Appendix Tables 12-13*). In addition, an example of the chromatogram of rat number 67 is demonstrated in **Figure 25**, where a very low signal of *S*-CE-123 was observed.

Table 12: Calculated concentrations of *S*-CE-123 in rat plasma samples (n=9 for each sample, mean ± SD; CV is shown) with the calibration curve for low concentrations.

Rat Number	Concentration of *S*-CE-123 [ng/mL]	CV* [%]
59	100	5.30
67	110	5.32

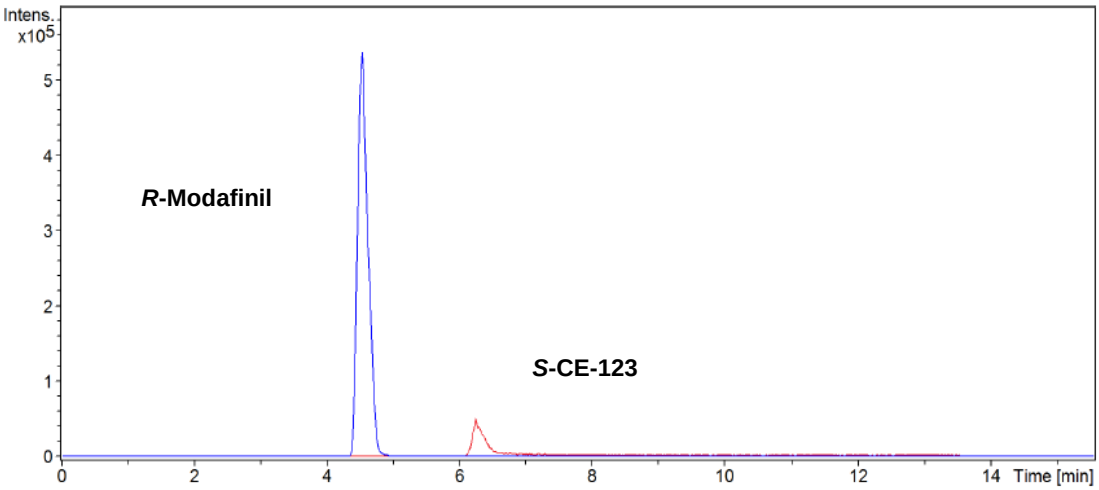

Figure 25: EIC of *R*-Modafinil (blue) (10000 ng/mL) as IS and *S*-CE-123 (red) as analyte of rat number 67. A low signal (I = 0.5 x 10^5) of S-CE-123 was observed.

In addition, **Figures 26 + 27** show EIC chromatograms of rat number 5B with an IS concentration of 10000 ng/mL. The signal-to-noise ratio of the applied *S*-CE-123 was found to be below the LLOQ of 10:1 applying the signal-to-noise approach. For this reason, the sample was not quantifiable and therefore the precise concentration of *S*-CE-123 was not determinable (ICH Expert Working Group, 1994). The same issue applies to samples from rats' number 6B, 50 and 55.

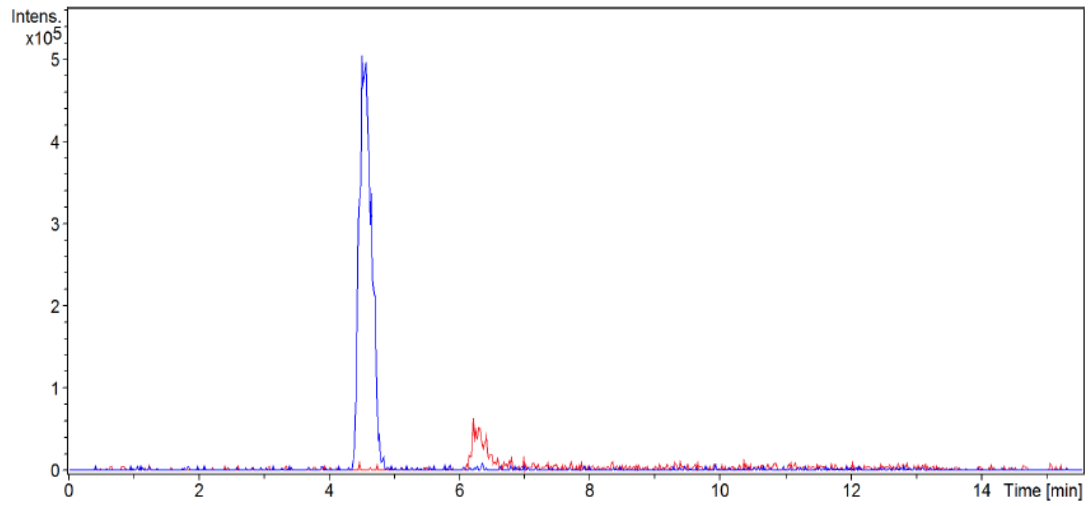

Figure 26: EIC of *R*-Modafinil (blue) (10000 ng/mL) as IS and *S*-CE-123 (red) as analyte of rat number 5B.

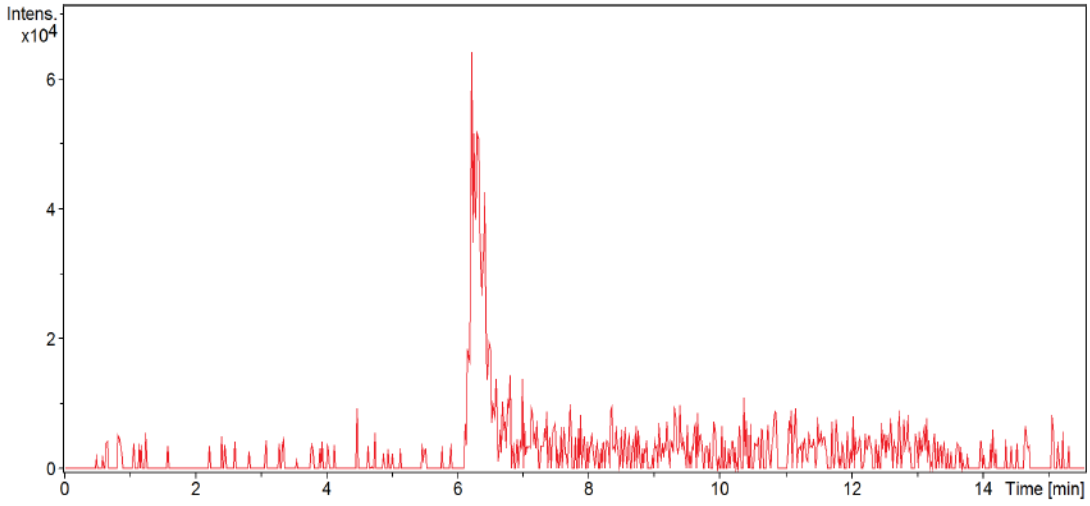

Figure 27: EIC of analyte *S*-CE-123 of rat number 5B. The signal was found to be below the LLOQ.

4.2 Validation

4.2.1 Linearity and Range

Linear correlation was given from 650 to 8700 ng/mL and the calibration curve also surpassed the minimum of 5 concentrations (6 different analyte concentrations prepared in duplicates). The calculated analyte concentrations of

treated rat plasma samples were between 1220 ng/mL and 4080 ng/ml. Therefore, the calibration range (650-8700 ng/mL standard analyte concentration) exceeded the required minimum of 80-120 % of analyte concentration.

4.2.2 Accuracy

The results containing the calculated and true values of analyte concentration (with a constant amount of IS with an initial concentration of 10600 ng/mL) with relative errors (ER) between the two values are depicted in **Table 13** and **14** (n=4 for each concentration). In the first table the accuracy within the validation range of the first calibration curve and in the second, the accuracy for the values of the calibration curve for lower concentrations are demonstrated. The calculated errors with values between -8.1% and 6.2%, were all below ± 15 % nominal concentrations, which is the acceptance criteria for accuracy (Food and Drug Administration Office, 2018).

Table 13: Accuracy was determined using the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365. The results containing the calculated and true values of the analyte concentration (with a constant amount of IS with a stock concentration of 10600 ng/mL) with relative errors (ER) between the two values are shown (n=4 for each concentration). The accuracy of all spiked plasma samples is accurate.

iC A [ng/mL]	calculated A [ng/mL]	% ER	
650	662	1.8	accurate
1300	1195	-8.1	accurate
2600	2431	-6.5	accurate
4200	4459	6.2	accurate
5200	5357	3.0	accurate
8700	8547	-1.8	accurate

Table 14: Accuracy was determined using the equation of the calibration curve with a slope of 3.8721 and an intercept of 0.0938. The results containing the calculated and true values of the analyte concentration (with a constant amount of IS with a stock concentration of 10600 ng/mL) with relative errors (ER) between the two values are shown (n=4 for each concentration). The accuracy of all spiked plasma samples is accurate.

iC A [ng/mL]	calculated A [ng/mL]	% ER	
96	100	-4.35	accurate
220	210	4.65	accurate
304	310	-1.93	accurate
421	420	0.14	accurate

Furthermore, the use of the equation of the first calibration curve was tested for accuracy studies for lower values, as shown in **Table 15** and in contrast, the calibration curve for lower concentrations was applied to higher concentrations, as depicted in **Table 16**. The results (for low and high concentrations, respectively) did not fulfil the acceptance criteria for accuracy for both methods reaching calculated errors between -81% and 527%.

Table 15: Accuracy was determined using the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365. The results for low concentrations (100 – 420 ng/m) did not fulfil the acceptance criteria for accuracy reaching calculated errors from -8% to 527%.

iC A [ng/mL]	calculated A [ng/mL]	% ER	
627	100	526.85	
799	210	280.41	
916	310	195.35	
1077	420	156.46	
662	650	1.80	accurate
1195	1300	-8.07	accurate
2431	2600	-6.49	accurate
4459	4200	6.16	accurate
5357	5200	3.01	accurate
8547	8700	-1.76	accurate

Table 16: Accuracy was determined using the equation of the calibration curve with a slope of 3.8721 and an intercept of 0.0938. The results for high concentrations (650 – 8700 ng/m) did not fulfil the acceptance criteria for accuracy reaching calculated errors from -81% to 5%.

iC A [ng/mL]	calculated A [ng/mL]	% ER	
96	100	-4.35	accurate
220	210	4.65	accurate
304	310	-1.93	accurate
421	420	0.14	accurate
121	650	-81.42	
506	1300	-61.10	
1398	2600	-46.24	
2861	4200	-31.88	
3509	5200	-32.52	
5811	8700	-33.21	

4.2.3 Precision

For intra-day precision, the prepared samples were measured twice within the same batch. In **Table 17** the values of initially measured samples and intra batch samples are shown. The relative standard deviation (%CV) of intra batch samples applying analyte concentrations of 2100 ng/mL, 4200 ng/mL and 8200 ng/mL was below 2.2%. Therefore, the samples fulfilled the acceptance criteria of ± 15 % %CV (Food and Drug Administration Office, 2018). The results for inter-day precision, measuring the same set of samples 3 days later, which also achieved the requirements for precision are depicted in **Table 18**. For inter-day precision a relative standard deviation below 2.3% was determined.

Table 17: For intra-day precision, the prepared samples were measured twice within the same batch. In this table the values of initially measured samples and intra batch samples are shown. Concentrations were determined applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365. Average concentrations of initially measured samples and intra-batch samples (mean intra) are shown (n=6 for each concentration, mean ± SD). Following the guidelines, a sufficient level of intra-day precision was accomplished (Food and Drug Administration Office, 2018).

Sample	iC A [ng/mL]	I A/IS	calculated A [ng/mL]	mean intra	SD	CV [%]
Initially						
1	2100	0.5723	2266			
2	2100	0.5935	2345			
3	2100	0.5794	2293			
1	4200	1.2267	4702			
2	4200	1.2091	4636			
3	4200	1.2442	4767			
1	8200	2.3432	8857			
2	8200	2.2411	8477			
3	8200	2.3543	8899			
Intra Batch						
1	2100	0.5705	2259	2294	30	1.3
2	2100	0.5867	2320			
3	2100	0.5766	2282			
1	4200	1.2113	4644	4690	52	1.1
2	4200	1.2115	4645			
3	4200	1.2381	4744			
1	8200	2.3415	8851	8743	191	2.2
2	8200	2.2398	8472			
3	8200	2.3555	8903			

Table 18: Inter-day precision was determined by remeasuring the same set of samples after three days (stored at 8°C). Average concentrations of initially measured samples and inter-day samples (mean inter) are shown (n=6 for each concentration, mean ± SD). Following the guidelines, a sufficient level of inter-day precision was accomplished (Food and Drug Administration Office, 2018).

Sample	iC A [ng/mL]	I A/IS	calculated A [ng/mL]	mean inter	S D	CV [%]
After 3 Days						
1	2100	0.5717	2264	2291	28	1.2
2	2100	0.5815	2300			
3	2100	0.5754	2277			
1	4200	1.2143	4655	4706	48	1.0
2	4200	1.2307	4716			
3	4200	1.2418	4758			
1	8200	2.3512	8887	8767	200	2.3
2	8200	2.2478	8502			
3	8200	2.3768	8982			

4.2.4 Long-term Stability

4.2.4.1 Samples in ACN

The outcome for samples in ACN which were stored in a refrigerator at 4 °C is shown in **Table 19**. The table demonstrates the average intensities of analyte and internal standard at their tested concentrations and compares the difference of intensities between the stored samples and the initially (t_0) measured ones. *S*-CE-123 was measured in a concentration of 10 ng/1005µL and 8300 ng/1005µL. The lower concentration was found to be below the LLOQ (Signal-to-noise-ratio

< 10:1) for all measurements after storing the samples and could therefore not be compared with the initially measured ones. This was the case for all storage temperatures for *S*-CE-123 10 ng/1005µL samples. A chromatogram of *S*-CE-123 (10 ng/1005µL) measured after 3 months of storage at 4°C, which was below the LLOQ is illustrated in **Figure 29**. After 3 months (3M) of storage the concentration increased compared to previous measurements, presumably caused by evaporation of solvent. This unfortunate issue happened at all three storage temperatures. *S*-CE-123 in a concentration of 8300 ng/1005µL fulfilled the acceptance criteria of ± 15% deviation from the initially measured samples (Δ A = +0.9 %) and was considered stable for up to 3 months of storage at 4 °C. *R*-Modafinil was measured at a concentration of 5300 ng/1005µL and was added to both sample types, containing the two different *S*-CE-123 concentrations. The internal standard was considered stable for up to one week (1W) of storage at 4 °C. After 3 months, the concentration of *R*-Modafinil had nearly halved (Δ IS = -42.1% / -45.5%), compared to initial measurements. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/1005µL and *R*-Modafinil in a concentration of 5300 ng/1005µL as internal standard for samples containing 10 ng/1005µL *S*-CE-123 and 8300 ng/1005µL are illustrated in **Figure 28**. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 28** are shown in **Table 19**.

Table 19: Long-term stability in ACN at a storage temperature of 4°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.3. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/1005 µL]	C IS [ng/1005 µL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
4 °C								
t_0								
	10	5300	10314	66292			100.0	100.0
	8300	5300	794323	73192			100.0	100.0
1W								
	10	5300	-	58438	-	-11.9	-	88.2
	8300	5300	764708	62504	-3.7	**-14.6**	96.3	85.4
1M								
	10	5300	-	48192	-	-27.3	-	72.7
	8300	5300	703541	53044	-11.4	-27.5	88.6	72.5
3M								
	10	5300	-	38355	-	-42.1	-	57.9
	8300	5300	801201	39911	**+0.9**	-45.5	100.9	54.5

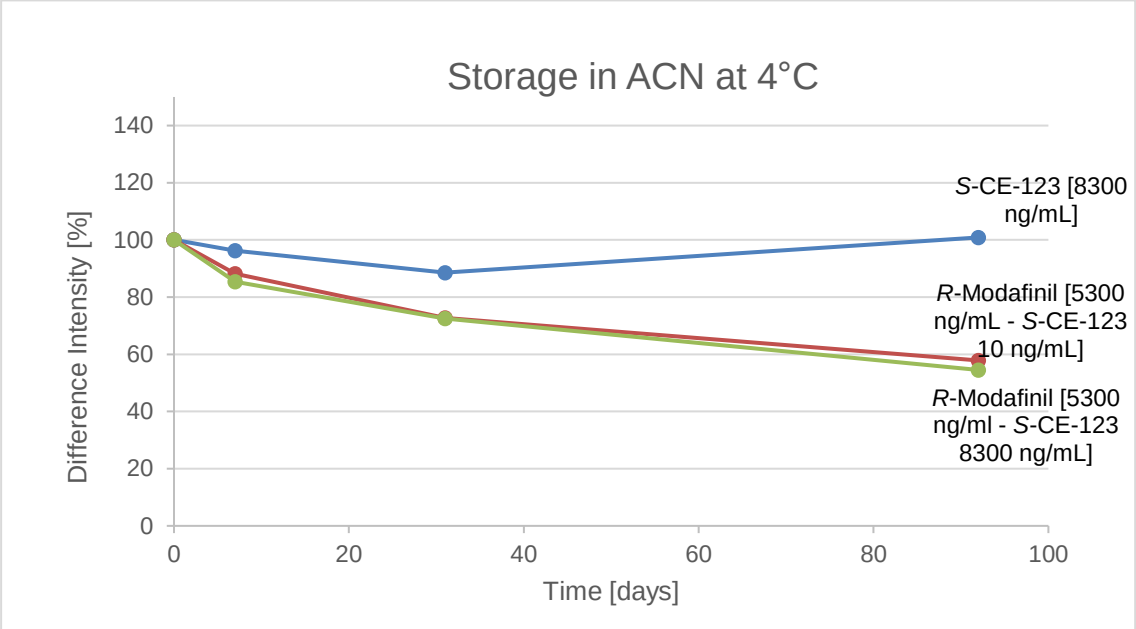

Figure 28: Progressive graphs of *S*-CE-123 (8300 ng/1005µL – blue) and *R*-Modafinil (5300 ng/1005µL) as internal standard for samples containing 10 ng/1005µL (red) *S*-CE-123 and 8300 ng/1005µL (green) are illustrated. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). *S*-CE-123 fulfilled the acceptance criteria of ± 15% deviation from the initially measured samples (Δ A = +0.9 %) and was considered stable for up to 3 months of storage at 4 °C. The internal standard was considered stable for up to one week (1W) of storage at 4 °C. After 3 months, the concentration of *R*-Modafinil had nearly halved (Δ IS = -42.1% / -45.5%), compared to initial measurements.

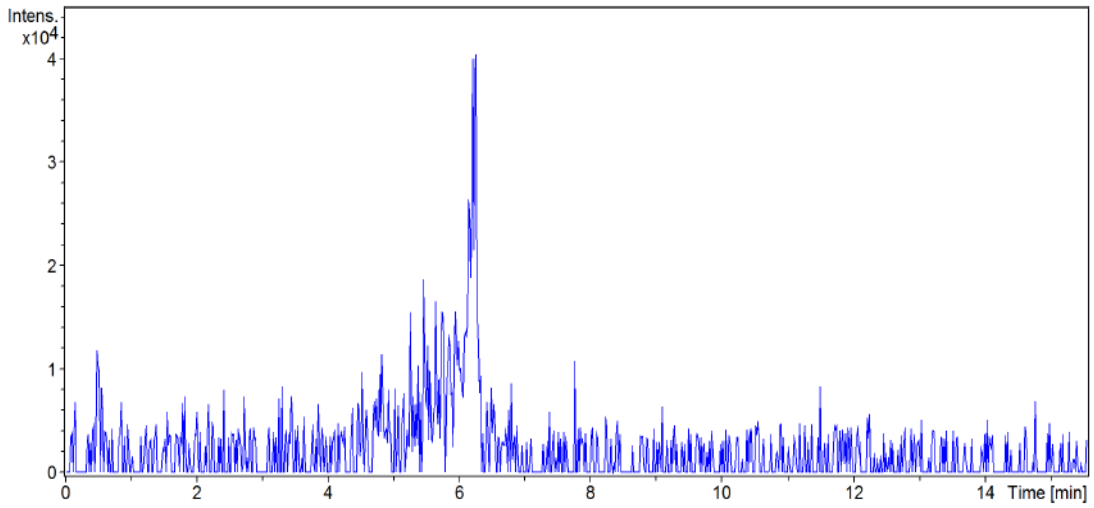

Figure 29: The EIC of *S*-CE-123 (10 ng/1005µL) measured after 3 months of storage at 4°C, which was below the LLOQ, is depicted.

Furthermore, the results for storage in a – 20 °C freezer were evaluated, which were similar to storage at 4 °C. **Table 20** shows that analyte and internal standard were equally stable for 3 months (Δ A = +0.3 %) and one week (Δ A = -14.0 %) of storage at – 20 °C, respectively. The table demonstrates the average intensities of analyte and internal standard at their tested concentrations and compares the difference of intensities between the stored samples and the initially (t_0) measured ones. *S*-CE-123 was measured in a concentration of 10 ng/mL and 8300 ng/mL. *R*-Modafinil was measured at a concentration of 5200 ng/mL and was added to both sample types, containing the two different *S*-CE-123 concentrations. After 3 months, the concentration of *R*-Modafinil had nearly halved (Δ IS = -44.9% / -41.1%), compared to initial measurements. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/1005µL and *R*-Modafinil in a concentration of 5300 ng/1005µL as internal standard for samples containing 10 ng/1005µL *S*-CE-123 and 8300 ng/1005µL are demonstrated in **Figure 30**. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 30** are shown in **Table 20**.

Table 20: Long-term stability in ACN at a storage temperature of -20°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.3. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/1005 µL]	C IS [ng/1005 µL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
-20 °C								
t_0								
	10	5300	10206	67662			100.0	100.0
	8300	5300	805044	67641			100.0	100.0
1W								
	10	5300	-	58829	-	-13.1	-	86.9
	8300	5300	782294	58181	-2.8	**-14.0**	97.2	86.0
1M								
	10	5300	-	50997	-	-24.7	-	75.4
	8300	5300	759828	50490	-5.6	-25.4	94.4	74.6
3M								
	10	5300	-	37291	-	-44.9	-	55.1
	8300	5300	807478	39845	**+0.3**	-41.1	100.3	58.9

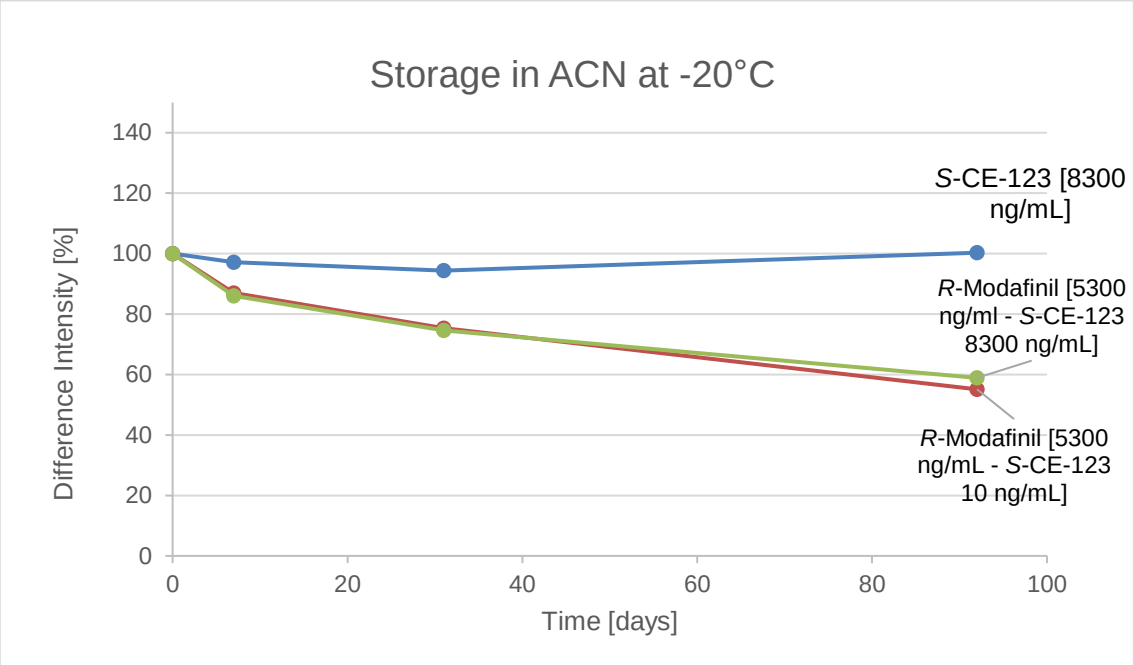

Figure 30: Progressive graphs of *S*-CE-123 (8300 ng/1005µL – blue) and *R*-Modafinil (5300 ng/1005µL) as internal standard for samples containing 10 ng/1005µL (red) *S*-CE-123 and 8300 ng/1005µL (green) are illustrated. The graphs display the change of intensity in percent (t0 = 100%) as a function of time (days). The analyte and internal standard were equally stable for 3 months (Δ A = +0.3 %) and one week (Δ A = -14.0 %) of storage at – 20 °C, respectively.

The evaluation of samples stored in a – 80 °C freezer, demonstrated in **Table 21**, shows, that *S*-CE-123 at a concentration of 8300 ng/mL was only considered stable for 1 week of storage at – 80 °C. After one month the deviation from the original value was already 30.1 % and therefore not fulfilling the acceptance criteria of ± 15 % deviation. *R*-Modafinil was equally considered stable for 1 week of storage, although only *R*-Modafinil samples containing an analyte concentration of 10 ng/mL, achieved the acceptance criteria (Δ A = -14.2 %). The table demonstrates the average intensities of analyte and internal standard at their tested concentrations and compares the difference of intensities between the stored samples and the initially (t_0) measured ones. *S*-CE-123 was measured

in a concentration of 10 ng/mL and 8300 ng/mL. *R*-Modafinil was measured at a concentration of 5200 ng/mL and was added to both sample types, containing the two different *S*-CE-123 concentrations. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/1005µL and *R*-Modafinil in a concentration of 5300 ng/1005µL as internal standard for samples containing 10 ng/1005µL *S*-CE-123 and 8300 ng/1005µL are given in **Figure 31**. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 31** are demonstrated in **Table 21**.

Table 21: Long-term stability in ACN at a storage temperature of -80°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.3. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/1005 µL]	C IS [ng/1005 µL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
-80 °C								
t_0								
	10	5300	10365	70879			100.0	100.0
	8300	5300	965394	70502			100.0	100.0
1W								
	10	5300	-	60824	-	**-14.2**	-	85.8
	8300	5300	908034	57203	**-5.9**	-18.9	94.1	81.1
1M								
	10	5300	-	51940	-	-26.7	-	73.3
	8300	5300	674963	50043	-30.1	-29.0	69.9	71.0
3M								
	10	5300	-	38939	-	-45.1	-	54.9
	8300	5300	971518	35402	+0.6	-49.8	100.6	50.2

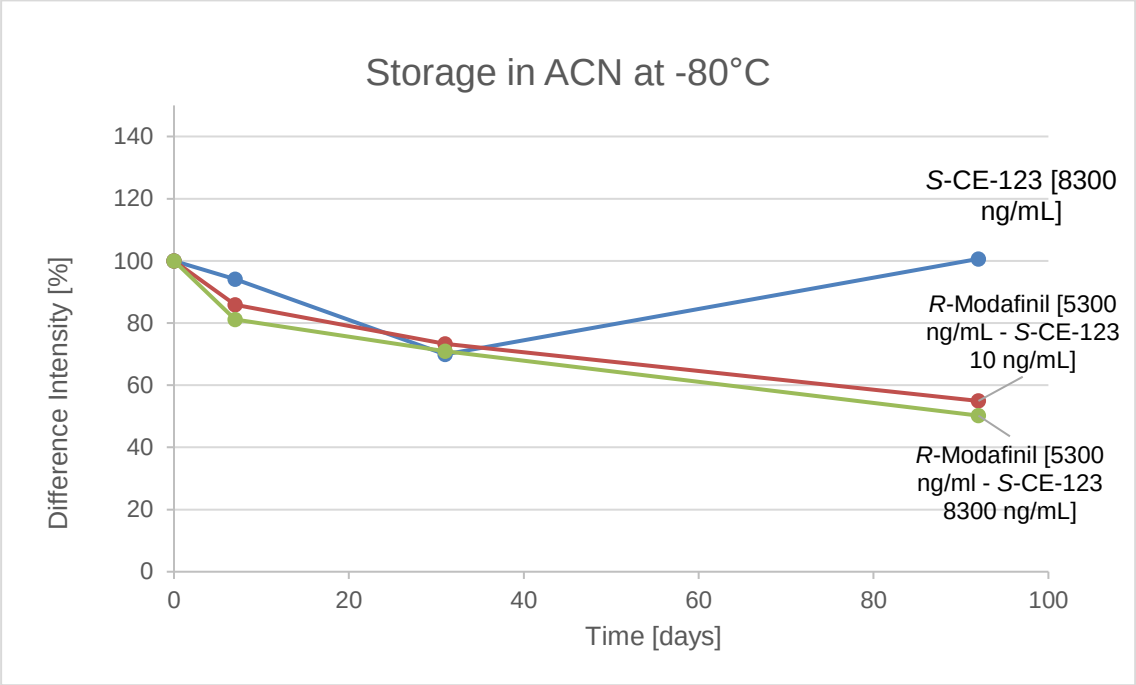

Figure 31: Progressive graphs of *S*-CE-123 (8300 ng/1005µL – blue) and *R*-Modafinil (5300 ng/1005µL) as internal standard for samples containing 10 ng/1005µL (red) *S*-CE-123 and 8300 ng/1005µL (green) are illustrated. The graphs display the change of intensity in percent (t_0 = 100%) as a function of time (days). *S*-CE-123 at a concentration of 8300 ng/mL was only considered stable for 1 week of storage at – 80 °C. After one month the deviation from the original value was already 30.1 % and therefore not fulfilling the acceptance criteria of ± 15 % deviation. *R*-Modafinil was equally considered stable for 1 week of storage, although only *R*-Modafinil samples containing an analyte concentration of 10 ng/mL, achieved the acceptance criteria (Δ A = -14.2 %).

4.2.4.2 Samples in Plasma

Equally to samples dissolved in ACN, samples dissolved in rat plasma were evaluated. The outcome for those stored in a refrigerator at 4 °C is shown in **Table 22**. *S*-CE-123 was measured in an initial concentration of 10 ng/mL and 8300 ng/mL. The lower concentration was found to be below the LLOQ (Signal-to-noise-ratio < 10:1) for measurements after one week and 1 month of storing the samples. After 3 months of storage, the signal-to-noise ratio was > 10:1 and

therefore a comparison to initially measured samples was not possible. This was the case for all storage temperatures of *S*-CE-123 samples with a concentration of 10 ng/mL. After 3 months of storage the concentration increased, compared to previous measurements, presumably caused by evaporation of solvent. This phenomenon was observed at all three storage temperatures (4°C, -20°C and -80°C). Both measured concentrations (10 ng/mL and 8300 ng/mL) of *S*-CE-123 fulfilled the acceptance criteria of ± 15 % deviation from the initially measured samples and were considered stable for up to 3 months of storage at 4 °C. *R*-Modafinil was measured in an initial concentration of 10100 ng/mL and was added to both sample types, containing the two different *S*-CE-123 concentrations. The internal standard was considered stable for up to one month of storage at 4 °C. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/mL and *R*-Modafinil in a concentration of 10100 ng/mL as internal standard for samples containing 10 ng/mL *S*-CE-123 and 8300 ng/mL are given in **Figure 32**. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 32** are shown in **Table 22**.

Table 22: Long-term stability for samples in rat plasma at a storage temperature of 4°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.6. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/mL]	C IS [ng/mL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
4 °C t_0								
	10	10100	8803	191080			100.0	100.0
	8300	10100	693960	201371			100.0	100.0
1W								
	10	10100	-	177943	-	-6.8	-	93.1
	8300	10100	679173	195797	-2.1	-2.7	97.9	97.2
1M								
	10	10100	-	165227	-	-13.5	-	86.5
	8300	10100	638046	182942	-8.1	**-9.2**	91.9	90.8
3M								
	10	10100	8547	156714	-2.9	-18.0	-	82.0
	8300	10100	748240	167645	**+7.8**	-16.8	107.8	83.3

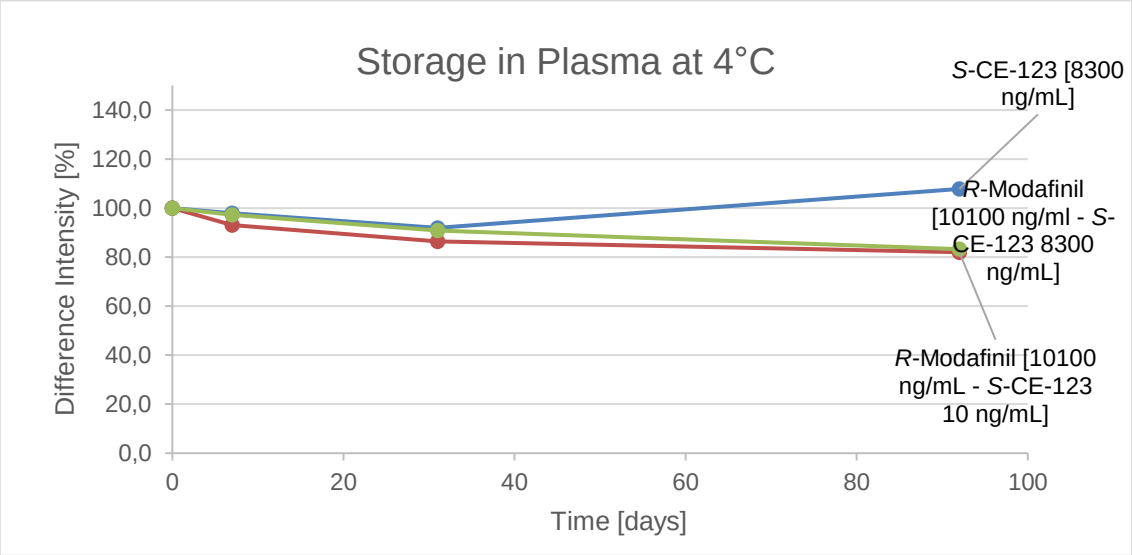

Figure 32: Progressive graphs of *S*-CE-123 (8300 ng/mL – blue) and *R*-Modafinil (10100 ng/mL) as internal standard for samples containing 10 ng/mL (red) *S*-CE-123 and 8300 ng/mL (green) are illustrated. The graphs display the change of intensity in percent (t_0 = 100%) as a function of time (days). Both measured concentrations (10 ng/mL and 8300 ng/mL) of *S*-CE-123 fulfilled the acceptance criteria of ± 15 % deviation from the initially measured samples and were considered stable for up to 3 months of storage at 4 °C. *R*-Modafinil was considered stable for up to one month of storage at 4 °C.

Furthermore, the stability of analytes for storage in a – 20 °C freezer was evaluated and the values are depicted in **Table 23**. *S*-CE-123 in a concentration of 8300 ng/mL was only considered stable for 1 week of storage at – 20 °C. After one month of storage at -20°C, the deviation from the original value was already found to be 18.1 % and therefore higher than the acceptance criteria of ± 15 % deviation. Although after 3 months the analyte at a concentration of 10 ng/mL was quantifiable, the deviation from the original samples did not fulfil the acceptance criteria. *R*-Modafinil was equally considered stable for 1 week of storage at – 20 °C. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/mL and *R*-Modafinil in a concentration of 10100 ng/mL as internal

standard for samples containing 10 ng/mL *S*-CE-123 and 8300 ng/mL are given in **Figure 33**. The graphs depict the change of intensity in percent (t0 = 100%) as a function of time (days).The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 33** are shown in **Table 23**.

Table 23: Long-term stability for samples in rat plasma at a storage temperature of -20°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.6. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/mL]	C IS [ng/mL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
-20 °C								
t_0								
	10	10100	10205	207103			100.0	100.0
	8300	10100	663560	202417			100.0	100.0
1W								
	10	10100	-	177062	-	-14.5	-	85.5
	8300	10100	622370	178872	**-6.2**	**-11.6**	93.8	88.4
1M								
	10	10100	-	127312	-	-38.5	-	61.5
	8300	10100	543555	136548	-18.1	-32.5	81.9	67.5
3M								
	10	10100	7125	69385	-30.2	-66.5	-	33.5
	8300	10100	677994	129310	+2.2	-36.1	102.2	63.9

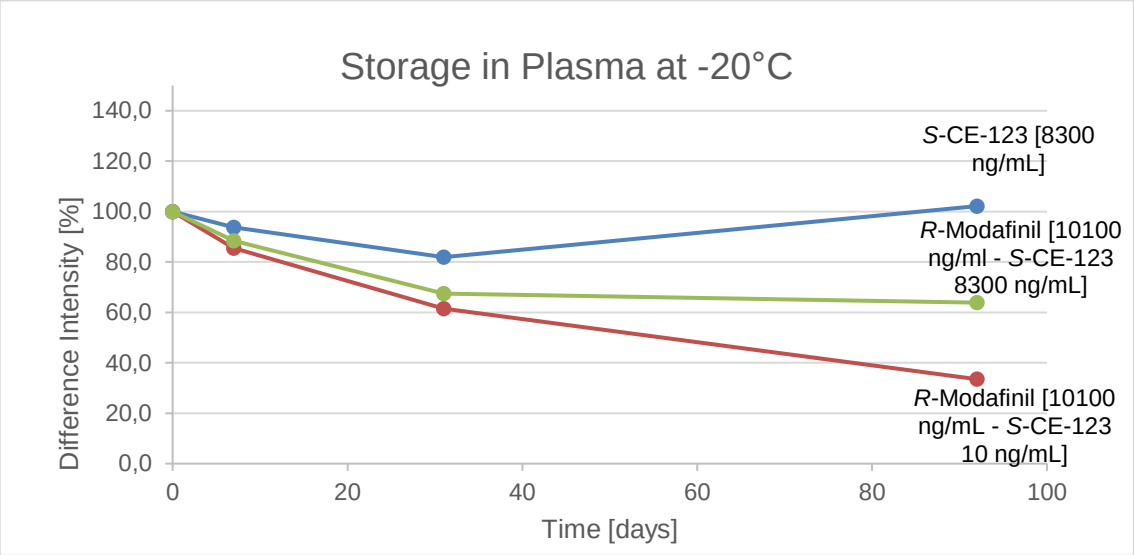

Figure 33: Progressive graphs of *S*-CE-123 (8300 ng/mL – blue) and *R*-Modafinil (10100 ng/mL) as internal standard for samples containing 10 ng/mL (red) *S*-CE-123 and 8300 ng/mL (green) are illustrated. The graphs demonstrate the change of intensity in percent (t0 = 100%) as a function of time (days). *S*-CE-123 was only considered stable for 1 week of storage at – 20 °C. After one month of storage at -20°C, the deviation from the original value was already found to be 18.1 % and therefore higher than the acceptance criteria of ± 15 % deviation. *R*-Modafinil was equally considered stable for 1 week of storage at – 20 °C.

Additionally, the results for storage in a – 80 °C freezer were evaluated, which were overall similar to storage at – 20 °C. **Table 24** shows that analyte and internal standard were equally stable for one week of storage at – 80 °C. The progressive graphs of *S*-CE-123 in a concentration of 8300 ng/mL and *R*-Modafinil in a concentration of 10100 ng/mL as internal standard for samples containing 10 ng/mL *S*-CE-123 and 8300 ng/mL are given in **Figure 34**. The graphs depict the change of intensity in percent (t_0 = 100%) as a function of time (days). The standardised (N) values (Δ A N [%], Δ IS N [%]) for the graphs of **Figure 34** are demonstrated in **Table 24**.

Table 24: Long-term stability for samples in rat plasma at a storage temperature of -80°C was established by preparing samples of two different initial analyte concentrations (10 ng/mL and 8300 ng/mL), which were located near the low and high end of the validation range. The low concentration was selected near the LLOQ. Three triplicates of each concentration were prepared. The samples were prepared following the protocol as described in 3.2.2.6. After preparation, they were measured initially and periodically assayed at specified intervals – after 1 week, 1 month and 3 months. They were considered stable, if the deviation is within ± 15 % (VICH Steering Committee, 2009).

	C A [ng/mL]	C IS [ng/mL]	Mean I A	Mean I IS	Δ A [%]	Δ IS [%]	Δ A N [%]	Δ IS N [%]
-80 °C								
t_0								
	10	10100	10004	174798			100.0	100.0
	8300	10100	601986	201214			100.0	100.0
1W								
	10	10100	-	167454	-	-4.2	-	95.8
	8300	10100	568043	177012	**-5.6**	**-12.0**	94.4	88.0
1M								
	10	10100	-	119124	-	-31.9	-	68.1
	8300	10100	488464	128104	-18.9	-36.3	81.1	63.7
3M								
	10	10100	7399	74325	-26.0	-57.5	-	42.5
	8300	10100	603870	118718	+0.3	-41.0	100.3	59.0

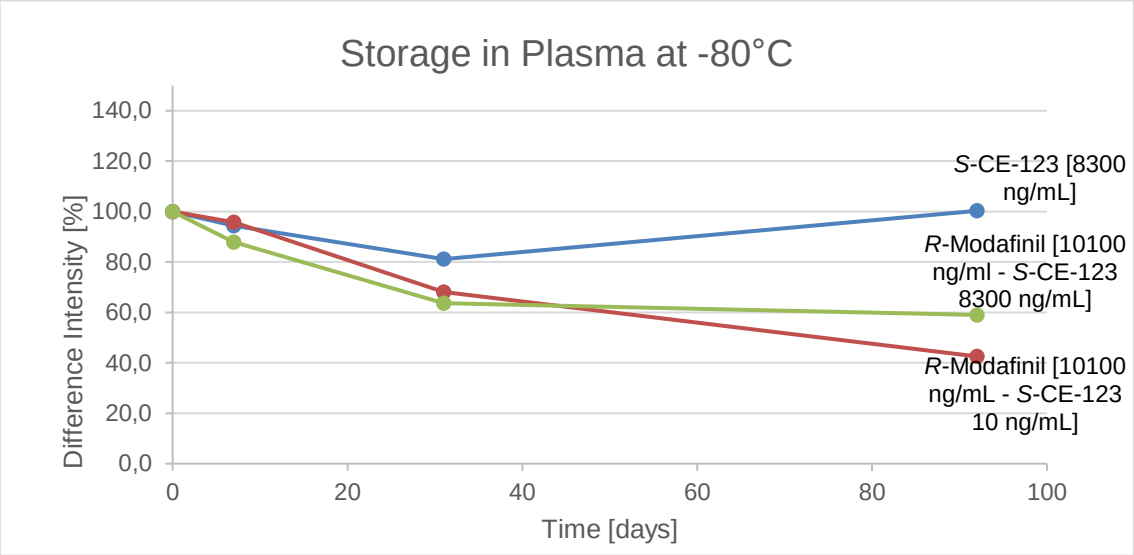

Figure 34: Progressive graphs of *S*-CE-123 (8300 ng/mL – blue) and *R*-Modafinil (10100 ng/mL) as internal standard for samples containing 10 ng/mL (red) *S*-CE-123 and 8300 ng/mL (green) are illustrated. The graphs demonstrate the change of intensity in percent (t0 = 100%) as a function of time (days). *S*-CE-123 and *R*-Modafinil were equally stable for one week of storage at – 80 °C.

4.2.4.3 Autosampler stability

Table 25 depicts the measured intensities of S-CE123 (I A) and *R*-Modafinil (I IS) as well as I A/IS determined from the $[M+H]^+$ and the $[M+Na]^+$ adduct, respectively. Concentrations of S-CE123 were determined applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365 as shown in 4.1. The results containing the true and calculated values of the analyte concentration (with a constant amount of IS with an initial concentration of 10400 ng/mL) with relative errors (ER) between the two values are shown (n=3 for each concentration) in **Table 26**. All samples achieved the acceptance criteria of an accuracy (% nominal) at a level of ± 15 % (Food and Drug Administration Office, 2018).

Table 25: Autosampler stability values: Autosampler stability was established by measuring control plasma samples in triplicates at two different initial concentrations of *S*-CE-123 within the validation range (4200 ng/mL and 8200 ng/mL), containing the IS at a constant initial concentration of 10400 ng/mL. After the first measurement, the samples were stored in the autosampler at 8 °C for 3 days and remeasured after this time period. This table depicts the measured intensities of *S*-CE-123 (I A) and *R*-*Modafinil* (I IS) as well as I A/IS determined from the $[M+H]^+$ and the $[M+Na]^+$ adduct, respectively.

Samples	iC A [ng/mL]	I A	I IS	I A/IS
Initially				
1	4200	321970	262459	1.2267
2	4200	323047	267190	1.2091
3	4200	324472	260784	1.2442
1	8200	609325	260042	2.3432
2	8200	599098	267324	2.2411
3	8200	615743	261539	2.3543
After 3 Days				
1	4200	316914	260993	1.2143
2	4200	317527	258006	1.2307
3	4200	320443	258041	1.2418
1	8200	606473	257939	2.3512
2	8200	594475	264466	2.2478
3	8200	612639	257758	2.3768

Table 26: Autosampler stability results: Concentrations of *S*-CE-123 were determined applying the equation of the calibration curve with a slope of 2.7942 and an intercept of 0.0365 (see Fig. S1). The results containing the true and calculated values of the analyte concentration (with a constant amount of IS with an initial concentration of 10400 ng/mL) with relative errors (ER) between the two values are shown (n=3 for each concentration). All samples achieved the acceptance criteria of an accuracy (% nominal) at each level of ±15 % (Food and Drug Administration Office, 2018).

measured	iC A [ng/mL]	calculated A [ng/mL]	% ER	
Initially	4200	4701	11.9	accurate
	8200	8742	6.6	accurate
After 3 Days	4200	4710	12.1	accurate
	8200	8788	7.2	accurate

4.2.5 LLOQ and LLOD

Using the Signal-to-Noise Approach LLOD and LLOQ were determined. The samples were prepared following the protocol described in 3.2.2.6. The acceptance criteria for LLOQ was a minimum signal-to-noise ratio of 10:1, whereas for the LLOD the signal-to-noise ratio had to exceed 3:1 (ICH Expert Working Group, 1994). For *R*-Modafinil a LLOD of 5 ng/mL was defined, which is depicted in **Figure 37**. The graph of the measured LLOQ for analyte *S*-CE-123 is illustrated in **Figure 35** and was defined as 6 ng/mL. Furthermore, for *S*-CE-123 a LLOD of 2 ng/mL was determined, which is shown in **Figure 36**. When comparing the two drugs, it can be noted that the LLOD of *S*-CE-123 is lower than of *R*-Modafinil. Therefore, lower concentrations of *S*-CE-123 than of *R*-Modafinil were still detectable and quantifiable.

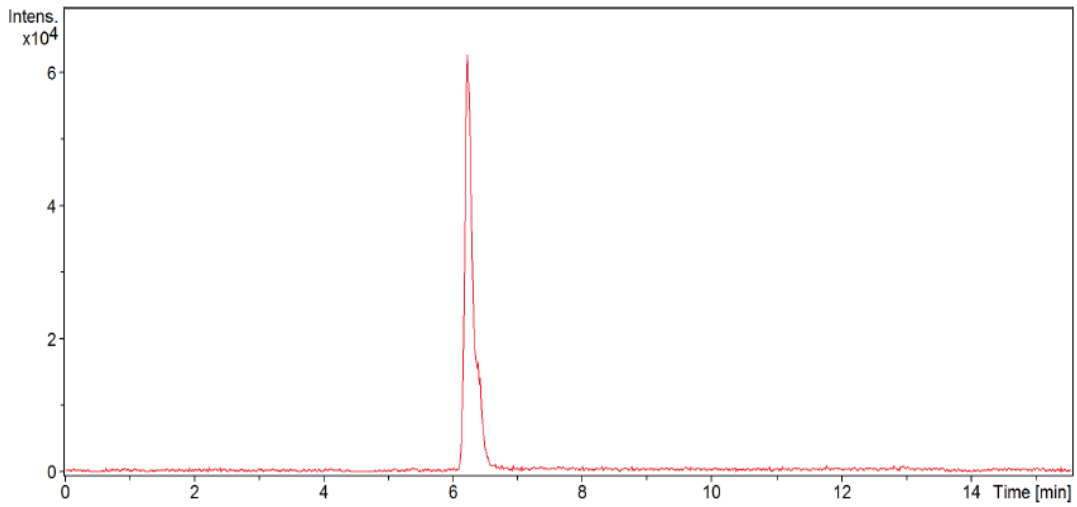

Figure 35: This chromatogram depicts the EIC of the measured LLOQ for analyte *S*-CE-123 (6 ng/mL). The acceptance criteria for LLOQ was a minimum signal-to-noise ratio of 10:1 (ICH Expert Working Group, 1994).

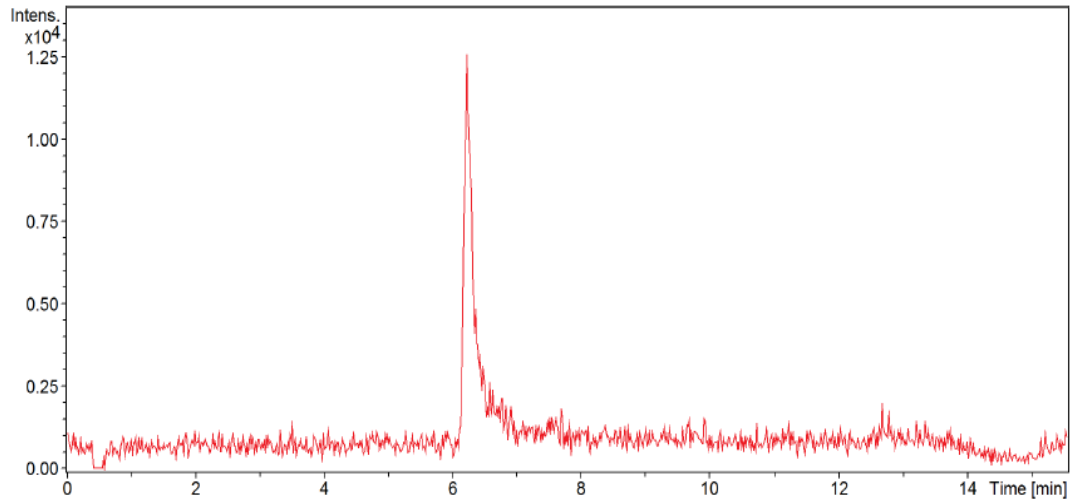

Figure 36: The EIC of the measured LLOD for analyte *S*-CE-123 (2 ng/mL) is depicted. The acceptance criteria for LLOD was a minimum signal-to-noise ratio of 3:1 (ICH Expert Working Group, 1994).

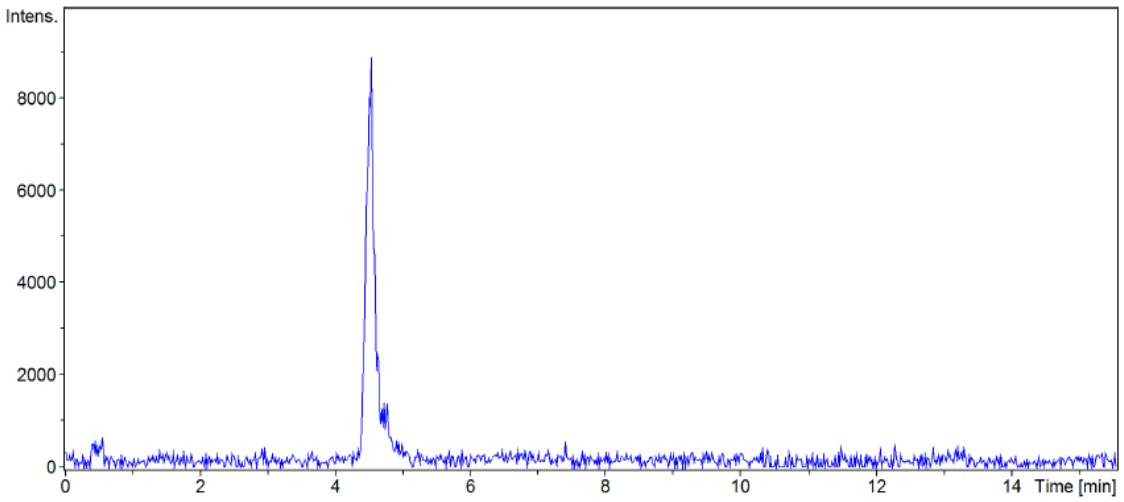

Figure 37: The EIC of the measured LLOD for internal standard *R*-Modafinil (5 ng/mL) is shown. The acceptance criteria for LLOD was a minimum signal-to-noise ratio of 3:1 (ICH Expert Working Group, 1994).

4.3 Adducts of *R*-Modafinil

Studies regarding the different intensities of the adducts of *R*-Modafinil were conducted. For sample preparation a stock solution of the drug *R*-Modafinil in ACN with a concentration of 1 mg/mL was prepared. The powder was weighed on an analytical balance (MC210P, Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany), transferred into a 10 mL volumetric flask and filled with acetonitrile. The solution was then diluted 1:10 with ACN (*R*-Modafinil concentration = 100 µg/mL). 15 µL of this dilution was mixed with 500 µL methanol and 50 µL of water containing 0.5 % formic acid (FA) (= sample 1). The MS chromatogram of sample 1 is depicted in **Figure 38**. Furthermore, 15 µL of the dilution, 500 µL methanol and 100 µL 0.5 % FA were combined (= sample 2). The MS chromatogram of sample 2 is illustrated in **Figure 39** Then, 15 µL dilution, 500 µL methanol and 500 µL 0.5 % FA were mixed (= sample 3). The MS-MS chromatogram of sample 3 is shown in **Figure 40** The three different samples were then measured *via* MS-MS direct injection. Mass spectra were

recorded with a maXis HD ESI—Qq-TOF mass spectrometer (Electrospray Ionisation – Double Quadrupole mass analyser – Time-of-flight mass analyser) (Bruker Corporation, Bremen, Germany) in full-scan positive mode.

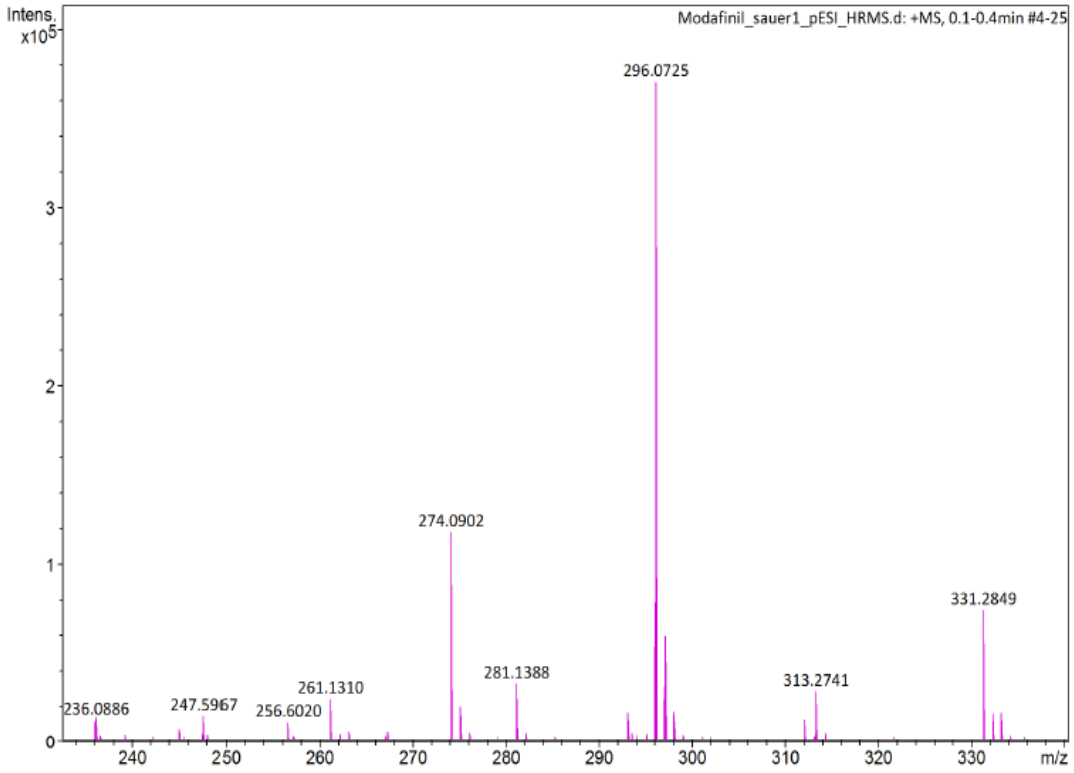

Figure 38: The MS chromatogram of sample 1 [15 µL dilution of R-Modafinil (100 µg/mL) + 500 µL methanol + 50 µL 0.5 % FA] is depicted in this table. The *m/z* of the hydrogen adduct of R-Modafinil is 274.0902 and has an intensity of approximately 1.2 x 10^5. The sodium adduct of R-Modafinil has a *m/z* of 296.0725 and an intensity of approximately 3.8 x 10^5. The result was a ratio of hydrogen adduct to sodium adduct of 1:3.2.

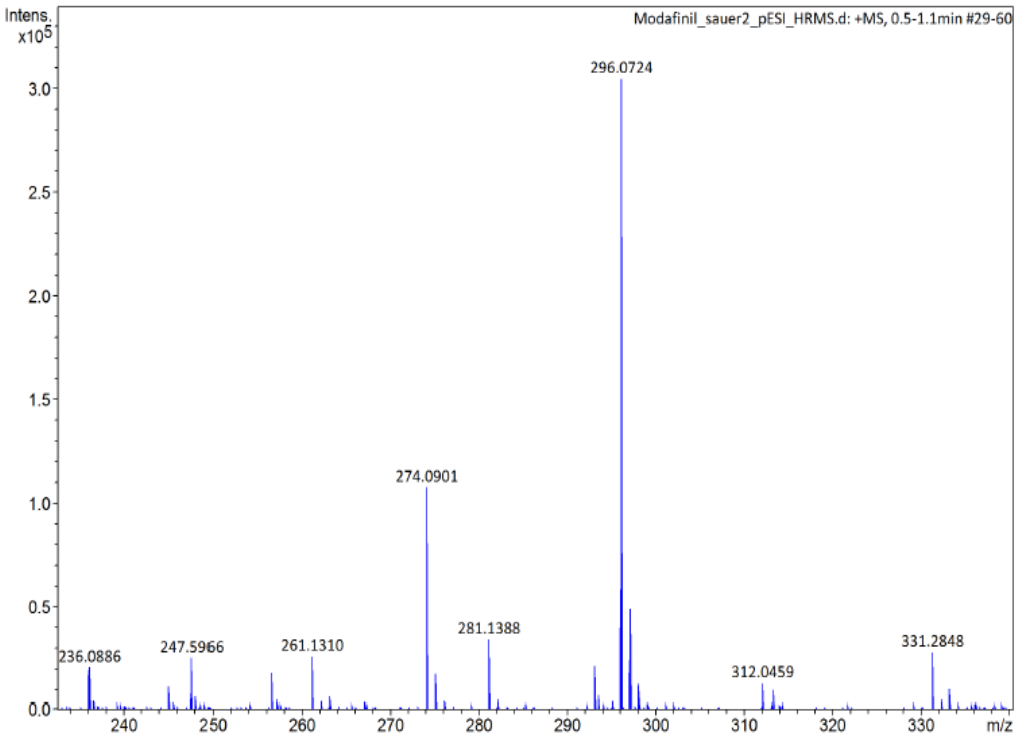

Figure 39: The MS chromatogram of sample 2 [15 µL dilution of R-Modafinil (100 µg/mL) + 500 µL methanol + 100 µL 0.5% FA] is depicted in this table. The *m/z* of the hydrogen adduct of R-Modafinil is 274.0901 and has an intensity of approximately 1.1 x 10^5. The sodium adduct of R-Modafinil has a *m/z* of 296.0724 and has an intensity of approximately 3.0 x 10^5. The ratio of H^+ to Na^+ was 1:2.7.

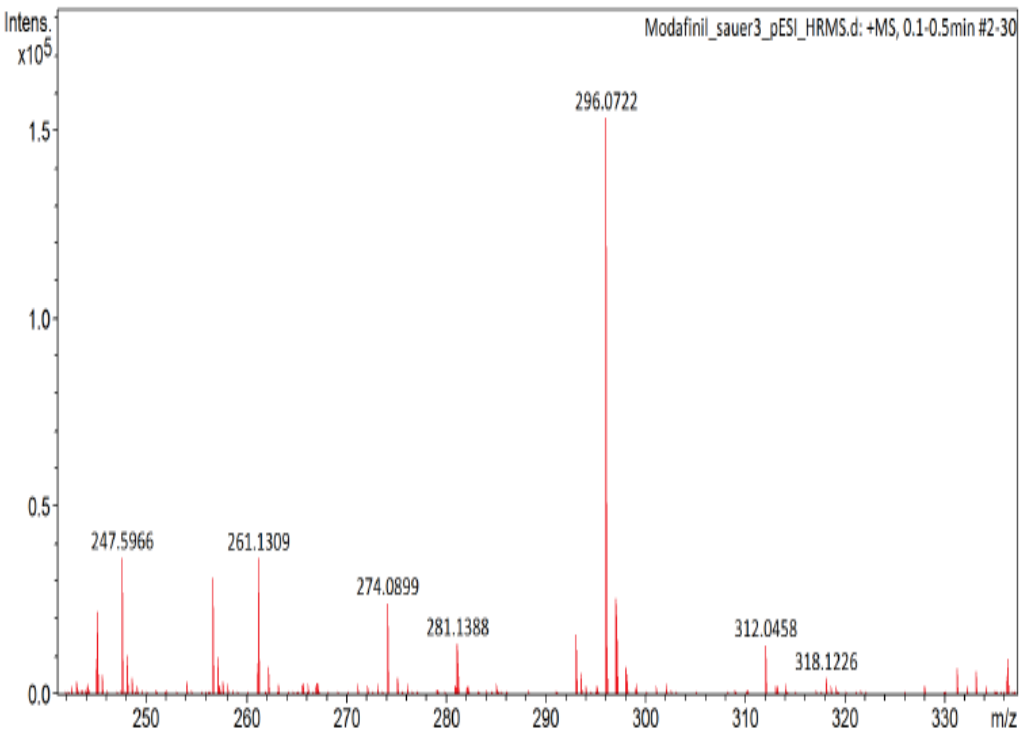

Figure 40: The MS chromatogram of sample 3 [15 µL dilution of R-Modafinil (100 µg/mL) + 500 µL methanol + 500 µL 0.5% FA] is depicted in this table. The *m/z* of the hydrogen adduct of R-Modafinil is 274.0899 and has an intensity of approximately 0.2 x 10^5. The sodium adduct of R-Modafinil has a *m/z* of 296.0722 and has an intensity of approximately 1.5 x 10^5. The ratio of hydrogen adduct to sodium adduct was 1:7.5.

The result for sample 1 was a ratio of hydrogen adduct to sodium adduct of 1:3.2. The ratio of H^+ to Na^+ of sample 2 was 1:2.7. The measure of sample 3 produced a ratio of hydrogen to sodium of 1:7.5.

The addition of acid (0.5 % FA) did not decrease the intensity of the sodium adduct of *R*-Modafinil applying ESI. In mass spectrometry, acids are added to increase the intensity of hydrogen adducts of the compounds. The reason is that acids in aqueous solution enhance the concentration of H^+ ions (Markgraf B., 2018).

5. Discussion

5.1 Results of rat samples

In the preceded pharmacokinetic study of Pharmacelsus (Saarbrücken, Germany) the plasma levels in Sprague Dawley rats with an age between 12 and 14 weeks of CE-123 1 hour after a single administration (10 mg/kg) was found to be 3000 ng/mL (%CV = 29) (Kristofova *et al.*, 2018). In our study we calculated the concentrations of the enantiomeric *S*-form of CE-123 in aged Sprague Dawley rats (2 years old) 2 hours after administration of 10 mg/kg. The concentrations from 8 out of 14 treated rat samples were found to be in a range of 1220-4080 ng/mL. The results of the other treated rat samples in the present study were found to be significantly lower (110 ng/mL *S*-CE-123). A probable reason was a mistake that occurred during the intraperitoneal injection of the analyte. Due to the time difference of the blood sampling of the Pharmacelsus study (1 hour after administration) and our study (2 hours after administration) and the application of racemic CE-123 (Pharmacelsus) and the application of *S*-CE-123, a direct comparison between the results of the studies is not possible. Nevertheless, the measured concentration of CE-123 in the Pharmacelsus study after one hour (3000 ng/mL CE-123) was found to be in a similar range compared to our calculated analyte concentrations after two hours (1220-4080 ng/mL) with a mean of 3098 ng/mL (%CV = 3.20).

5.2 Validation

The developed LC-HRMS method was evaluated for linearity in an initial analyte concentration range of 650-8700 ng/mL *S*-CE-123 in plasma with a correlation coefficient (R^2) of 0.9965. The minimum calibration range necessary for drug substance assays (80 to 120 percent of test concentration) was achieved (ICH

Expert Working Group, 1994). Accuracy was estimated using control plasma samples at concentrations within the validation range, prepared in duplicates. The acceptance criteria for accuracy of ± 15 % nominal concentration was achieved (Food and Drug Administration Office, 2018). Following the guidelines, a sufficient level of intra- and inter-day precision (± 15 % relative standard deviation (%CV) of the average concentrations) was accomplished (Food and Drug Administration Office, 2018). Furthermore, autosampler stability studies were conducted and all samples achieved the respective acceptance criteria (accuracy (% nominal) at each level within ± 15 %) (Food and Drug Administration Office, 2018). Applying the Signal-to-Noise Approach for *R*-Modafinil a LLOD of 5 ng/mL and for *S*-CE-123 a LLOQ of 6 ng/mL and a LLOD of 2 ng/mL were determined. Regarding the long-term stability studies, a selection of a higher analyte (*S*-CE-123) concentration than 10 ng/mL would have been necessary. The EIC of *S*-CE-123 was below the LLOQ, therefore this concentration could not be included. Furthermore, after 3 months of storage, the concentrations of analyte and IS increased, presumably caused by evaporation of solvent. Therefore, the value of *S*-CE-123 (Δ A [%]), for example for samples in rat plasma in a concentration of 8300 ng/mL and a storage temperature of 4°C exceeded the one of previous measurements (t_{1M} = 8.1%, t_{3M} = - 7.8%), whereas the value of *R*-Modafinil (Δ A [%]) did not decrease in the expected amount (t_{1M} = 9.2%, t_{3M} = 16.8%). The results of long-term stability studies for samples dissolved in ACN showed the lowest decrease of concentration (Δ A [%]) when applying storage temperatures of + 4 °C [*S*-CE-123 (8300 ng/1005µL): t_{3M} = +0.9%, *R*-Modafinil (5300 ng/1005 µL): t_{3M} = -42.1% / -45.5%] and – 20 °C [*S*-CE-123 (8300 ng/1005µL): t_{3M} = +0.3%, *R*-Modafinil (5300 ng/1005 µL): t_{3M} = -44.9% / -41.1%]. Furthermore, the lowest decrease in concentration of samples

prepared in rat plasma was obtained when storing the samples in the refrigerator at + 4 °C. Resulting from the stability studies, it can be determined that *S*-CE-123 is more stable than *R*-Modafinil, which is plausible considering the chemical structure of the two compounds, because of the substitution of the thiazole of CE-123 instead of the carboxyl-amide group of Modafinil (Kristofova *et al.*, 2018).

5.3 Signal-to-noise ratio

During the work of this project, a significant difference concerning the signal-to-noise ratio of samples containing low analyte concentrations was noticed. It was observed that the signal-to-noise ratio of *S*-CE-123 at an initial analyte concentration of 2 ng/mL was either below or above 10:1 when measuring the same set of samples at different days. Every calibration of the mass spectrometer was conducted immediately before measuring, therefore a calibration drift is not likely to be the reason for the deviation. This inconstancy was also observed at low concentrations of the IS (concentrations below 5 ng/mL), which were either below or above 3:1 when measuring the same set of samples at different days. As a result, this inexplicable variance should be considered, when performing LC-HRMS measurements with *S*-CE-123 and *R*-Modafinil at low concentrations.

5.4 Adducts of *R*-Modafinil

During studies with *R*-Modafinil, it was noticed, that the addition of acid (50 µL, 100 µL and 500 µL 0.5% FA) did not increase the concentration of hydrogen adduct applying ESI. In mass spectrometry, acids are added to increase the intensity of hydrogen adducts of the compounds (Markgraf B., 2018). However, *R*-Modafinil was found not to follow this presumption. Furthermore, experiments measuring *R*-Modafinil exposed to higher concentrations of formic acid (500 µL

0.5 % FA) were conducted. However, the intensity of the *R*-Modafinil hydrogen adduct did not increase and the intensity of the sodium adduct did not decrease. As a result, quantification applying *R*-Modafinil as IS was executed with its sodium adduct.

6. References

1. Altuntas E., Kempe K., Crecelius A., Hoogenboom R., Schubert U. S., 2015. ESI-Q-TOF MS/MS study of poly(2-oxazoline)s. Bruker Daltonics, Inc. http://bruker.poznan.pl/images/stories/Daltonics/noty/et25.pdf (accessed 2019.30.01)
2. Binder M.D., Hirokawa N., Windhorst U., 2009. Sleep-onset REM Period. https://link.springer.com/referenceworkentry/10.1007%2F978-3-540-29678-2_5445 (accessed 2018.10.06)
3. Bruker Daltonics Training Document., Bruker Corporation, Bremen, Germany
4. Burgess C. R., Tse G., Gillis L., Peever J. H., 2010. Dopaminergic regulation of sleep and cataplexy in a murine model of narcolepsy. SLEEP 33(10):1295-1304.
5. Deutsche Gesellschaft für Neurologie, 2012. Narkolepsie. Leitlinien für Diagnostik und Therapie in der Neurologie. https://www.dgn.org/images/red_leitlinien/LL_2012/pdf/030-056l_S1_Narkolepsie_2012_verlaengert.pdf (accessed 2018.10.08)
6. Fachinformation: Modasomil 100 mg Tabletten, 2017
7. Fachinformation: Ritalin 10 mg, 20 mg, 30 mg, 40 mg Kapseln, 2018
8. Fachinformation: Xyrem 500 mg/mL Lösung zum Einnehmen, 2015
9. Fachinformation: Waxis 18 mg Filmtabletten/ 4.5 mg Filmtabletten, 2018
10. Food and Drug Administration Office, 2018. USFDA: Bioanalytical Method Validation: Guidance for Industry https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf (accessed 2018.09.18)
11. Fox M. A., Whitesell J. K., 1994. Organic Chemistry, Third Edition. Jones and Bartlett Publishers, Inc

12. ICH Expert Working Group, 1994. ICH Harmonised Tripartite Guideline: Validation of analytical procedures: Text and Methodology Q2(R1) https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (accessed 2018.09.18)
13. Jung J-C., Lee Y., Son J-Y., Lim E., Jung M., Oh S., 2012. Simple Synthesis of Modafinil Derivatives and Their Anti-inflammatory Activity. Molecules 17, 10446-10458
14. Kalaba P., Aher N. Y., Ilić M., Dragačević V., Wieder M., Miklosi A. G., Zehl M., Wackerlig J., Roller A., Beryozkina T., Radoman B., Saroja S. R., Lindner W., Gonzalez E. P., Bakulev V., Leban J. J., Sitte H. H., Urban E., Langer T., Lubec G., 2017. Heterocyclic Analogues of Modafinil as Novel, Atypical Dopamine Transporter Inhibitors. Journal of Medicinal Chemistry 60 (22), 9330-9348
15. Kristofova M., Aher Y. D., Ilic M., Radoman B., Kalaba P., Dragacevic V., Aher N. Y., Leban J., Korz V., Zanon L., Neuhaus W., Wieder M., Langer T., Urban E., Sitte H. H., Hoeger H., Lubec G., Aradska J., 2018. A daily single dose of a novel modafinil analogue CE-123 improves memory acquisition and memory retrieval. Elsevier B.V. 0166-4328
16. Lavagnini I., Magno F., Seraglia R., Traldi P., 2006. Quantitative Applications of Mass Spectrometry. John Wiley & Sons Ltd, West Sussex, England
17. Loland C. J., Mereu M., Okunola O. M., Cao J., Prisinzano T. E., Mazier S., Kopajtic T., Shi L., Katz J. L., Tanda G., Hauck Newman A., 2012. *R-Modafinil* (Armodafinil): A unique dopamine uptake inhibitor and potential

medication for psychostimulant abuse. Biol Psychiatry 2012. 72(5): 405-413.

18. Lough W. J., Wainer I. W., 1996. High Performance Liquid Chromatography. Fundamental Principles and Practice. Blackie Academic & Professional, an imprint of Chapman & Hall, Glasgow
19. Lundanes E., Reubsaet L., Greibokk T., 2014. Chromatography - Basic Principles, Sample Preparations and Related Methods. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany
20. Markgraf B., 2018. What is an Arrhenius Acid? - Sciencing, https://sciencing.com/what-is-an-arrhenius-acid-13712171.html (accessed 2019.01.28)
21. Markowski Wojciech, 2002. Automation and Robotics in Planar Chromatography, in: Cazes Jack, Encyclopedia of Chromatography. Marcel Dekker Inc., New York
22. National Center for Biotechnology Information, 2019. PubChem Compound Database https://pubchem.ncbi.nlm.nih.gov/compound/4236 (accessed 2019.01.28)
23. Nikiforuk A., Kalaba P., Ilic M., Korz V., Dragačević V., Wackerlig J., Langer T., Höger H., Golebiowska J., Popik P., Lubec G., 2017. A Novel Dopamine Transporter Inhibitor CE-123 Improves Cognitive Flexibility and Maintains Impulsivity in Healthy Male Rats. Front. Behav. Neurosci. 11:222
24. Prasenjit M., Satla S., Raparla R., 2016. Quantification of Blonanserin in Human Plasma Using Liquid Chromatography- Electrospray Ionization-Tandem Mass Spectrophotometry-Application to Pharmacokinetic Study. Journal of Young Pharmacists, Vol 8, Issue 4

25. Rodenbeck, A., Geisler, P., Schulz, H., 2014. Internationale Klassifikation von Schlafstörungen, 3. Version (ICSD-3). https://www.researchgate.net/profile/Hartmut_Schulz/publication/278681401_Internationale_Klassifikation_der_Schlafstorungen_Ubersicht_uber_die_Anderungen_in_der_ICSD-3/links/55fd3c7f08ae07629e209d1e/Internationale-Klassifikation-der-Schlafstoerungen-Uebersicht-ueber-die-Aenderungen-in-der-ICSD-3.pdf (accessed 2018.10.08)
26. Scammell T., Willie J., Guilleminault C., Siegel J., 2009. A Consensus Definition of Cataplexy in Mouse Models of Narcolepsy. SLEEP 32(1):111-116.
27. Schmitt Kyle C., Reith Maarten E. A., 2011. The Atypical Stimulant and Nootropic Modafinil Interacts with the Dopamine Transporter in a Different Manner than Classical Cocaine-Like Inhibitors. PLoS ONE 6(10): e25790.
28. Teva Pharmaceuticals USA, Inc., 2017. Highlights of prescribing information - Nuvigil®. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/021875s023lbl.pdf (accessed 2019.01.29)
29. The U.S. Modafinil in Narcolepsy Multicenter Study Group authors, 1998. Randomized Trial of Modafinil for the Treatment of Pathological Somnolence in Narcolepsy. Annals of Neurology. 43:88-97
30. VICH Steering Committee, 2009. Guidelines for the validation of analytical methods used in residue depletion studies. https://www.ema.europa.eu/documents/scientific-guideline/vich-topic-gl49-step-4-guidelines-validation-analytical-methods-used-residue-depletion-studies_en.pdf (accessed 2018.10.08)

31. Wang W., Xiang S., Zhou X., Ji Y., Xiang B., 2012. Enantiomeric Separation and Determination of the Enantiomeric Impurity of Armodafinil by Capillary Electrophoresis with Sulfobutyl Ether-β-cyclodextrin as Chiral Selector. Molecules. 17, 303-314
32. Wong Y. N., King S. P., Simcoe D., Gorman S., Laughton W., McCormick G. C., Grebow P., 1999. Open-Label, Single-Dose Pharmacokinetic Study of Modafinil Tablets: Influence of Age and Gender in Normal Subjects. Journal of Clinical Pharmacology, 1999;39:281-288
33. Waters Ges.m.b.H. High-Performance Liquid Chromatography [HPLC] System http://www.waters.com/waters/de_AT/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=de_AT (accessed 2018.10.05)
34. Wisor J. P., Nishino S., Sora I., Uhl G. H., Mignot E., Edgar D. M., 2001. Dopaminergic Role in Stimulant-Induced Wakefulness. The Journal of Neuroscience. 21(5):1787-1794

7. Appendix

Appendix Table 1: Calibration curve in ACN.

Sample	iC of A [ng/mL]	I A $[M+H]^+$	Mean	I IS $[M+Na]^+$	Mean	C A/IS	I A/IS	Mean	SD	CV [%]
1a	260	22244	22644	125432	124676	0.052	0.177	0.182	0.005	2.8
1b	260	23668		126496			0.187			
1c	260	22019		122100			0.180			
2 a	530	63326	64929	130631	132933	0.106	0.485	0.488	0.024	4.9
2 b	530	68486		133274			0.514			
2 c	530	62975		134895			0.467			
3 a	1100	168573	162808	138513	134818	0.220	1.217	1.208	0.022	1.8
3 b	1100	159920		135250			1.182			
3 c	1100	159930		130691			1.224			
4 a	2600	284129	281464	134135	134241	0.520	2.118	2.097	0.088	4.2
4 b	2600	270888		135415			2.000			
4 c	2600	289374		133173			2.173			
5 a	6600	668423	666227	132607	131730	1.320	5.041	5.063	0.209	4.1
5 b	6600	664501		136562			4.866			
5 c	6600	665757		126020			5.283			
6 a	10600	954936	956950	136012	133260	2.120	7.021	7.183	0.141	2.0
6 b	10600	952092		131277			7.253			
6 c	10600	963823		132490			7.275			

Appendix Table 2: Calibration curve in Plasma.

Sample	iC of A [ng/mL]	iC of A rounded [ng/mL]	I A $[M+H]^+$	Mean	I IS $[M+Na]^+$	Mean	C A/IS	I A/IS	Mean	SD	CV [%]
1 a	645	650	42943	40481	303926	293453	0.0613	0.1413	0.1379	0.004	3.1
1 a	645	650	41463		293305			0.1414			
1 b	650	650	38550		294526			0.1309			
1 b	650	650	38969		282054			0.1382			
2 a	1289	1300	78626	80238	288455	288114	0.1226	0.2726	0.2785	0.007	2.4
2 a	1289	1300	83822		301686			0.2778			
2 b	1301	1300	81482		281561			0.2894			
2 b	1301	1300	77020		280754			0.2743			
3 a	2578	2600	183450	178529	297988	295366	0.2453	0.6156	0.6044	0.029	4.8
3 a	2578	2600	187333		292180			0.6412			
3 b	2602	2600	163934		292197			0.5610			
3 b	2602	2600	179399		299097			0.5998			
4 a	4150	4200	316646	330723	284966	290358	0.3962	1.1112	1.1389	0.025	2.2
4 a	4150	4200	345237		294198			1.1735			
4 b	4164	4200	331632		287760			1.1525			
4 b	4164	4200	329379		294508			1.1184			
5 a	5155	5200	391745	402714	290312	292712	0.4906	1.3494	1.3755	0.021	1.6
5 a	5155	5200	425767		303421			1.4032			
5 b	5205	5200	398646		287139			1.3883			
5 b	5205	5200	394697		289974			1.3611			
6 a	8591	8700	627111	649102	294821	292840	0.8208	2.1271	2.2165	0.056	2.5
6 a	8591	8700	662611		292977			2.2617			
6 b	8675	8700	632303		285818			2.2123			
6 b	8675	8700	674384		297745			2.2650			

Appendix Table 3: Calibration curve for lower analyte concentrations (below 650 ng/mL *S*-CE-123) in Plasma.

Sample	iC of A [ng/mL]	iC of A rounded [ng/mL]	I A [M+H]+	Mean	I IS [M+Na]+	Mean	C A/IS	I A/IS	Mean	SD	CV [%]
1 a	103	100	30782	29084	233428	225966	0.009	0.132	0.129	0.005	3.6
1 b	104	100	28632		216619			0.132			
1 b	104	100	27838		227853			0.122			
2 a	208	210	38678	39097	234586	224808	0.020	0.165	0.174	0.006	3.7
2 a	208	210	39727		217492			0.183			
2 b	214	210	38521		222469			0.173			
2 b	214	210	39463		224685			0.176			
3 a	307	310	50064	50457	249133	246356	0.029	0.201	0.205	0.004	1.9
3 a	307	310	50858		242572			0.210			
3 b	313	310	49839		248037			0.201			
3 b	313	310	51066		245680			0.208			
4 a	415	420	57395	56174	222907	227379	0.040	0.257	0.247	0.011	4.6
4 a	415	420	56309		216569			0.260			
4 b	424	420	54796		233624			0.235			
4 b	424	420	56195		236414			0.238			

Appendix Table 4: Results Rat 51.

Rat 51	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	129829	117759	1.1025	4076
2	129495	114120	1.1347	4192
3	128817	113158	1.1384	4205
4	127780	117907	1.0837	4009
5	126973	115343	1.1008	4070
6	125399	117346	1.0686	3955
7	124782	119642	1.0430	3863
8	127928	113224	1.1299	4174
9	127810	113426	1.1268	4163

Mean [ng/mL]	SD [ng/mL]	CV [%]
4079	111	2.7

Appendix Table 5: Results Rat 52.

Rat 52	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	63311	106425	0.5949	2260
2	59151	107702	0.5492	2096
3	61152	103710	0.5896	2241
4	65542	111555	0.5875	2233
5	65470	106271	0.6161	2335
6	60850	108172	0.5625	2144
7	62031	107246	0.5784	2201
8	65014	103710	0.6269	2374
9	64455	109883	0.5866	2230

Mean [ng/mL]	SD [ng/mL]	CV [%]
2235	81	3.6

Appendix Table 6: Results Rat 53.

Rat 53	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	61824	102904	0.6008	2281
2	59508	107704	0.5525	2108
3	56003	107320	0.5218	1998
4	61627	102400	0.6018	2284
5	59782	102583	0.5828	2216
6	61599	112130	0.5494	2097
7	60054	100699	0.5964	2265
8	60312	104562	0.5768	2195
9	60052	107708	0.5575	2126

Mean [ng/mL]	SD [ng/mL]	CV [%]
2174	93	4.3

Appendix Table 7: Results Rat 54.

Rat 54	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	113583	112929	1.0058	3730
2	104648	107851	0.9703	3603
3	112101	116427	0.9628	3576
4	109607	112651	0.9730	3613
5	107239	102469	1.0466	3876
6	114178	114036	1.0012	3714
7	119010	132250	0.8999	3351
8	118565	123599	0.9593	3564
9	126429	129747	0.9744	3618

Mean [ng/mL]	SD [ng/mL]	CV [%]
3627	135	3.7

Appendix Table 8: Results Rat 57.

Rat 57	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	123032	114568	1.0739	3974
2	119288	108833	1.0961	4053
3	116967	113824	1.0276	3808
4	114042	110113	1.0357	3837
5	120901	108980	1.1094	4101
6	116463	115650	1.0070	3735
7	120338	114278	1.0530	3899
8	122959	114936	1.0698	3959
9	120276	117948	1.0197	3780

Mean [ng/mL]	SD [ng/mL]	CV [%]
3905	119	3.0

Appendix Table 9: Results Rat 58.

Rat 58	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	33332	115052	0.2897	1167
2	38273	123863	0.3090	1236
3	34128	111338	0.3065	1228
4	36502	117117	0.3117	1246
5	31906	110294	0.2893	1166
6	31912	109090	0.2925	1178
7	33729	111543	0.3024	1213
8	37521	115100	0.3260	1297
9	38094	121956	0.3124	1249

Mean [ng/mL]	SD [ng/mL]	CV [%]
1220	41	3.4

Appendix Table 10: Results Rat 65.

Rat 65	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	100271	105412	0.9512	3535
2	103093	101986	1.0109	3748
3	109816	111055	0.9888	3670
4	109416	113401	0.9649	3584
5	106797	105389	1.0134	3757
6	102592	103657	0.9897	3673
7	100421	101814	0.9863	3661
8	111750	107479	1.0397	3852
9	109652	112304	0.9764	3625

Mean [ng/mL]	SD [ng/mL]	CV [%]
3678	91	2.5

Appendix Table 11: Results Rat 66.

Rat 66	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	106619	105503	1.0106	3747
2	109404	101568	1.0772	3986
3	107333	104136	1.0307	3819
4	110159	110049	1.0010	3713
5	109596	106859	1.0256	3801
6	110182	105944	1.0400	3853
7	110231	102889	1.0714	3965
8	106712	100422	1.0626	3934
9	109618	107338	1.0212	3785

Mean [ng/mL]	SD [ng/mL]	CV [%]
3845	91	2.4

Appendix Table 12: Results Rat 59.

Rat 59	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	14163	109007	0.13	93
2	14393	109987	0.13	96
3	14003	106951	0.13	96
4	14268	108232	0.13	98
5	14343	105783	0.14	108
6	14385	105957	0.14	108
7	14336	106307	0.13	106
8	14340	108624	0.13	99
9	14366	107499	0.13	103

Mean [ng/mL]	SD [ng/mL]	CV [%]
101	5	5.3

Appendix Table 13: Results Rat 67.

Rat 67	Intensity of A	Intensity of IS	Intensity A/IS	*S*-CE123 [ng/mL]
1	14132	108005	0.13	96
2	14681	108497	0.14	107
3	14162	104438	0.14	108
4	14498	108499	0.13	103
5	14632	105414	0.14	116
6	14436	105387	0.14	112
7	14469	106325	0.14	109
8	14430	107261	0.13	105
9	14408	108282	0.13	101

Mean [ng/mL]	SD [ng/mL]	CV [%]
106	6	5.3