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Dear Clemens, to quote a great friend: *"Indeed, we truly is experts!"*

I have learned a great deal during my trials and tribulations and do not wish to have missed any of them. As Shakespeare said: "All's well that ends well".

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1 Abstract

Hepatitis C virus (HCV) is responsible for causing acute hepatitis and chronic liver disease, which subsequently can lead to the development of cirrhosis and hepatocellular carcinoma. Worldwide, approximately 170 million people suffer from chronic HCV infections and the incidence of new infections each year is 3 to 4 million. Current treatment strategies of Hepatitis C are based on pegylated interferon-alpha (PEG-IFN-α) therapy given either alone or in combination with ribavirin. However, due to severe side effects and low response rates there is a strong need for new therapy approaches in Hepatitis C treatment.

A novel therapeutic approach to target HCV is the development of a therapeutic vaccine consisting of short peptides in conjunction with Human Leukocyte Antigen (HLA) molecules. This strategy aims to provoke not an antibody, but a T cell based immune response in affected individuals. T cells are the main HCV-effector cells and antibodies do not protect against HCV due to its high antigenic variability. The biotechnology company Valneva Austria GmbH (formerly Intercell AG) focuses on vaccines and antibodies for the treatment of infectious diseases.

In the current diploma thesis performed at the company in 2007/2008, an exploratory prototype formulation for a therapeutic HCV vaccine which consists of 9 synthetic HCV-derived peptides being the active substance and Valneva's licensed adjuvant IC31® as a strong stimulator of immune cells was developed.

The nine candidate peptides which cover structural and non-structural viral regions of HCV were synthesized using Fmoc chemistry in order to avail them for the development and optimization of an analytical reversed-phase HPLC method for subsequent studies. Different solvents were analyzed to define optimal solubility conditions for each peptide. The interaction between each of the nine HCV-derived peptides and IC31® was investigated by binding studies in order to draw conclusion on a possible depot effect of IC31®. Results acquired from solubility studies led to the finding that peptides can be divided into two groups; the water soluble and those requiring DMSO for solubilization. A procedure for an exploratory therapeutic Hepatitis C vaccine formulation, consisting of peptides solubilized in stocks using either water or DMSO as solvent, respectively, combined with IC31® as potent immunizer was designed.

2 Zusammenfassung

Das Hepatitis C Virus (HCV) ist verantwortlich für akute und chronisch-verlaufende Leberentzündungen, die in Folge zur Entwicklung von Leberzirrhose und hepatozellulären Karzinomen führen können. Weltweit leiden ca. 170 Millionen Menschen an chronischer HCV-Infektion und die Inzidenz der jährlichen Neuerkrankungen beträgt 3 bis 4 Millionen. Die derzeitige Behandlung von Hepatitis C basiert auf einer Therapie mit pegyliertem Interferon-Alpha (PEG-IFN-α), welches entweder alleine oder in Kombination mit Ribavirin verabreicht wird. Aufgrund der Nebenwirkungen und geringen Ansprechraten besteht jedoch großer Bedarf an neuen Therapeutika zur Behandlung von Hepatitis C.

Ein neuer Ansatz Hepatitis C zu behandeln ist die Entwicklung eines therapeutischen Impfstoffes, der aus kurzen viralen Peptiden konjugiert mit HLA (Human Leukozyten Antigen) besteht. Diese Strategie zielt nicht darauf ab, eine Antikörper- sondern eine T-Zell basierte Immunantwort in betroffenen Individuen hervorzurufen, da hauptsächlich T-Zellen für die Eliminierung des Hepatitis C Virus verantwortlich sind. Antikörper bieten aufgrund der hohen Antigenvariabilität keinen Schutz gegen das Hepatitis C Virus. Das Biotechnologie-Unternehmen Valneva Austria GmbH (ehemals Intercell AG) ist spezialisiert auf Impfstoffe und Antikörper zur Behandlung von infektiösen Krankheiten.

In der 2007/2008 bei Valneva Austria GmbH durchgeführten, vorliegenden Diplomarbeit wurden experimentelle Prototypformulierungen eines therapeutischen Hepatitis C Impfstoffs, bestehend aus neun synthetischen viralen Peptiden als aktive Komponente und Valnevas lizensiertem Adjuvans IC31® als starkem Stimulator von Immunzellen, entwickelt.

Die Peptide, die strukturelle und nicht-strukturelle Abschnitte des Hepatitis C Virus enthalten, wurden mittels Festphasensynthese nach der Fmoc-Strategie hergestellt und für die Entwicklung und Optimierung einer analytischen RP-HPLC Methode verwendet, die nachfolgend Anwendung fand. Verschiedene Lösungsmittel wurden zur Ermittlung optimaler Löslichkeitsbedingungen für jedes Peptid getestet. Die Interaktion zwischen jedem der neun viralen Peptide und IC31® wurde in Bindungsstudien untersucht, um einen möglichen Depoteffekt von IC31® zu erklären. Die Ergebnisse der Löslichkeitsstudie zeigten, dass die Peptide in zwei Gruppen eingeteilt werden können; die wasserlöslichen Peptide und jene, die DMSO als Lösungsmittel benötigen. Ein experimenteller Ansatz zur Formulierung eines therapeutischen Hepatitis C Impfstoffs, bestehend aus den aktiven Peptiden, gelöst vorliegend in entsprechenden Stocks, und IC31® als immunstimulierendem Bestandteil wurde entwickelt.

3 Introduction

3.1 The Hepatitis C Disease

Hepatitis C is a severe disease primarily affecting the liver caused by hepatitis C virus (HCV). Hepatitis C infections can be self-limiting, but the majority of patients develop viral persistence following an acute infection [1]. Hepatitis C persisting longer than six months is seen as chronic [2].

Acute and chronic HCV infection mostly occur asymptomatic, however, persisting infections can cause severe liver disease, including fibrosis, cirrhosis and subsequently hepatocellular carcinoma (HCC) [2]. These end-stage liver diseases make hepatitis C the most common indication for liver transplantation [3].

Regarding the mechanism leading to liver destruction, it is currently assumed that hepatocellular injury is not mediated by cellular destruction due to the action of HCV, but rather by the immune response of the host after HCV infection. Disease progression is influenced by factors like male gender, age, alcohol abuse and duration of virus infection. [4]

3.2 The Hepatitis C Virus

The Hepatitis C virus is part of the genus *Hepacivirus* and belongs to the family of *Flaviviridae* [5]. It was first described in 1989 as a viral hepatitis which was neither classified as Hepatitis A nor B [6]. The only hosts of HCV are humans and chimpanzees [7]. Hepatitis C virus is thought to have an icosahedral structure and principally consists of the core protein and the envelope glycoproteins E1 and E2. The two glycoproteins are anchored to a host cell-derived double layer lipid envelope that surrounds the single-stranded RNA genome and multiple copies of the core protein. [7, 8]

Viral entry into host cells (hepatocytes) is mediated by interaction between virions (viral particles) and several specific surface receptors. Viral and cellular membranes fuse via clathrin-mediated endocytosis and the virus enters the cell in an endosome. The low pH in the endosome leads to decomposition of the viral particle and subsequent release of the single-stranded (ss), positive-sense RNA genome in the cytoplasm of the newly infected host cell. Viral replication takes place in the hepatocytes of the liver and follows three steps: translation, replication and virion assembly. [9]

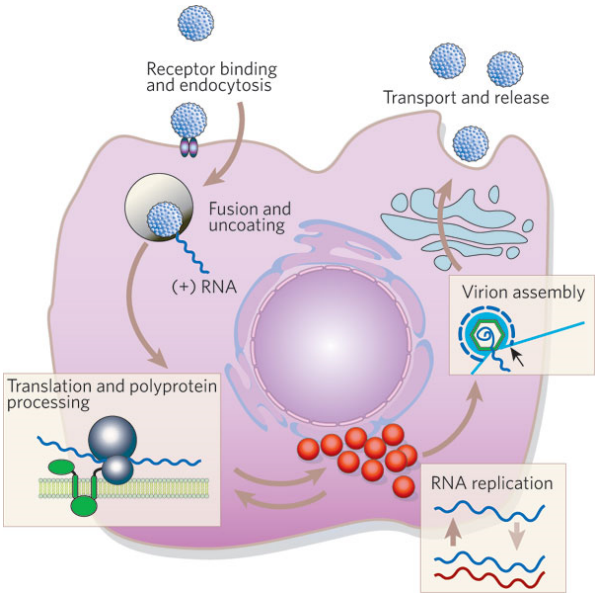


Figure 1: Hepatitis C virus life cycle. After receptor binding, endocytosis and viral uncoating, viral mRNA is translated into a polyprotein, cleaved by cellular and viral proteases; viral RNA is replicated and packed into new virions. Virions are assembled, enter the secretory pathway and are released from infected cells. [figure taken from [9]]

After uncoating, the single-stranded positive-sense RNA genome serves as mRNA for translation of viral proteins. It is 9600 bp long and contains an internal ribosome entry site (IRES) within the 5-noncoding region (NCR). This IRES binds the 40S ribosomal subunit of the host at the ER and recruits other translation initiating factors, thus promoting viral translation. Upon induction of translation, viral mRNA is translated into one single polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into the viral proteins. The amino-terminus of the polyprotein encodes structural proteins, the highly basic core (C) protein which forms the viral nucleocapsid, and glycoproteins E1 and E2 which build the viral envelope. Then, a small integral membrane protein, p7, is translated, followed by non-structural (NS) proteins NS2, NS4A, NS4B, NS5A and NS5B, which are important for intracellular processes of the viral life cycle.

Cleavage of proteins occurs by signal peptidases and processing within the NS region is mediated by NS2, a cysteine autoprotease, and NS3-4A, a serine protease. HCV can also produce a small protein called F (frame shift) or ARFP (alternative reading frame protein) by ribosomal frame shifting into an alternative reading frame, although its function is not entirely clear. The NS proteins recruit the viral genome into an RNA replication complex and RNA is replicated by the RNA polymerase NS5B to form a negative strand RNA intermediate. This intermediate RNA serves as template for the production of positive strand viral genomes, which can be translated, replicated or packaged within new virus

particles. Virions then enter the lipoprotein secretory pathway and are released at the cell surface. [7, 9]

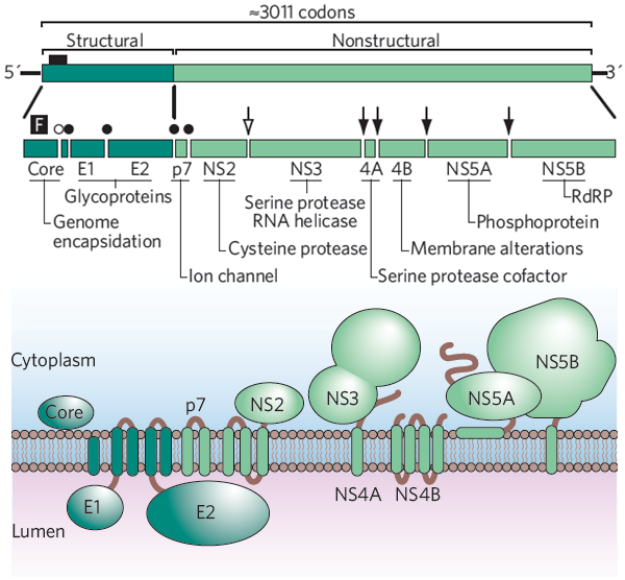


Figure 2: The structure of the viral genome. The long open reading frame encoding structural and nonstructural genes and 5' and 3' NCRs is shown. The polyprotein processing scheme is depicted below. Closed circles (black) refer to signal peptidase cleavage sites; the open circle (white) refers to the signal peptide peptidase cleavage site. Below, the topology of HCV proteins with respect to a cellular membrane is shown. [figure taken from [9]]

Due to the high error rate of the viral RNA-dependent RNA polymerase (RdRp) NS5B, HCV rapidly mutates and many variants of the virus exist [10]. Currently, HCV is categorized into six major genotypes and numerous subtypes that are designated as a, b, c, etc. Genotypes 1, 2 and to some extent 3 are found across the globe, whereas genotypes 4 and 5 are mainly distributed in African countries and genotype 6 is found predominant in Asia. [11, 12]

Figure 3: HCV genotype and subtype prevalence. [figure taken from [11]]

It is also important to distinguish between different genotypes in respect of successful therapy response of chronic Hepatitis C. HCV genotypes are correlated with interferon-based therapy response; whereas patients persistently infected with genotypes 1 and 4 are less responsive, treatment of patients persistently infected with genotypes 2 and 3 leads to sustained response. [13, 14]

3.3 Epidemiology

It is estimated that approximately 170 million people suffer from chronic HCV infections worldwide, and 3 to 4 million people are newly infected each year [4]. There is a pronounced difference between areas and countries [15]. In Austria, about 60.000 individuals are infected with HCV, which corresponds to a prevalence of 0.7% [16]. HCV may be transmitted sexually and perinatally but is primarily transmitted percutaneously, i.e. by needle sharing of drug users. The transmission by blood and blood products hardly occurs anymore because of stringent screening tests. [17, 18]

3.4 Current Therapy of Hepatitis C

Patients chronically infected with HCV currently receive a combination of pegylated interferon-alpha (PEG-IFN-α), either 2a or 2b, and ribavirin [19-21].

Interferon-alpha (IFN-α) is a naturally occurring glycoprotein produced by the immune system to stimulate a strong antiviral response [22, 23]. It functions by binding to cellular receptors and triggers a signal transduction cascade by activating receptor associated Janus-kinases. These, in turn, phosphorylate and activate STAT (signal transducer and activator of transcription) transcription factors which then dimerize, translocate into the nucleus and activate the expression of interferon-induced genes that contribute in viral defense mechanisms. [22, 24]

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is a synthetic guanosine analogue with antiviral properties. By being incorporated into the viral genome, ribavirin induces error catastrophe by the HCV RNA-dependent RNA polymerase NS5B. It also inhibits inosine monophosphate dehydrogenase (IMPDH), which leads to depletion of the intracellular GTP pool. Furthermore, ribavirin is thought to modulate the immune response by enhancing the expression of interferon stimulated genes (ISG) and inducing a HCV-specific Th1 immune response. However, ribavirin as single agent is not effective as treatment for Hepatitis C. [25]

Before the late 1990s, IFN-α was used as monotherapy for patients chronically infected with hepatitis C but had little impact on the viral load [26]. The combination of IFN-α and ribavirin has proven to be more effective than IFN-α monotherapy and enhanced sustained virologic response rates (SVR), defined as the loss of detectable HCV RNA during treatment and continued absence for at least 6 months after stopping therapy, to the range of about 40%. The use of polyethylene glycol (PEG)-modified IFN-α, which has an increased half-life, in combination with ribavirin, further improved SVR up to 56 %. [25, 27]

The combination treatment consisting of ribavirin and PEG-IFN-α has achieved the best clinical results so far. Ribavirin dosing schedules depend on body weight of the patient and on the HCV genotype. Patients infected with genotype 1, 4, 5 or 6 require the highest ribavirin doses and should be treated for 48 weeks. Patients infected with HCV genotypes 2 or 3 are more sensitive to interferon-based therapy therefore receiving therapy for 24 weeks with considerably lower ribavirin doses. [26]

However, current therapy has significant side effects that remarkably reduce the quality of life of patients and their adherence to treatment [28]. Major side effects of combination therapy include influenza-like symptoms, hematologic abnormalities, and neuropsychiatric symptoms [19]. Another disadvantage is that not all infected patients can be treated because of numerous exclusion criteria [27, 29].

3.5 Areas of novel therapeutic research

As stated above, current therapy is of limited success for which reason the need for new, safe and efficacious treatment options is tremendous and vaccines may play an important role in the development of such medications. Efforts to design an effective and safe prophylactic or therapeutic vaccine have been hampered by the fact that there are numerous HCV genotypes and quasi-species present. [23]

Traditionally, vaccines are designed to induce high levels of antibodies [30, 31]. In the case of hepatitis C, a vaccine strategy aiming at inducing antibodies will likely fail due to the high variability of HCV [32]. To be successful, a therapeutic vaccine will need to induce a strong cytotoxic T lymphocyte and T helper cell response [33].

3.6 Cellular response to HCV infection

An important part in defeating the Hepatitis C virus and HCV infection is attributed to cellular immunity, in particular to T-cells. Acute and chronic HCV infection cause both $CD4^+$ and $CD8^+$ T cell responses. [33]

In case of a strong and persistent activation of $CD8^+$ and helper $CD4^+$ T cells responding to dominant T cell epitopes acute HCV infection can become self-limiting due to spontaneous rejection of the virus [34, 35]. Persistence of HCV and thereof occurrence of chronic Hepatitis C anyhow are associated with impaired $CD8^+$ and $CD4^+$ T cell responses [36-39]. Furthermore, clearance of HCV from peripheral blood and curing of chronic Hepatitis C after antiviral treatment correlates with strong $CD4^+$ T cell responses to several HLA (Human Leukocyte Antigen) class II HCV epitopes, which suggest that HLA class II T cell epitopes also play an important role in addition to HLA class I epitopes from HCV when searching for a therapeutic vaccine against the virus [40].

3.7 The therapeutic HCV vaccine IC45®

T cells recognize antigens in form of short epitopes (peptides) in conjunction with HLA expressed on the surface of antigen presenting cells (APCs). In particular, the outcome of infection with HCV virus is determined by T cells responding to HLA class I and II restricted epitopes from HCV [39-42].

To be successful, a therapeutic vaccine has to be able to induce strong cytotoxic class I ($CD8^+$) and helper class II ($CD4^+$) T cell responses [43]. IC45® is a HCV peptide-based vaccine with the adjuvant IC31® being developed for the treatment of chronic Hepatitis C.

3.7.1 HCV-derived peptides

As the virus is highly variable, only peptide antigens that are highly conserved in the most common HCV genotypes are used. It has been shown that in patients able to eliminate the virus, at least three viral proteins are recognized by the patients' T cells: a structural protein, the non-structural protein NS3, and the non-structural protein NS4 [34].

The therapeutic IC45® contains nine conserved peptides with amino acid chain lengths from 9 to 26 amino acids derived from 4 different proteins from HCV core, E2, NS3 and NS4.

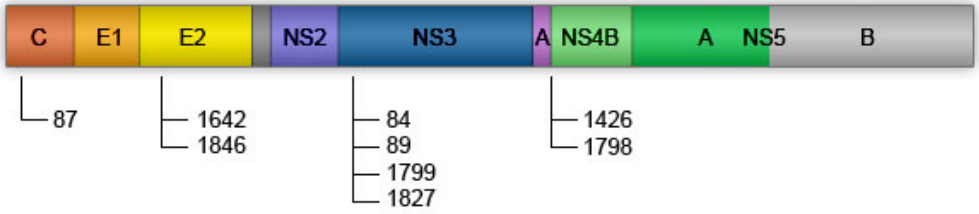


Figure 4: HCV-derived peptides of IC45®. IC45® contains nine conserved peptides derived from structural and non-structural HCV proteins. Peptides 87, 1624 and 1846 originate from structural components, whereas peptides 84, 89, 1799, 1827, 1426 and 1789 belong to non-structural parts of the translated HCV genome.

The selection of T cell epitopes was based on published reports showing a strong and persistent T cell response to these epitopes in patients spontaneously rejecting HCV and/or Valneva Austria GmbH (formerly Intercell) has characterized the suitability of the peptides selected for IC45® *in vitro* and *in vivo*. The selected peptides have the potential to induce $CD8^+$ and $CD4^+$ T cell responses but are restricted by HLA class I (HLA-A2 epitopes) but also HLA class II (multiple HLA-DR epitopes). [44, 45]

3.7.2 IC31®

However, the HCV-derived peptides need a potent immunizer to induce a T cell response in individuals expressing relevant HLA alleles [46]. Therefore, Valneva's licensed adjuvant IC31® is also part of the therapeutic HCV vaccine IC45® [47].

Valneva's adjuvant IC31® consists of two synergizing components, a specific oligodeoxynucleotide [ODN1a; $(dIdC)_{13}$] as Toll-like receptor 9 (TLR9) agonist and an antibacterial peptide [KLK; KLKLLLLLKLK] [47].

Toll-like receptor agonists represent pathogen associated molecular patterns (PAMPs), such as CpG-ODNs. ODN1a consists of dimeric repeats of deoxy-inosine / deoxy-cytosine linked by a natural phosphodiester DNA backbone. ODN1a does not contain guanine (CpG) motifs, but is able to activate the intracellularly localized TLR9 inducing type 1 responses. Most probably due to its short half-life based on its phosphodiester backbone, ODN1a does not activate the immune system by itself. [47] The cationic antimicrobial peptide (CAMP) KLK induces a depot at the injection site, therefore favoring continuous, long-term antigen and adjuvant release in order to permanently stimulate the induction of antigen specific immune response [47, 48]. The combination of ODN1a and KLK forms a stable complex via ionic and hydrophobic interactions, in which the nuclease sensitive

ODN1a and the vaccine antigen are protected against degradation [47]. KLK serves as transporter of ODN1a into the cytoplasm/endosome, where it gains access to TLR9 [48]. IC31® strongly induces antigen-specific cellular and humoral immune responses due to the synergistic effects of ODN1a and KLK [47]. KLK induces potent type 2 immune responses and enhances the uptake of antigen and ODN1a by APCs [49]. ODN1a exerts its immunostimulatory effects via the TLR9/MyD88 signaling pathway inducing a type 1 immune response [47].

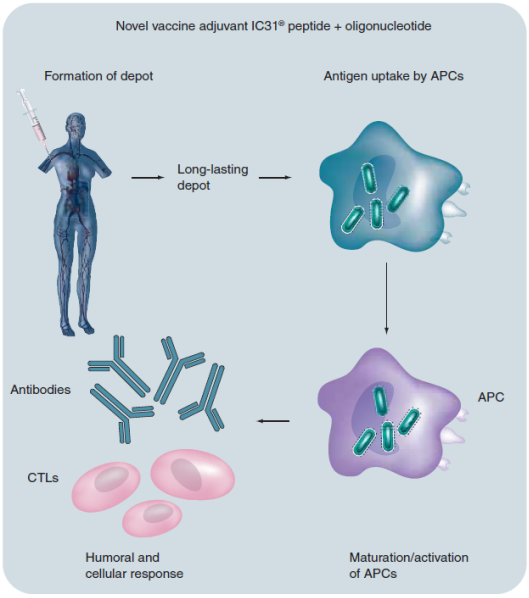


Figure 5: Valneva's adjuvant IC31®. APC: antigen presenting cell, CTL: cytotoxic T-lymphocyte. [figure taken from [47]]

3.7.3 The therapeutic HCV vaccine

The immunogenicity and efficacy of peptide-based vaccines combined with a potent immunizer in which T cells are they key effector cell types has been demonstrated in numerous preclinical animal models including cancer models [46, 50].

IC45® is designed as a therapeutic vaccine based on nine synthetic peptides in conjunction with IC31® as immunizer to elicit a T cell based (cellular) immune response in individuals chronically infected with HCV. Both, the inability of short peptide antigens of 9-26 amino acids to induce antibodies and the adjuvant properties of IC31® focus the immune response towards a cellular rather than an antibody response.

The final formulation containing nine synthetic HCV-derived peptides and IC31® is expected to follow the mechanism described in Figure 6.

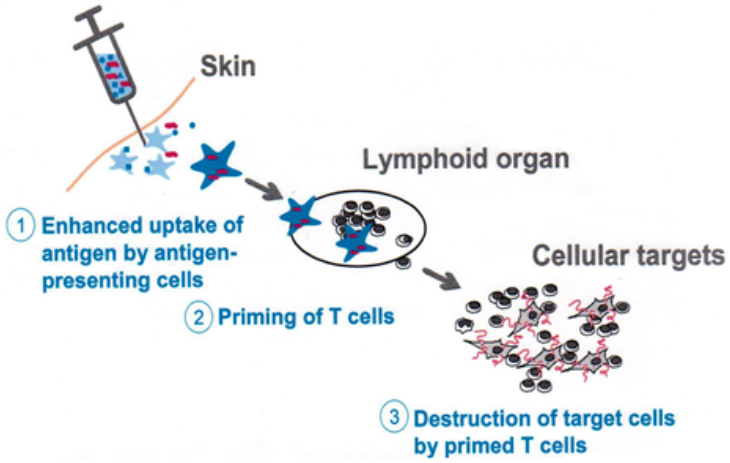


Figure 6: Mode of application and action of the therapeutic HCV vaccine IC45®. [© Valneva Austria GmbH]

The IC45® vaccine is designed to be administered subcutaneously. Antigens (peptides representing T cell epitopes) and IC31® are taken up by APCs present in the skin. Antigen-charged APCs migrate to local lymph nodes where they prime (activate) peptide-specific T cells and lead to their proliferation and differentiation to effector T cells (cytotoxic $CD8^+$ T-lymphocytes). The activated effector T cells then leave the lymphoid organ and patrol the periphery until they encounter cellular targets expressing the appropriate viral antigens (e.g. hepatocytes) which will be eliminated upon recognition by the T cells.

3.8 Aim

The aim of this diploma thesis was to develop exploratory prototype formulations for nine synthetic HCV-derived peptides with the adjuvant IC31®.

Pharmaceutical development started with the set-up and optimization of a reversed-phase HPLC method for separation and quantification of individual peptides synthesized by solid phase peptide synthesis using Fmoc chemistry. After determining optimal elution conditions and setting up calibration curves for each peptide, solubility studies of the various peptides were performed. To solubilize the HCV-derived peptides at the desired concentration, different solvents and mixtures of various solvents were tested depending on the chemical properties of the peptides (i.e. hydrophobicity, charge).

Additionally, the interaction between the peptides and IC31® was studied for drawing a conclusion on a possible depot effect.

With this, a procedure for an exploratory therapeutic Hepatitis C vaccine formulation was designed which might help to battle HCV infections in the future.

4 Materials and Methods

4.1 Drug substances

4.1.1 HCV-derived peptides

Hepatitis C virus-derived peptides with a length from 9-26 amino acids are the active substance contained in prototype formulations for a therapeutic HCV vaccine. HCV antigens from which the peptides were derived, sequences and molecular weights are listed in Figure 7 below.

Antigen	Sequence	peptide length	molecular weight [Da]	**Peptide #**	remarks
HCV core	DLMGYIPAV	9	978.1	Ipep 87	
HCV E2	LEDRDRSELSPLLLSTTEW	19	2260.4	Ipep 1624	
HCV E2	DYPYRLWHYPCTVNFTIFKV	20	2562.9	Ipep 1846	Cys
HCV NS3	GYKVLVLNPSVAAT	14	1431.7	Ipep 84	
HCV NS3	CINGVCWTV	9	994.1	Ipep 89	Cys
HCV NS3	AAWYELTPAETTVRLR	16	1877.1	Ipep 1799	
HCV NS3	TAYSQQTRGLLG	12	1294.4	Ipep 1827	
HCV NS4	HMWNFISGIQYLAGLSTLPGNPA	23	2487.8	Ipep 1426	
HCV NS4	IGLGKVLVDILAGYGAGVAGALVAFK	26	2473.0	Ipep 1798	

Figure 7: Characteristics of HCV-derived peptides. Peptides are shown in their respective amino acid (single) code: G: Glycine; P: Proline; A: Alanine; V: Valine; L: Leucine; I: Isoleucine; M: Methionine; C: Cysteine; F: Phenylalanine; Y: Tyrosine; W: Tryptophan; H: Histidine; K: Lysine; R: Arginine; Q: Glutamine; N: Asparagine; E: Glutamic Acid; D: Aspartic Acid; S: Serine; T: Threonine.

Two peptides, Ipep 1846 and 89, contain cysteine residues and therefore have the tendency to dimerize or form disulfide bonds which has to be monitored by RP-HPLC analysis to avoid unwanted reactions.

Peptides for preliminary solubility studies, RP-HPLC method development and optimization were produced in-house (Valneva GmbH, Austria). Peptides for calibration, solubility studies, binding studies and formulation development were purchased from BACHEM (Switzerland).

4.1.2 IC31®

Either TRIS (10 mM) or phosphate buffered (5 mM) IC31® formulations, both in-house produced (Valneva GmbH, Austria), containing 2000 nmol/ml KLK and 80 nmol/ml ODN1a were included in prototype formulations as potent immunizer for a therapeutic peptide-based vaccine. Phosphate and TRIS buffered IC31® formulations were provided containing 0 mM, 25 mM, 50 mM, 75 mM, 100 mM or 135 mM NaCl (sodium chloride).

4.2 Peptide Synthesis

4.2.1 Solid Phase Peptide synthesis

HCV-derived peptides were synthesized using standard Fmoc chemistry on an automated Syro II synthesizer (Multisyntech).

Peptide synthesis starts at the C-terminal end of the peptide towards the N-terminus. The α-carboxyl group of the first amino acid is attached to a Whatman 540 cellulose membrane as hydroxyl-functionalized support via ester linkage. The membrane was therefore incubated with a 0.2 M solution of DIC (diisopropylcarbodiimide)/NH^i activated Fmoc-β-Ala-OH in DMF (dimethylformamide) for 3 hours prior to attachment. The resin used was HMP (4-hydroxymethyl-phenoxymethyl-copolystyrene-1% divinylbenzene)-resin. The carboxyl groups of the Fmoc-protected amino acids were activated with HOBt/DCC (1-hydroxy-benzotriazole/dicyclohexylcarbodiimide) in NMP (N-Methyl-Morpholin). For the first C-terminal amino acid prolonged coupling time was used and DMAP added as a catalyst. The Fmoc group protecting the amino terminus was removed by 20 % piperidin in NMP. The process was repeated until final peptide assembly and side chain protecting groups were cleaved by treatment with scavenger mixtures A, B or C (as listed in Table 1) for at least 1.5 hours depending on the sequence of the peptides. Deprotection was prolonged for 30 minutes for each arginine in the sequence, but without extending 3 hours of total cleavage time.

The resin was drained over a filter funnel followed by washing with DCM (dichlormethane) and evaporation of TFA (trifluoracetic acid) and DCM in a Rotavapor. The crude peptides were subsequently purified by preparative RP-HPLC.

For peptides containing	Scavenger Mixture
Arginine (Arg), Methionine (Met)	A 0.5 ml H_2O 9.5 ml TFA (trifluoracetic acid)
Tryptophan (Trp)	B 0.75 g crystallized phenol 0.5 ml thioanisole 0.25 ml EDT (1,2 ethanedithiol) 0.5 ml H_2O 10 ml TFA
none of the mentioned amino acids	C 0.25 ml EDT 9.5 ml TFA

Table 1: Scavenger mixtures. Scavenger mixtures are used for deprotection of amino acid side chains in peptides synthesized using Fmoc chemistry.

4.2.2 Peptide purification and quality control

Purification of synthetic peptides was carried out on a BioCAD preparative RP-HPLC system (Applied Biosystems) using C18 columns and a 45 min 2-45 % gradient of 0.1 % (v/v) TFA in sterile-filtered water and 0.1 % TFA (v/v) TFA in acetonitrile. Each peptide was checked for identity and purity by MALDI-TOF on a Reflex III mass-spectrometer (Voyager-DE STR, Applied Biosystems). Peptides were finally lyophilized and stored as dry powder at -20 °C.

4.3 Preliminary solubility studies

1 mg/ml and 3 mg/ml of each synthesized peptide were dissolved in water, dimethyl sulfoxide (DMSO, 10 %, 50 and 100 %), 50 % acetic acid (HAc) and ethanol (EtOH, 10 %, 20 % and 50 %). Peptides were solubilized by vortexing for a few seconds and afterwards transferred to glass cuvettes. The analysis of solubility was performed using an UV-Vis spectrophotometer by measuring extinction at 600 nm.

4.4 RP-HPLC method development and optimization

An UltiMate 3000 HPLC system (Dionex, USA) was used, which consists of a degasser, binary pump, autosampler and a UV detector, to measure UV absorbance at a wavelength of 214 nm. Reversed-phase HPLC method set-up was performed at room temperature using a 100 Å, 3.5 µm, 4.6 mm × 75 mm Symmetry C 18 column (Waters, USA). The mobile phase used for separation was a mixture of HPLC grade solvents A: 0.1 % (v/v) TFA in sterile-filtered water and B: 0.1 % (v/v) TFA in acetonitrile. The flow rate was kept at 1.0 ml/min. Gradients were adjusted to achieve separation of the peptides as depicted in Figure 8. Blank gradients (without sample injection) were run after about 10 sample injections.

Peptide samples were prepared in 50 % acetic acid at concentrations of 1 mg/ml for each peptide separately. The sample injection volume was 10 µl in all cases. Chromatograms were evaluated using Dionex™ Chromeleon™ Chromatography Data System (CDS) software.

gradient #	Time [min.]	A [%] *water/TFA*	B [%] *acetonitrile/TFA*
(1)	0	95	5
	30	10	90
	30	95	5
	35	95	5

gradient #	Time [min.]	A [%] *water/TFA*	B [%] *acetonitrile/TFA*
(2)	0	95	5
	60	50	50
	60	95	5
	65	95	5

gradient #	Time [min.]	A [%] *water/TFA*	B [%] *acetonitrile/TFA*
(3)	0	82	18
	3	82	18
	8	73	27
	14	67	33
	19	60	40
	21	50	50
	22	82	18
	25	82	18

gradient #	Time [min.]	A [%] *water/TFA*	B [%] *acetonitrile/TFA*
(4)	0	82	18
	5	82	18
	8	73	27
	14	70	30
	15	67	33
	17	60	40
	17	50	50
	21	40	60
	22	82	18
	25	82	18

gradient for final RP-HPLC method			
gradient #	Time [min.]	A [%] *water/TFA*	B [%] *acetonitrile/TFA*
(5)	0	86	14
	4	86	14
	7	73	27
	10	71	29
	16	30	70
	17	5	95
	19	5	95
	20	86	14
	25	86	14

Figure 8: Gradients used for analytical RP-HPLC method development and optimization.

Gradients started with a period in which the eluent composition is held at the initial % B, to equilibrate the column and facilitate transfer of gradients between different instruments. Afterwards, the elution strength of the mobile phase was increased over time.

For the final gradient the column was flushed with high concentrations of organic solvent at the end of the run to ensure complete elution. Finally, gradients returned to the initial eluent concentrations to ensure that the column was conditioned for the next run.

4.4.1 Peptide-based calibration

For the generation of calibration curves, each peptide was solubilized in 50 % acetic acid and dimethylsulfoxide (DMSO) containing 0.1 % TFA at a concentration of 0.1 mg/ml. For RP-HPLC analysis the optimized gradient (5) described in section 4.4. was used. Varying sample volumes (20, 30, 50 and 70 µl) of either 0.22 µm filtered or unfiltered peptide solutions were injected.

Peak areas (y-axis) were blotted against peptide amount loaded (x-axis) and calibration curves obtained by calculating linear equations and corresponding correlation coefficients (R^2). R^2 had to be ≥ 0.99 for calibration curves to be valid.

4.5 Solubility studies

To define solubility characteristics of each individual peptide initially the grand average of hyrophopathicity index (GRAVY) and theoretical isoelectric points (pI) were calculated using ExPASy ProtParam tool (http://web.expasy.org/protparam/) [51, 52].

Preliminary solubility studies were performed with water (H_2O), 25 % ethanol (EtOH), 25 % acetic acid (HAc) and 25 % DMSO as solvent for 1 mg/ml of each peptide. Resulting solutions were analyzed by RP-HPLC as described above.

A detailed solubility screening was performed with water, HAc (1 %, 3 %, 7 %, 10 %, 25 % and 50 %) and DMSO (2.5 %, 5 %, 10 %, 15 %, 25 %, 50 % and 100 %) as solvent for 1 mg/ml of each peptide. Solubility was tested at different temperatures (4 °C, 25 °C and 37 °C) with incubation times of 0 h, 2 h, 4 h, 8 h and 12 h, respectively. Peptide solutions were put in a sonication bath during the incubation time and filtered through 0.22 µl filters before 50 µl were injected onto C18 column for RP-HPLC analysis.

4.6 Binding studies

Binding studies were performed with phosphate and TRIS buffered IC31® formulations containing 0 mM, 25 mM, 50 mM, 75 mM, 100 mM or 135 mM NaCl, respectively (described in section 4.1.2). Ipeps 87, 1624, 84, 1799, 1827 and 89 were solubilized in water, Ipeps 1846, 89 and 1798 in 10 % DMSO and Ipep 1426 in 100 % DMSO, each at a concentration of 2 mg/ml. 200 µl of each of the peptide solutions was mixed with 200 µl of each of the IC31® formulations and incubated for 120 minutes. Samples were then centrifuged at 16,100 rcf for 10 minutes and 50 µl of the respective supernatants analyzed by RP-HPLC.

5 Results

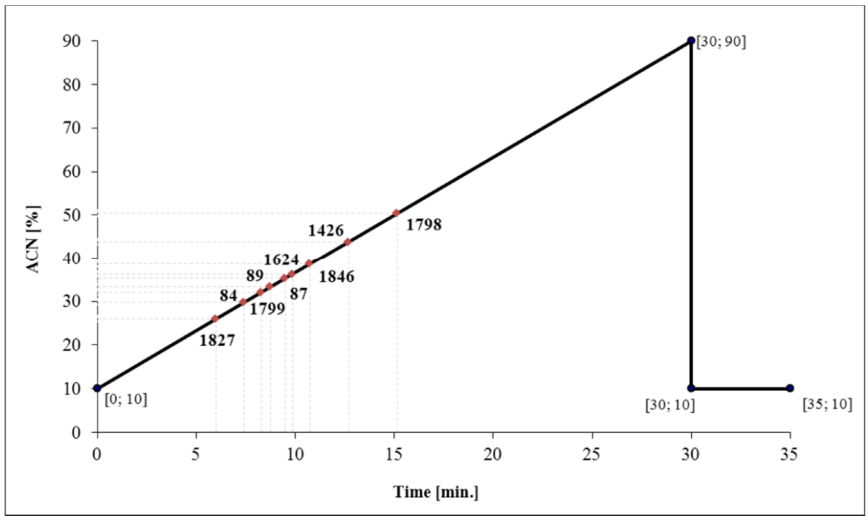
5.1 Preliminary solubility studies

Preliminary solubility studies were performed by measuring the OD (optical density) of 1 mg/ml and 3 mg/ml peptide solutions in water, DMSO (10 %, 50 % and 100 %), 50 % HAc and EtOH (10 %, 20 % and 50 %). Solutions were mixed thoroughly and peptide solubility analyzed by measuring absorption at 600 nm using a UV-Vis spectrophotometer. This preliminary solubility study unfortunately did not lead to feasible results. Peptide precipitates tended to sink fast to the bottom of the cuvette and therefore it was not possible to measure the clouding of the solutions or suspensions. Since we got no prediction about the solubility characteristics of the single peptides with this method, data is not shown. However, we observed supposedly complete solubility of each peptide using 50 % acetic acid as solvent. 50 % acetic acid is, for logical reason, not feasible to be used as a solvent for peptides in formulations to be applicable for humans. But complete solubility is a favorable condition for the set-up of an analytical reversed-phase HPLC method and necessary for monitoring recovery of single peptides in future formulations.

5.2 RP-HPLC method development and optimization

A high performance liquid chromatographic (HPLC) method based on a reversed-phase C18 column was set-up for the simultaneous separation, identification and quantification of the nine HCV-derived peptides. A Symmetry C18 column was chosen because of its good applicability for peptide separation with acetonitrile/water eluent at different compositions. Each solubilized peptide was separately injected and graphs designed by merging the individual results to defined gradients, respectively.

Gradient #1. As depicted in Figure 9, initially a linear gradient was set-up to increase mobile phase B from 10 % to 90 %. All nine peptides were eluted within 16 minutes. In reversed-phase separation, the most polar peptide elutes first while the most hydrophobic peptide elutes last. Ipep 1798, designated the most hydrophobic peptide, eluted at a concentration of 50.4 % B. This indicates that a maximum concentration of approximately 50 % acetonitrile is sufficient for elution of all peptides. Regarding separation, four peptides, Ipep 1799, 89, 87 and 1624, appeared at similar retention times and eluted within 1.6 minutes.

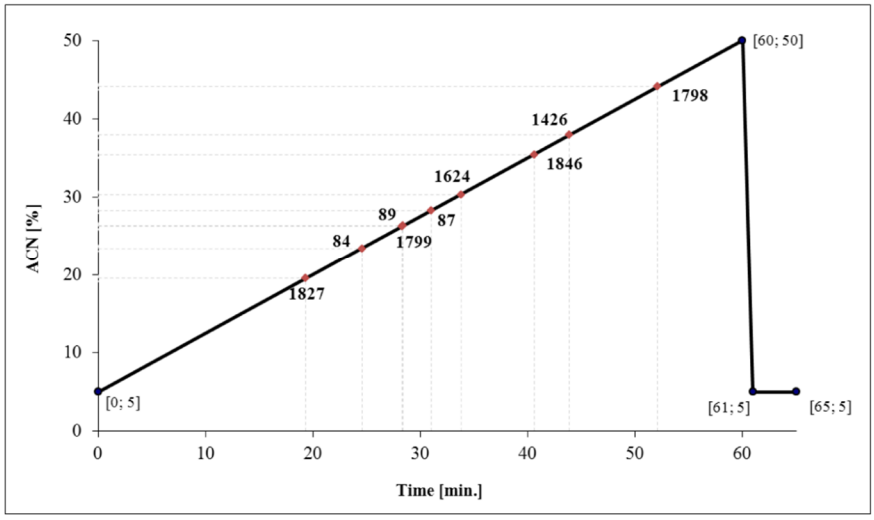


Gradient # 1

Ipep	Ret. Time [min.]	Peak #	% ACN
87	9,489	5	35,3
1624	9,852	6	36,3
1846	10,74	7	38,6
84	7,429	2	29,8
89	8,762	4	33,4
1799	8,287	3	32,1
1827	6,006	1	26,0
1426	12,69	8	43,8
1798	15,156	9	50,4

Figure 9: RP-HPLC method development: gradient #1. Mobile phase B was increased from 10 % to 90 % acetonitrile in 30 minutes. Peaks eluted in order of increasing hydrophobicity of corresponding peptides.

Gradient #2. As shown in Figure 10, a next step in developing an applicable RP-HPLC method, a linear gradient to increase mobile phase B from 5 % to 50 % was set up, thereby reducing final acetonitrile concentration in the mobile phase. The run time was extended to 65 minutes to achieve a better separation of the nine peptides. All peptides eluted within 52 minutes, indicating that adjustment of mobile phase regarding acetonitrile concentration was sufficient. Separation efficacy for peptides 89 and 1799 again was very low as both eluted at retention times within one minute therefore requiring further improvement. Also, a total run time of 65 minutes was not suitable as a high-throughput of samples was required.

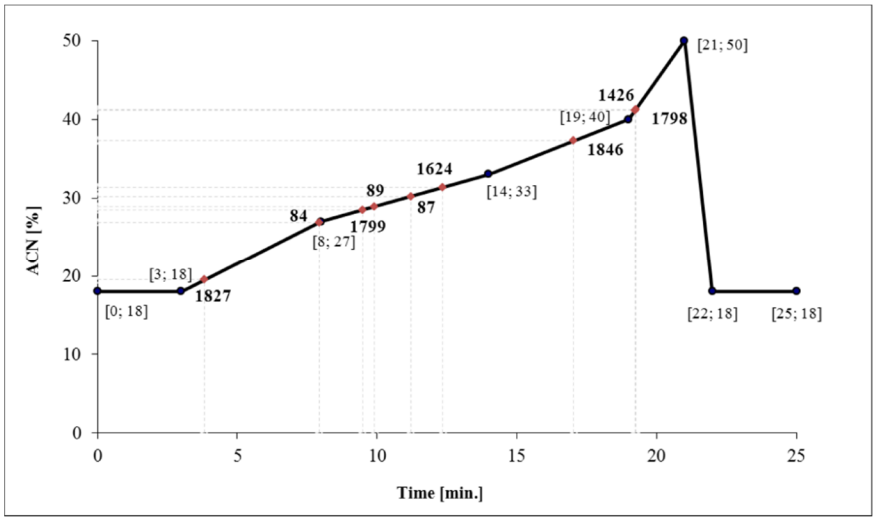


Gradient # 2

Ipep	Ret. Time [min.]	Peak #	% ACN
87	31,022	5	28,3
1624	33,797	6	30,3
1846	40,591	7	35,4
84	24,595	2	23,4
89	28,402	4	26,3
1799	28,333	3	26,2
1827	19,288	1	19,5
1426	43,849	8	37,9
1798	52,078	9	44,1

Figure 10: RP-HPLC method development: gradient #2. Mobile phase B was increased from 5 % to 50 % acetonitrile in 60 minutes. Peaks eluted in order of increasing hydrophobicity of corresponding peptides.

Gradient #3. In the following trial, as can be seen in Figure 11, run time was reduced to 25 minutes and a stepped gradient developed to achieve a more efficient separation of Ipeps 89 and 1799. Initial acetonitrile concentration was set to 18 % and held for 3 minutes. Ipep 1827, the designated most hydrophilic peptide, was shown not to elute below this concentration of the nonpolar component in the mobile phase. Raising the acetonitrile concentration from 27 % to 33 % within 6 minutes afterwards led, as expected, to a better separation of peaks corresponding to Ipep 1799 and 89. Further decrease of the polarity of the mobile phase using 50 % acetonitrile unfortunately led to appearance of peptides 1426 and 1798 both with approximate retention times of 19 minutes.

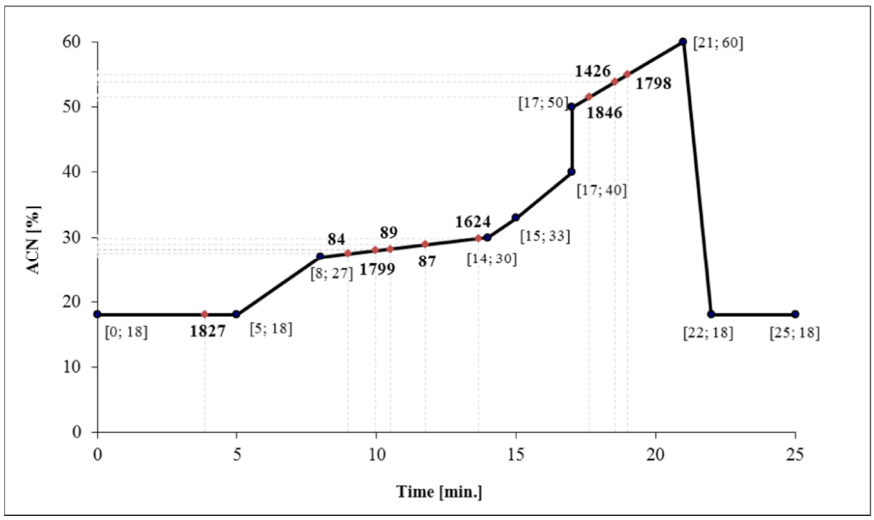


Gradient # 3

Ipep	Ret. Time [min.]	Peak #	% ACN
87	11,201	5	30,2
1624	12,361	6	31,4
1846	17,039	7	37,3
84	7,927	2	26,9
89	9,915	4	28,9
1799	9,475	3	28,5
1827	3,811	1	19,5
1426	19,22	8	41,1
1798	19,26	9	41,3

Figure 11: RP-HPLC method development: gradient #3. Initial acetonitrile concentration of 18 % was increased to 27 % within 5 minutes. Acetonitrile concentration subsequently was increased to 33 % within 6 minutes. Further decrease of the polarity of the mobile phase was achieved by stepping the gradient to 40 % acetonitrile within 5 minutes followed by an increase of acetonitrile concentration to 50 minutes in 3 minutes at the end of the gradient.

Gradient #4. As illustrated in Figure 12, further optimization was achieved by stepping the gradient from 27 % B to 30 % in 6 minutes and then from 30 % B to 33 % in 1 minute, leading to efficient separation of Ipeps 84, 1799, 89, 87 and 1624. A gradient step from 33 % B to 40 % B in 2 minutes and following increase from 50 % B to 60 % B in 4 minutes resulted in efficient separation of peaks corresponding to Ipep 1846, 1426 and 1798. The most hydrophilic peptide Ipep 1827 eluted first, as in the gradients tested before. The initial period held at 18 % B was extended to 5 minutes.



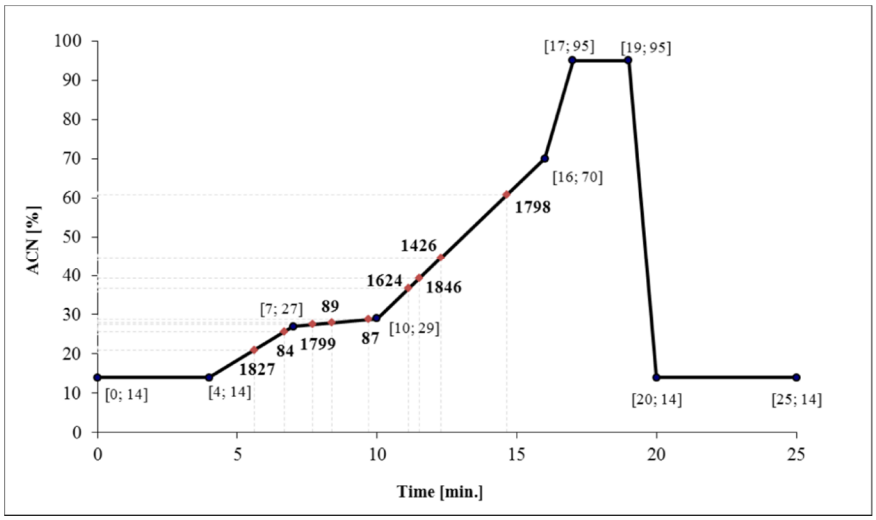
Gradient # 4

Ipep	Ret. Time [min.]	Peak #	% ACN
87	11,756	5	28,9
1624	13,646	6	29,8
1846	17,609	7	51,5
84	8,986	2	27,5
89	10,496	4	28,2
1799	9,964	3	28
1827	3,865	1	18
1426	18,555	8	53,9
1798	18,981	9	55

Figure 12: **RP-HPLC method development: gradient #4.** The stepped gradient included an additional step, first increasing the acetonitrile concentration from 27 % to 30 % within 6 minutes, followed by a further increase to 33 %. Increasing the acetonitrile concentration to 60 % instead of 50 % at the end of the gradient led to more efficient separation of the two most hydrophobic peptides.

Gradient #5. The results from preceding experiments led to definition of the final, optimized gradient shown in Figure 13 used for RP-HPLC analysis of HCV-derived peptides in subsequent studies.

The gradient started with a period in which the eluent composition is held at the initial concentration of 14 % acetonitrile over 4 minutes. Then a change in acetonitrile concentration to 27 % in 3 minutes led to elution of peaks corresponding to Ipeps 1827 and 84. Ipeps 1799, 89 and 87 were efficiently separated in the following gradient step from 27 % B to 29 % B in 3 minutes. A steep gradient step leading to increase of acetonitrile concentration from 29 % to 70 % in 6 minutes finally separated the late-eluting peaks corresponding to Ipeps 1624, 1846, 1426 and 1798. At the end of the run, the column was flushed with 95 % acetonitrile for 2 minutes and the gradient returned to initial eluent concentrations of 14 % B.



Gradient # 5

Ipep	Ret. Time [min.]	Peak #	% ACN
87	9,696	5	29,4
1624	11,119	6	37,7
1846	11,498	7	39,7
84	6,699	2	25,4
89	8,396	4	28,3
1799	17,702	3	27,3
1827	5,624	1	21,4
1426	12,297	8	45,4
1798	14,638	9	60,2

Figure 13: RP-HPLC method development: gradient #5. The stepped gradient optimized regarding separation of all nine HCV-derived peptides starts with an increase of acetonitrile concentration from 14 % to 27 % within 3 minutes. Next, acetonitrile concentration was increased to 29 % within another 3 minutes. Further decrease of the polarity of the mobile phase by enhancing acetonitrile concentrations to 70 % was achieved within 6 minutes. Adding a wash step at the end and equilibration steps at the beginning and the end of the gradient led to a total run time of 25 minutes.

The analytical reversed-phase HPLC method running gradient elution, whereby the amount of the organic solvent acetonitrile is increased from 14 % to 70 % over a period of 16 minutes, is sufficient to elute the nine HCV-derived peptides as illustrated in Figure 14. A total run time of 25 minutes including wash steps and equilibration at the beginning and the end of the gradient allowed for more frequent sample throughput. In addition, the method was transferable to a different HPLC system (HPLC System Alliance, Waters, USA).

This RP-HPC method gave the possibility to identify peptides using determined retention times as a result of efficient peak separation.

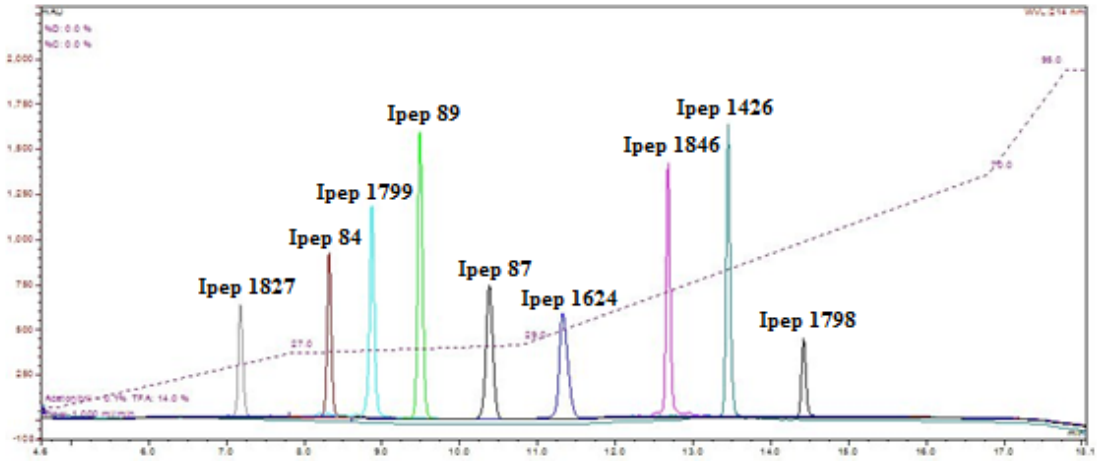


Figure 14: Overlay of RP-HPLC chromatograms. The overlay illustrates efficient peak separation of respective HCV-derived peptides. x-axis: retention times [min], y-axis: peak height [mAU]

5.2.1 Peptide-based calibration

In order to monitor recovery of peptides during formulation development, quantification of peptides is necessary. Therefore, calibration curves were generated for each peptide at a concentration of 0.1 mg/ml. In addition to 50 % acetic acid, DMSO containing 0.1 % TFA was tested as solvent. After solubilization, samples were analyzed by RP-HPLC, 0.22 µm filtered or unfiltered to test whether complete solubilization was successful.

However, for generation of calibration curves, 50 % acetic acid was used as solvent since DMSO led to inconsistent data and incomplete peptide solubilization. A comparison between DMSO and acetic acid as solvent for Ipep 87 calibration is shown as an example in Figure 15.

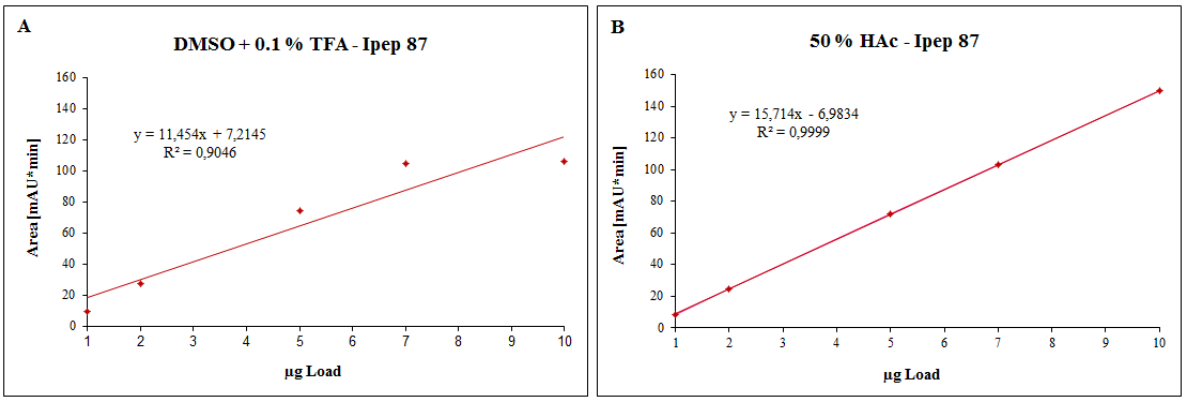


Figure 15: Comparison between DMSO and 50 % HAc as solvent for Ipep 87. A: For DMSO containing 0.1 % TFA as solvent for Ipep 87 no linear dependence between peak area and the amount peptide loaded can be shown indicated by a correlation coefficient (R^2) of ≤ 0.99. **B:** A linear dependence between peak area and the amount Ipep 87 loaded can be seen when 50 % acetic acid is used as solvent indicated by $R^2 \geq 0.99$.

Calibration was performed by injecting different volumes (20, 30, 50 and 70 µl) of 0.1 mg/ml solutions of each peptide onto the RP-HPLC column. The amount of peptide loaded (x-axis) was blotted against the corresponding peak area (y-axis) to give a calibration curve for each peptide, respectively. Equations of the resulting regression lines were calculated as well as the correlation coefficients R^2 (R-squared) which describes how well the data fits. Calibration curves of all peptides showed correlation coefficients (R^2) ≥ 0.99. We assumed that single point calibration was suitable for calculation of peptide concentrations thereby monitoring recovery of peptides in prototype formulations.

Accuracy and reproducibility of the method were determined by performing independent recovery experiments. To test for complete solubilization, 0.22 µm filtered peptide solutions were analyzed. Particles retained by the filter membrane would lead to reduced

amount of peptide in the filtrate which would result in lower peak areas obtained by RP-HPLC analysis. One recovery experiment for 0.22 µm filtered and one for unfiltered peptide solutions is shown in Figure 16 and Table 2 and Figure 17 and Table 3, respectively.

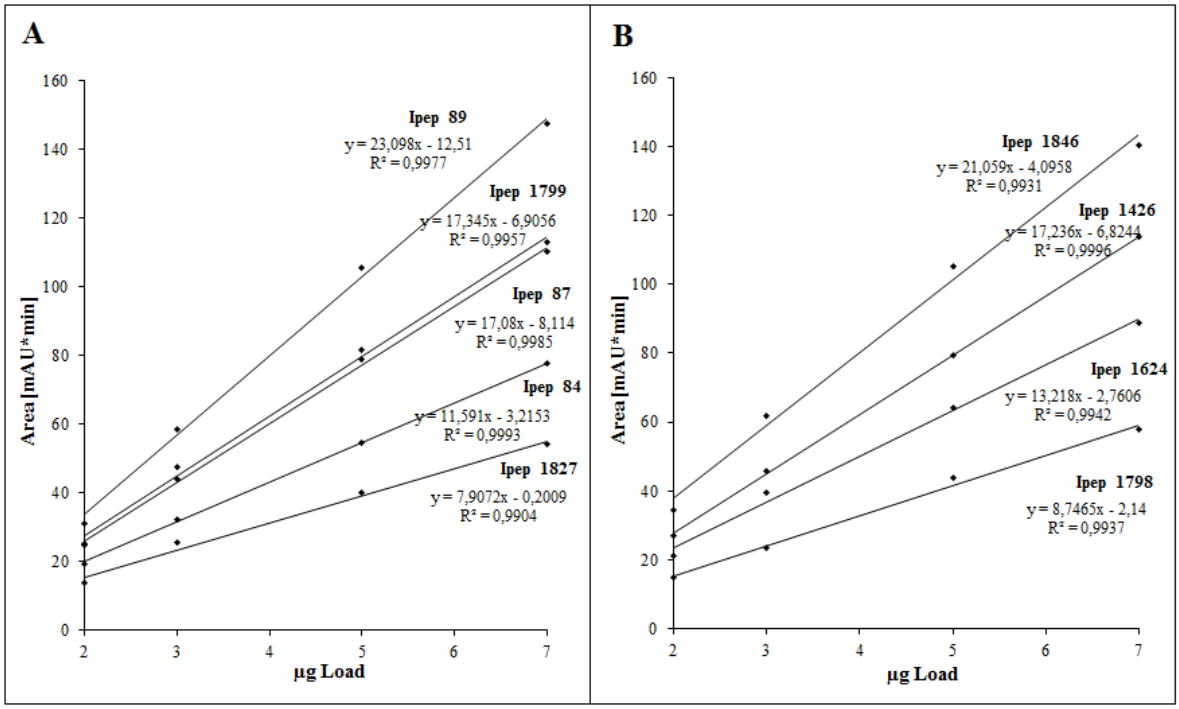


Figure 16: Calibration curves for 0.2 µm filtered peptide solutions. A: linear equations and corresponding correlation coefficients for Ipeps 89, 1799, 87, 84 and 1827. **B:** linear equations and corresponding R^2 for Ipeps 1846, 1426, 1624 and 1798.

Ipep 87				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	10,73	24,744
1.0	30	3	10,72	43,926
1.0	50	5	10,71	78,941
1.0	70	7	10,70	110,293

Ipep 1624				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	11,57	21,206
1.0	30	3	11,56	39,543
1.0	50	5	11,55	64,205
1.0	70	7	11,55	88,711

Ipep 1846				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	12,26	34,339
1.0	30	3	12,25	61,665
1.0	50	5	12,24	105,236
1.0	70	7	12,23	140,376

Ipep 84				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	7,73	19,22
1.0	30	3	7,73	32,46
1.0	50	5	7,73	54,79
1.0	70	7	7,72	77,71

Ipep 89				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	9,48	31,259
1.0	30	3	9,47	58,611
1.0	50	5	9,45	105,401
1.0	70	7	9,44	147,363

Ipep 1799				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	8,79	25,10
1.0	30	3	8,78	47,58
1.0	50	5	8,72	81,62
1.0	70	7	8,71	112,93

Ipep 1827				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	6,58	13,709
1.0	30	3	6,58	25,545
1.0	50	5	6,56	40,048
1.0	70	7	6,58	54,317

Ipep 1426				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	14,00	26,84
1.0	30	3	14,00	45,95
1.0	50	5	13,99	79,24
1.0	70	7	13,98	113,69

Ipep 1798				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	15,60	15,063
1.0	30	3	15,59	23,342
1.0	50	5	15,58	43,833
1.0	70	7	15,58	57,893

Table 2: Retention times and peak areas for calculating linear regression in Figure 16 for all nine peptides.

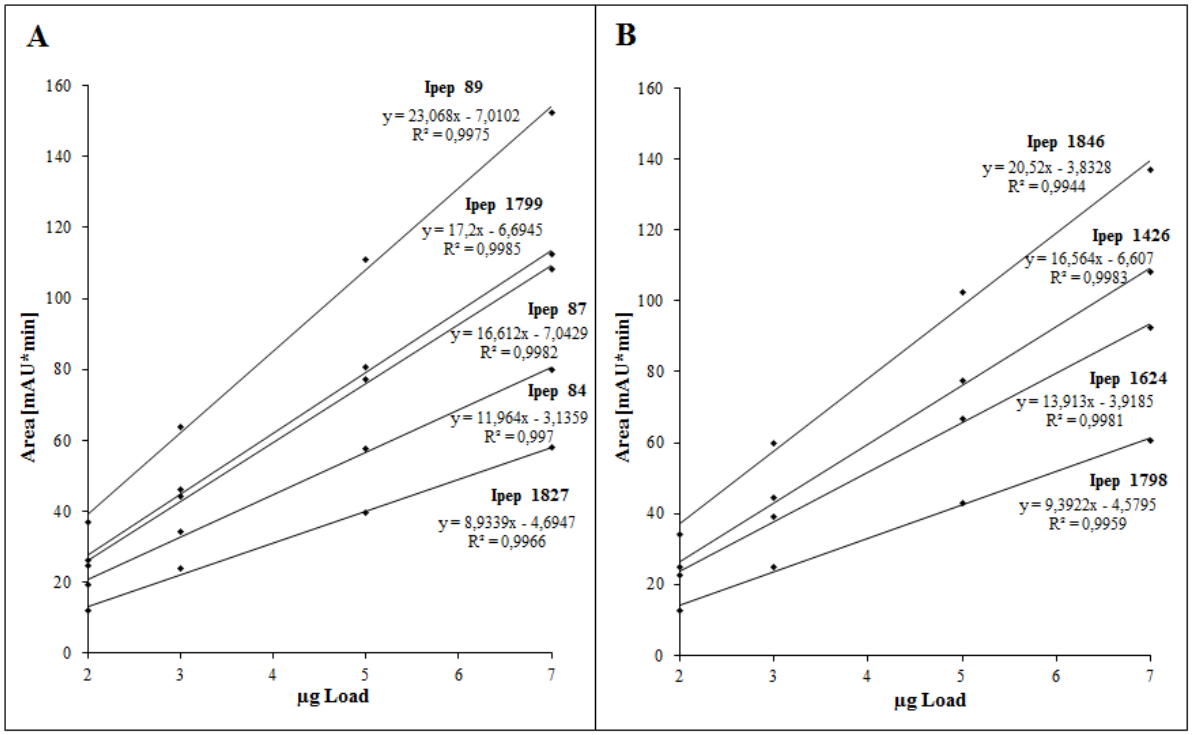


Figure 17: Calibration curves for unfiltered peptide solutions. A: linear Equations and corresponding correlation coefficients for Ipeps 89, 1799, 87, 84 and 1827. **B:** linear equations and corresponding R^2 for Ipeps 1846, 1426, 1624 and 1798.

Ipep 87				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	9,28	24,483
1.0	30	3	9,27	44,383
1.0	50	5	9,25	77,084
1.0	70	7	9,23	108,284

Ipep 1624				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	10,40	22,473
1.0	30	3	10,40	39,083
1.0	50	5	10,39	66,703
1.0	70	7	10,37	92,583

Ipep 1846				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	11,08	34,053
1.0	30	3	11,08	59,819
1.0	50	5	11,06	102,475
1.0	70	7	11,05	137,168

Ipep 84				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	6,53	19,20
1.0	30	3	6,52	34,33
1.0	50	5	6,52	57,52
1.0	70	7	6,52	79,80

Ipep 89				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	8,09	36,735
1.0	30	3	8,08	63,852
1.0	50	5	8,06	110,995
1.0	70	7	8,05	152,538

Ipep 1799				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	7,42	26,20
1.0	30	3	7,41	46,12
1.0	50	5	7,39	80,66
1.0	70	7	7,38	112,65

Ipep 1827				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	5,42	12,106
1.0	30	3	5,41	23,723
1.0	50	5	5,40	39,41
1.0	70	7	5,41	57,858

Ipep 1426				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	12,79	24,96
1.0	30	3	12,78	44,36
1.0	50	5	12,78	77,57
1.0	70	7	12,77	108,27

Ipep 1798				
conc. [mg/ml]	injection vol. [µl]	load [µg]	Ret. Time [min.]	Area [mAU*min]
1.0	20	2	14,54	12,741
1.0	30	3	14,53	25,07
1.0	50	5	14,53	43,095
1.0	70	7	14,53	60,443

Table 3: Retention times and peak areas for calculation linear regression in Figure 17 for all nine peptides.

Results of RP-HPLC analysis show matching peak areas for the same amount load so we conclude that solubilization of 0.1. mg/ml of each peptide in 50 % HAc was complete.

5.3 Solubility studies

5.3.1 Hypothesized solubility characteristics

To define optimal formulation conditions, in particular solvent compositions, solubility characteristics of the HCV-derived peptides had to be evaluated.

As the grand average of hydropathicity index (GRAVY) is commonly used to indicate the solubility characteristics of proteins, we first calculated it for each of the nine peptides as can be seen in Figure 18. Positive GRAVY was thought to tag hydrophobic peptides and negative GRAVY was thought to be related to hydrophilic behavior. In addition, we calculated isoelectric points (pI) for each of the nine peptides (Figure 18). As the solubility of a protein will be minimal near the isoelectric point, the theoretical pI of each peptide could give additional information on their solubility characteristics.

Peptide #	Sequence	order of elution	GRAVY	theoretical pI
Ipep 87	DLMGYIPAV	5	1.044	3.80
Ipep 1624	LEDRDRSELSPLLLSTTEW	6	-0.726	4.18
Ipep 1846	DYPYRLWHYPCTVNFTIFKV	7	-0.160	8.16
Ipep 84	GYKVLVLNPSVAAT	2	0.829	8.59
Ipep 89	CINGVCWTV	4	1.378	5.51
Ipep 1799	AAWYELTPAETTVRLR	3	-0.294	6.19
Ipep 1827	TAYSQQTRGLLG	1	-0.533	8.41
Ipep 1426	HMWNFISGIQYLAGLSTLPGNPA	8	0.265	6.74
Ipep 1798	IGLGKVLVDILAGYGAGVAGALVAFK	9	1.456	8.50

Figure 18: GRAVY and theoretical pI calculated for HCV-derived peptides.

It was apparent from the beginning that the order in which GRAVY would list the peptides regarding their hydrophobicity and the order of chromatographic elution did not match perfectly. In addition we observed no correlation between calculated isoelectric points, GRAVY and order of elution. Despite this initial finding, solubility characteristics for each peptide were experimentally tested.

5.3.2 Solubility screening

Prior to detailed solubility screening, water, 25 % ethanol, 25 % acetic acid and 25 % DMSO were tested as solvents for 1 mg/ml of each peptide. Results of RP-HPLC analysis depicted in Figure 19 and Table 4 showed that ethanol did not bring any advantage compared to water, DMSO or acetic acid. Therefore, we abandoned ethanol as possible solvent used in prototype formulations and excluded it from further detailed solubility studies.

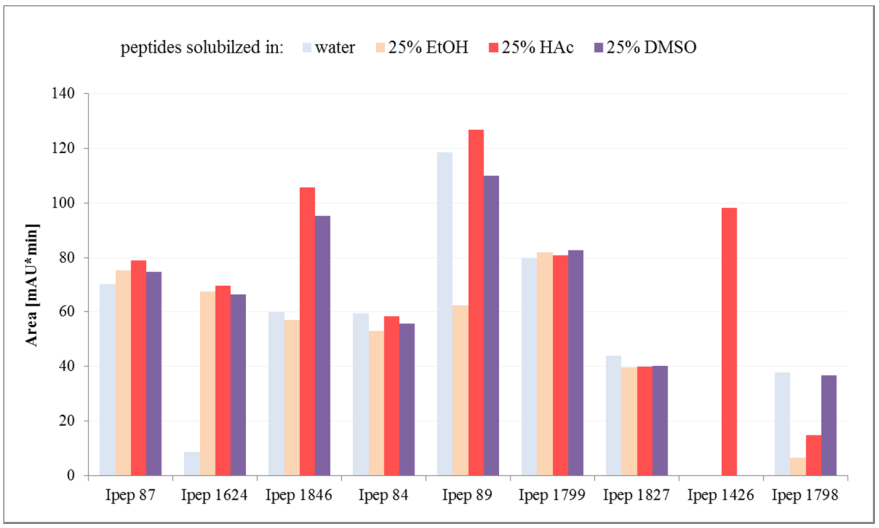


Figure 19: Solubility study testing water, 25 % EtOH, 25 % HAc and 25 % DMSO as solvents for HCV-derived peptides.

		H_2O		25 % EtOH		25 % HAc		25 % DMSO	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
peptide	Ipep 87	10,582	70,152	10,549	75,014	10,544	79,101	10,528	74,646
	Ipep 1624	11,579	8,788	11,563	67,402	11,577	69,470	11,577	66,374
	Ipep 1846	12,569	60,028	12,567	57,086	12,573	105,900	12,713	95,304
	Ipep 84	8,357	59,367	8,356	53,112	8,369	58,438	8,352	55,757
	Ipep 89	9,575	118,559	9,577	62,461	9,589	126,940	9,569	110,052
	Ipep 1799	8,912	79,854	8,932	81,990	8,914	80,964	8,910	82,984
	Ipep 1827	7,205	43,891	7,203	39,639	7,205	39,873	7,216	40,314
	Ipep 1426					13,460	98,380		
	Ipep 1798	14,420	37,812	14,450	6,524	14,420	14,922	14,410	36,771

Table 4: Retention times and peak areas for preliminary solubility studies to Figure 19

Detailed solubility studies therefore were performed with water, 1 %, 3 %, 5 %, 7 %, 10 %, 25 % and 50 % acetic acid and 2.5 %, 5 %, 10 %, 15 %, 25 %, 50 % and 100 % DMSO. Peptide solubility was testes in respective solvents at different temperatures (4 °C, 25 °C and 37 °C) with varying incubation times (0 h, 2 h, 4 h, 8 h and 12 h). Peptides were solubilized at concentrations of 1 mg/ml and put in a sonication bath during their incubation time for a possible further enhancement of solubilization. Afterwards peptide solutions were filtered through 0.22 µm filters and 50 µl analyzed by RP-HPLC.

An overview of the results from RP-HPLC analysis for the solubility screening is shown in Figure 20.

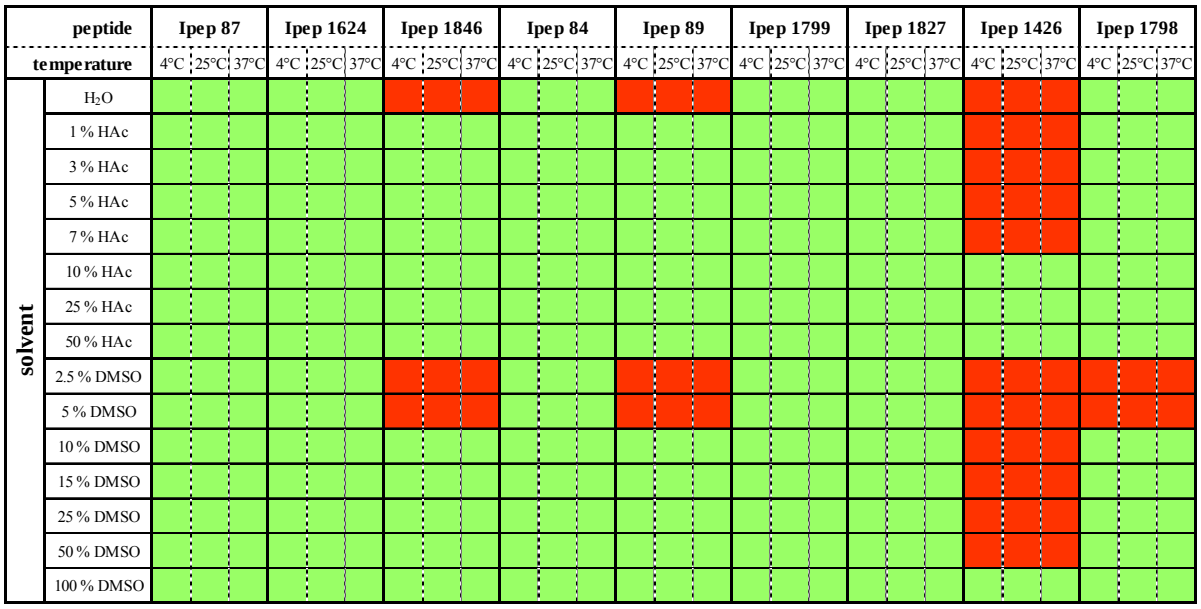


Figure 20: Solubility Screening. Green squares indicate solubility, red squares show that the respective peptide is not soluble in the tested solvent.

As shown in Figure 20, the temperature had no effect on the solubility of the peptides. We therefore conclude that further preformulation studies can be performed at room temperature, since temperature had no influence on solubility results obtained for 4 °C, 25 °C and 37 ° C.

Results of RP-HPLC analysis of the relevant steps to determine requirements of each peptide in order to be completely dissolved are shown below.

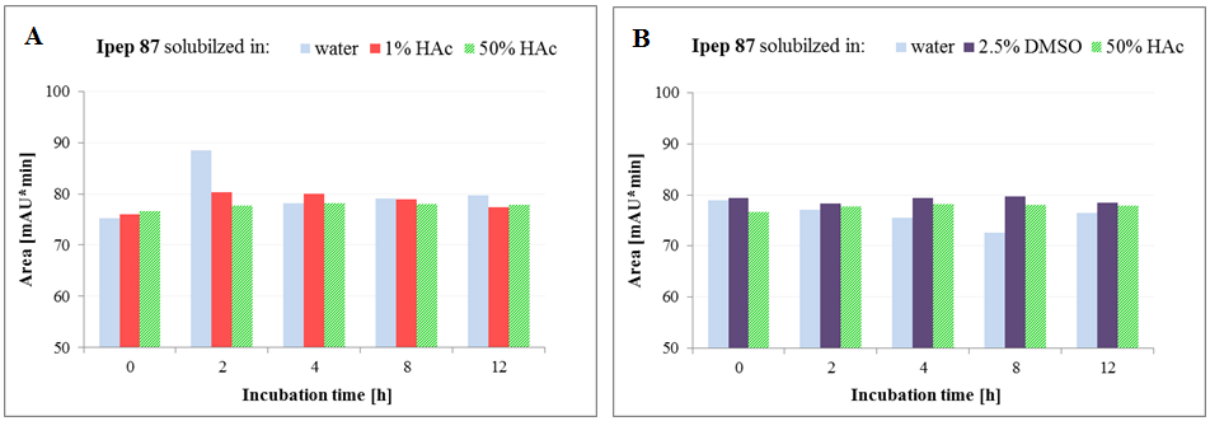


Figure 21: Solubility characteristics of Ipep 87. A: Ipep 87 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 87 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 87		0 h incubation time		2 h incubation time		4 h incubation time		8 h incubation time		12 h incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	10,550	75,217	10,549	88,520	10,560	78,170	10,557	79,089	10,553	79,635
	1 % HAc	10,547	76,045	10,542	80,377	10,535	80,015	10,549	78,867	10,565	77,344
	H_2O	10,391	79,000	10,432	77,052	10,396	75,616	10,462	72,687	10,572	76,432
	2.5 % DMSO	10,566	79,364	10,411	78,373	10,400	79,331	10,556	79,634	10,510	78,431
	50 % HAc	10,548	76,628	10,538	77,652	10,546	78,077	10,554	77,969	10,563	77,865

Table 5: Retention times and peak areas for Ipep 87 to Figure 21.

As depicted in Figure 21 and Table 5, Ipep 87 had been shown to be soluble in water. Addition of neither DMSO nor acetic acid led to enhanced solubilization and thereby greater peak areas. Solubilization was better after a short incubation period but not further enhanced through a prolonged incubation period. Deviations seen are due to variations caused by the method or operator.

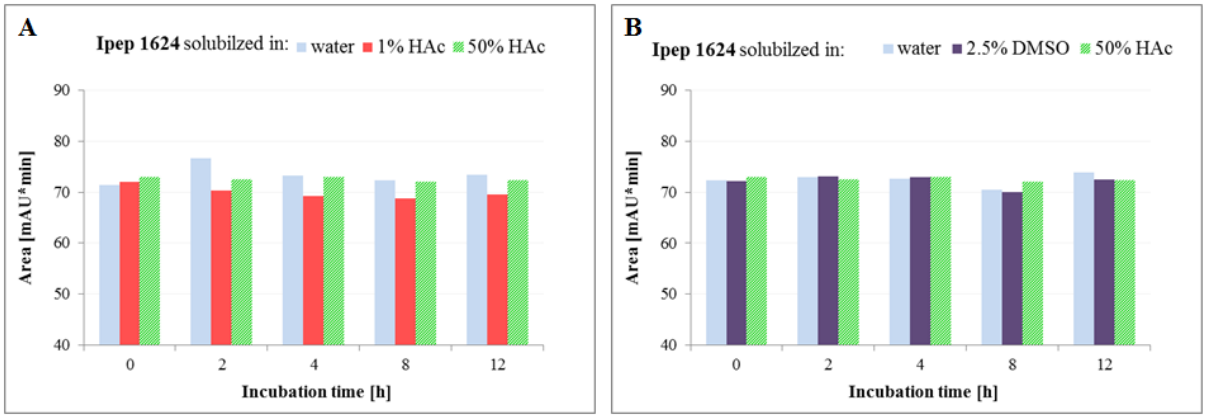


Figure 22: Solubility characteristics of Ipep 1624. A: Ipep 1624 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1624 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1624		0 h incubation time		2 h incubation time		4 h incubation time		8 h incubation time		12 h incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	11,682	71,460	11,665	76,704	11,664	73,242	11,664	72,287	11,660	73,426
	1 % HAc	11,686	71,999	11,680	70,325	11,681	69,193	11,652	68,854	11,658	69,561
	H_2O	11,344	72,292	11,388	72,959	11,305	72,689	11,437	70,426	11,506	73,791
	2.5 % DMSO	11,348	72,104	11,383	73,138	11,278	72,941	11,444	70,075	11,518	72,452
	50 % HAc	11,670	72,963	11,670	72,464	11,670	72,875	11,652	71,955	11,662	72,241

Table 6: Retention times and peak areas for Ipep 1624 to Figure 22.

Ipep 1624 had been shown to be water soluble as depicted in Figure 22 and Table 6. Neither DMSO nor acetic acid used as solvents led to further increase of solubility.

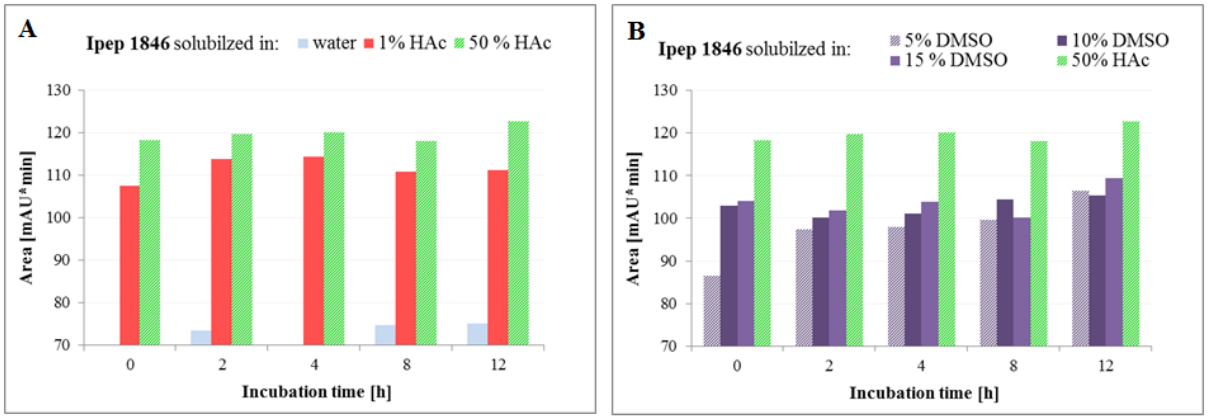


Figure 23: Solubility characteristics of Ipep 1846. A: Ipep 1846 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1846 solubilized in 5 % DMSO, 10 % DMSO and 15 % DSMO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1846		**0 h** incubation time		**2 h** incubation time		**4 h** incubation time		**8 h** incubation time		**12 h** incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	12,616	68,790	12,609	73,448	12,623	69,932	12,627	74,688	12,618	75,050
	1 % HAc	12,626	107,538	12,603	113,691	12,619	114,260	12,618	110,852	12,617	111,164
	5 % DMSO	12,674	86,444	12,700	97,395	12,685	97,947	12,700	99,530	12,733	106,303
	10 % DMSO	12,679	102,898	12,705	100,223	12,689	101,037	12,703	104,380	12,728	105,307
	15 % DMSO	12,674	104,066	12,700	101,806	12,673	103,790	12,718	100,129	12,736	109,491
	50 % HAc	12,626	118,111	12,621	119,603	12,619	120,015	12,633	118,041	12,621	122,530

Table 7: Retention times and peak areas for Ipep 1846 to Figure 23.

As depicted in Figure 23 and Table 7, Ipep 1846 has shown to need an organic modifier or acidic conditions to get in solution. 1 % HAc was sufficient to reproduce peak areas big enough to be valid compared with those seen using 50 % HAc as solvent. 10 % DMSO was not sufficient to completely solubilize Ipep 1846 but a further increase of DMSO concentration did not significantly increase solubility. Therefore 10 % DMSO was confirmed to be a valid solvent for Ipep 1846.

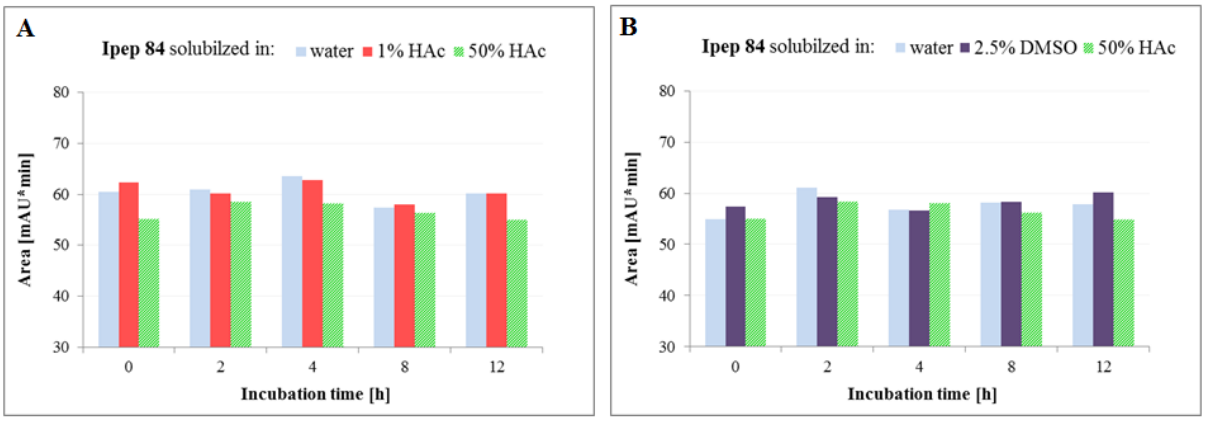


Figure 24: Solubility characteristics of Ipep 84. A: Ipep 84 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 84 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 84		**0 h** incubation time		**2 h** incubation time		**4 h** incubation time		**8 h** incubation time		**12 h** incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	8,417	60,433	8,412	60,876	8,404	63,508	8,421	57,409	8,412	60,114
	1 % HAc	8,400	62,341	8,390	60,107	8,403	62,755	8,417	57,986	8,408	60,186
	H_2O	8,341	54,942	8,347	61,159	8,321	56,787	8,363	58,239	8,363	57,847
	2.5 % DMSO	8,328	57,342	8,334	59,314	8,333	56,567	8,350	58,287	8,378	60,189
	50 % HAc	8,404	55,091	8,414	58,394	8,416	58,101	8,409	56,318	8,399	54,903

Table 8: Retention times and peak areas for Ipep 84 to Figure 24.

As depicted in Figure 24 and Table 8, Ipep 84 had been shown to be soluble in water. Addition of neither DMSO nor acetic acid led to enhanced solubilization and thereby greater peak areas. Duration of incubation seemed to have no influence on the solubility of Ipep 84. Deviations seen are due to variations caused by the method or operator.

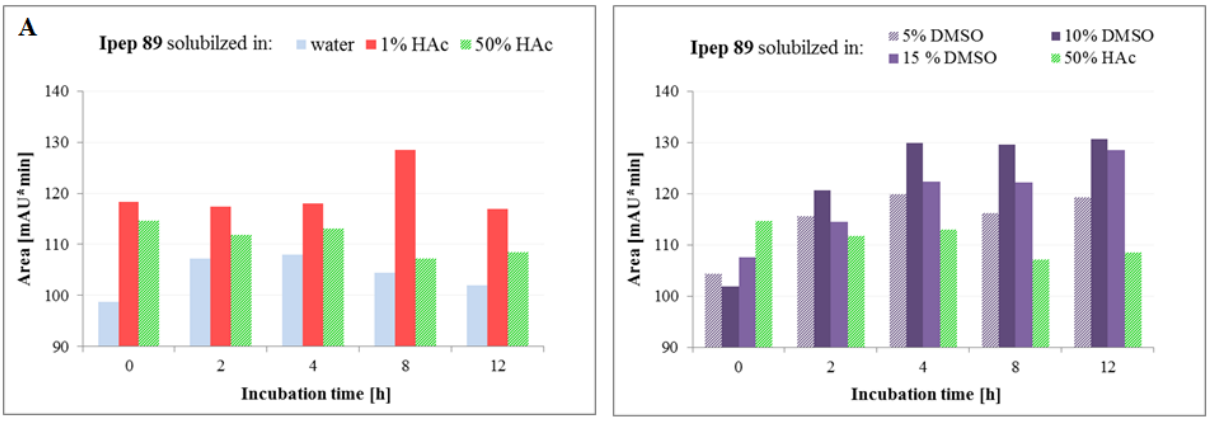


Figure 25: Solubility characteristics of Ipep 89. A: Ipep 89 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B**: Ipep 89 solubilized in 5 % DMSO, 10 % DMSO and 15 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 89		0 h incubation time		2 h incubation time		4 h incubation time		8 h incubation time		12 h incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	9,637	98,826	9,607	107,182	9,602	108,046	9,563	104,441	9,588	101,993
	1 % HAc	9,637	118,382	9,633	117,447	9,606	117,957	9,562	128,424	9,608	116,909
	5 % DMSO	9,512	104,340	9,483	115,583	9,501	119,879	9,531	116,097	9,589	119,231
	10 % DMSO	9,501	101,971	9,489	120,689	9,507	129,949	9,533	129,642	9,586	130,754
	15 % DMSO	9,509	107,630	9,477	114,588	9,513	122,394	9,540	122,271	9,605	128,458
	50 % HAc	9,605	114,617	9,616	111,773	9,612	112,966	9,608	107,148	9,623	108,408

Table 9: Retention times and peak areas for Ipep 89 to Figure 25.

Solubilization of Ipep 89 clearly requires the addition of DMSO, an organic modifier, as can be seen in Figure 25 and Table 9. As shown for Ipep 87 and 1624 before, a short incubation period led to better solubilization. Addition of 1 % acetic acid was sufficient to reproduce peak areas obtained using 50 % HAc as solvent. Indeed using 10 % DMSO led to further enhancement of solubility of Ipep 89 and therefore had been chosen to be a valid solvent. DMSO concentrations over 10 % have been shown to have no effect on solubility. Although not relevant for the previously described set up of the RP-HPLC method, Figure 25 additionally shows that 50 % HAc is not sufficient for complete solubilization of Ipep 89 at concentrations of 1 mg/ml.

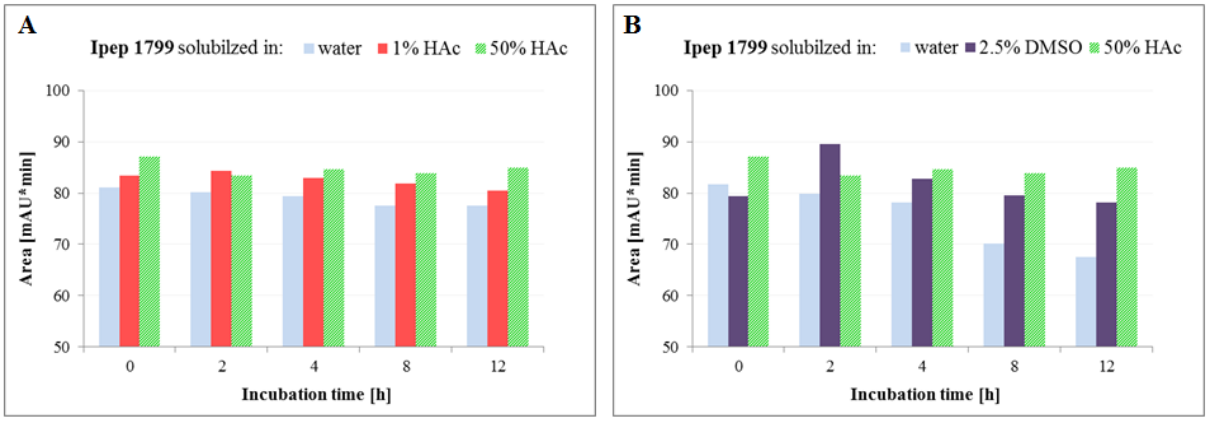


Figure 26: Solubility characteristics of Ipep 1799. A: Ipep 1799 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1799 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1799		0 h incubation time		2 h incubation time		4 h incubation time		8 h incubation time		12 h incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	8,963	81,039	8,951	80,192	8,958	79,422	8,957	77,543	8,970	77,508
	1 % HAc	8,951	83,466	8,960	84,357	8,968	82,865	8,950	81,871	8,963	80,501
	H_2O	8,866	81,660	8,850	79,930	8,839	78,097	8,872	70,087	8,943	67,460
	2.5 % DMSO	8,867	79,324	8,860	89,486	8,846	82,760	8,875	79,518	8,924	78,171
	50 % HAc	8,943	87,091	8,941	83,380	8,960	84,644	8,941	83,862	8,960	84,915

Table 10: Retention times and peak areas for Ipep 1799 to Figure 26.

Ipep 1799 had been shown to be soluble in water (see Figure 26 and Table 10). Addition of neither DMSO nor acetic acid led to enhanced solubilization and thereby greater peak areas. Duration of incubation seemed to have no influence on the solubility of Ipep 1799. Deviations seen for Ipep 1799 solubility in water are probably due to variations caused by the method or operator but have to be further evaluated because also stability problems could cause decreased recovery over time.

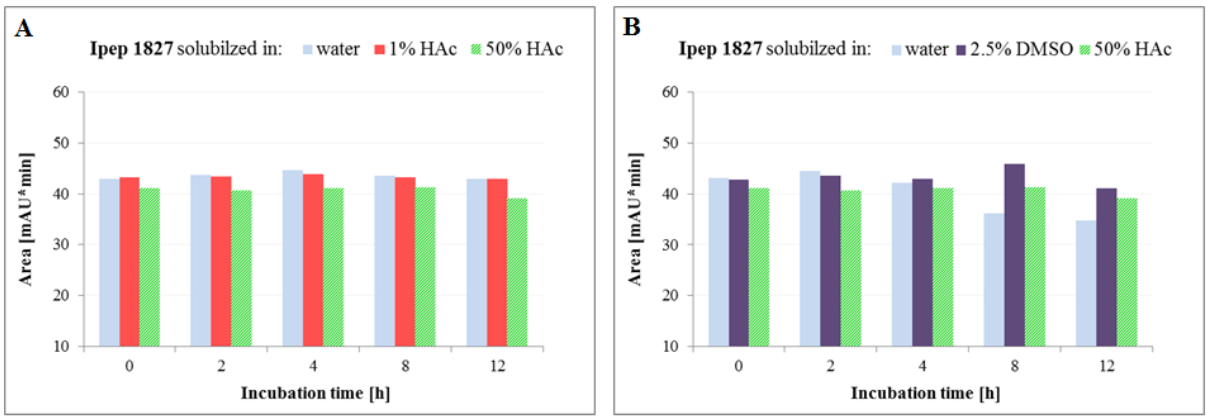


Figure 27: Solubility characteristics of Ipep 1827. A: Ipep 1827 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1827 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1827		0 h incubation time		2 h incubation time		4 h incubation time		8 h incubation time		12 h incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	7,250	42,914	7,250	43,635	7,267	44,583	7,283	43,588	7,253	42,947
	1 % HAc	7,243	43,292	7,259	43,460	7,263	43,881	7,269	43,276	7,265	43,001
	H_2O	7,175	43,135	7,168	44,494	7,192	42,100	7,197	36,222	7,241	34,700
	2.5 % DMSO	7,176	42,735	7,161	43,479	7,185	42,959	7,194	45,834	7,235	41,065
	50 % HAc	7,251	41,096	7,262	40,620	7,250	41,064	7,267	41,181	7,253	39,088

Table 11: Retention times and peak areas for Ipep 1827 to Figure 27.

As depicted in Figure 27 and Table 11 Ipep 1827 had been shown to be water soluble. Addition of acetic acid or DMSO did not increase the solubility. Extension of the incubation period also had no effect. Peak areas obtained using water, 0.1 % HAc or 2.5 % DMSO as solvent were slightly bigger compared to those of the reference obtained using 50 % HAc as solvent.

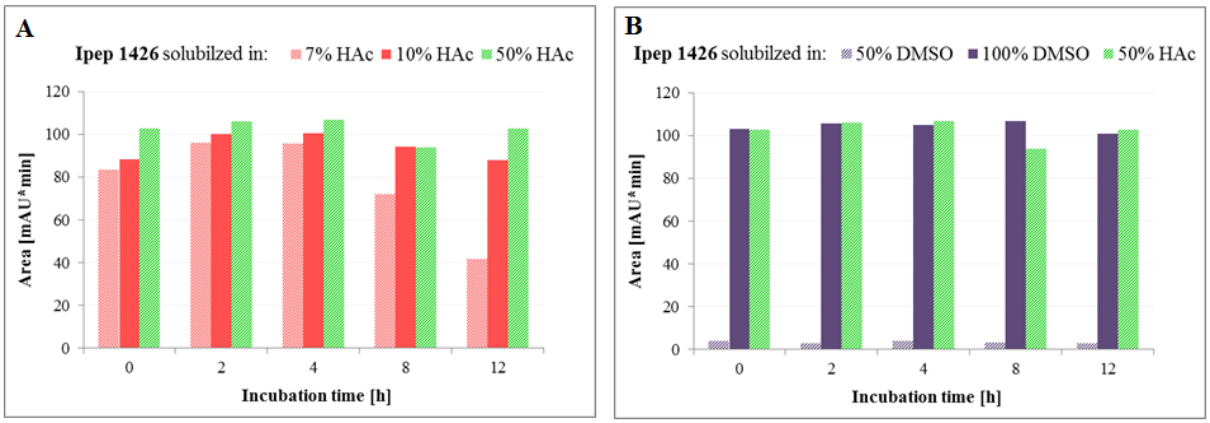


Figure 28: Solubility characteristics of Ipep 1426. A: Ipep 1426 solubilized in 7 % HAc and 10 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1426 solubilized in 50% DMSO and 100 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1426		**0 h** incubation time		**2 h** incubation time		**4 h** incubation time		**8 h** incubation time		**12 h** incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	7 % HAc	13,500	83,378	13,490	95,889	13,489	95,613	13,492	71,804	13,500	41,732
	10 % HAc	13,491	88,370	13,483	100,100	13,486	100,667	13,487	94,145	13,490	87,950
	50 % DMSO	13,450	3,732	13,420	2,928	13,467	3,789	13,442	3,146	13,485	2,908
	100 % DMSO	13,450	102,967	13,401	105,614	13,447	105,048	13,456	106,656	13,473	100,977
	50 % HAc	13,492	102,673	13,491	106,042	13,492	106,733	13,477	93,797	13,500	102,511

Table 12: Retention times and peak areas for Ipep 1426 to Figure 28.

Ipep 1426 was shown to be soluble only using 10 % acetic acid and 100 % DMSO (see Figure 28 and Table 12). Both were sufficient to reproduce peak area of the reference (50 % HAc). Hence acidic conditions or the addition of 100 % DMSO are required to get Ipep 1426 in solution at desired concentrations of 1 mg/ml.

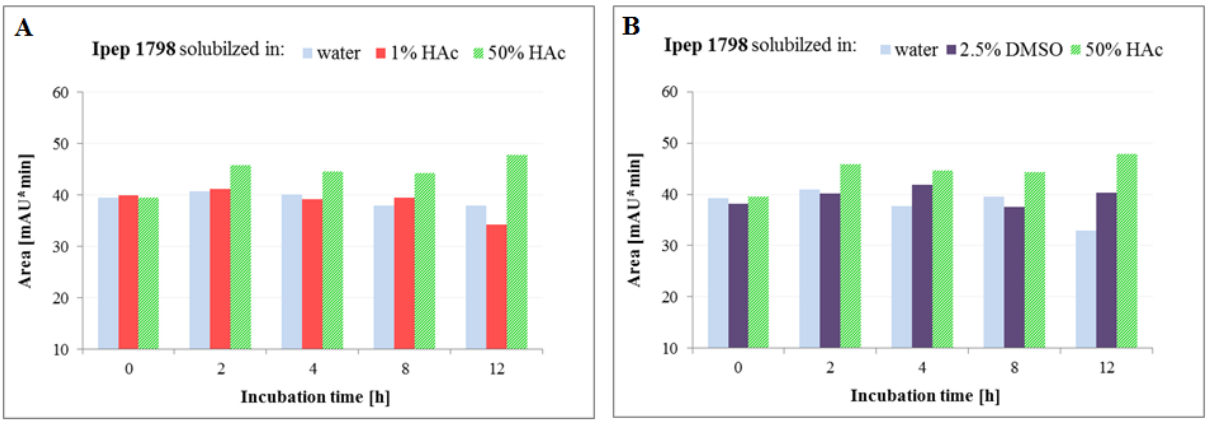


Figure 29: Solubility characteristics of Ipep 1798. A: Ipep 1798 solubilized in water and 1 % acetic acid. 50 % HAc is shown as a reference, since complete solubility was assumed. **B:** Ipep 1798 solubilized in water and 2.5 % DMSO. 50 % HAc is shown as a reference since complete solubility was assumed.

Ipep 1798		**0 h** incubation time		**2 h** incubation time		**4 h** incubation time		**8 h** incubation time		**12 h** incubation time	
		Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]	Ret. Time [min]	Area [mAU*min]
solvent	H_2O	14,499	39,508	14,487	40,607	14,480	40,081	14,494	37,955	14,486	37,962
	1 % HAc	14,489	39,862	14,484	41,068	14,492	39,107	14,508	39,397	14,491	34,229
	H_2O	14,410	39,165	14,400	40,988	14,408	37,730	14,423	39,522	14,432	33,000
	2.5 % DMSO	14,418	38,199	14,387	40,143	14,392	41,856	14,421	37,548	14,446	40,335
	50 % HAc	14,496	39,584	14,475	45,808	14,486	44,601	14,503	44,257	14,486	47,864

Table 13: Retention times and peak areas for Ipep 1798 to Figure 29.

As depicted in Figure 29 and Table 13, Ipep 1798 had been shown to be soluble in water. Addition of neither DMSO nor acetic acid led to enhanced solubilization and thereby greater peak areas. Duration of incubation seemed to have no influence on the solubility of Ipep 1798. Decreasing peak areas after 12 h incubation could indicate low stability of the peptide in aqueous solutions at room temperature.

Regarding the solvents tested, we concluded that acidic conditions, provided by adding acetic acid, were not necessary for formulations containing the nine tested HCV-derived peptides. In contrast, DMSO had been shown to be crucial for solubilization of at least one peptide, Ipep 1426. DMSO was also sufficient for solubilization of the other non-water soluble peptides.

Summarizing the results of the solubility screening (see Figure 20), we concluded that peptides can be divided into 3 groups. The water soluble peptides: Ipep 87, 1624, 84, 1799, 1827 and 98; those soluble in 10 % DMSO: Ipep 1846, 89 and 1798 and Ipep 1426 which was soluble only in 100 % DMSO. For three peptides (Ipep 87, 1624, 89) it was quite

evident that a short incubation time enhances solubility, so we therefore conclude that it is beneficial to allow solubilization for at least 15 minutes in a sonication bath.

Referring to Figure 18, the results of the solubility screening punctuate that there is no obvious correlation between hydrophobicity index, pI and solubility characteristics.
Therefore, we conclude that it is not possible to predict the solubility characteristics of a peptide only by knowing the amino acid sequence but has to be tested experimentally.
Remarkably, Ipep 1798 which eluted as the last one in RP-HPLC analysis and is also the last listed in the calculated hydrophobicity indices had been shown to be water soluble (see Figure 30).

Peptide #	Sequence	order of elution	GRAVY	theoretical	water soluble ?
Ipep 87	DLMGYIPAV	5	1.044	3.80	**YES**
Ipep 1624	LEDRDRSELSPLLLSTTEW	6	-0.726	4.18	**YES**
Ipep 1846	DYPYRLWHYPCTVNFTIFKV	7	-0.160	8.16	
Ipep 84	GYKVLVLNPSVAAT	2	0.829	8.59	**YES**
Ipep 89	CINGVCWTV	4	1.378	5.51	
Ipep 1799	AAWYELTPAETTVRLR	3	-0.294	6.19	**YES**
Ipep 1827	TAYSQQTRGLLG	1	-0.533	8.41	**YES**
Ipep 1426	HMWNFISGIQYLAGLSTLPGNPA	8	0.265	6.74	
Ipep 1798	IGLGKVLVDILAGYGAGVAGALVAFK	9	1.456	8.50	**YES**

Figure 30: Solubility characteristics of HCV-derived peptides. Hydrophobic amino acids are marked in red.

5.4 Binding studies

As IC31® is part of prototype formulations we next addressed the interaction between IC31® and the HCV-derived peptides. In particular, we studied whether the peptides bind to IC31® to draw conclusions on a possible depot-effect when IC31® is co-administered with the active peptides in a therapeutic HCV vaccine.
Phosphate buffered and TRIS buffered IC31® formulations with increasing amounts NaCl (0 mM, 25 mM, 50 mM, 75 mM, 100 mM or 135 mM NaCl) were mixed with equal parts of each peptide solubilized in the solvent chosen to be sufficient for complete solubilization (see section 5.3.2) at a concentration of 2 mg/ml. The resulting formulations containing IC31® and one peptide respectively, were incubated for 120 minutes to allow binding of peptides to IC31® particles. Afterwards, samples were centrifuged to precipitate IC31® and bound peptides and the supernatants analyzed by RP-HPLC for unbound

peptide. Comparing areas of peptides injected without IC31® (reference sample) allowed calculation of percentage peptide remaining in solution, thereby not bound to IC31®.

Results of RP-HPLC analysis of the supernatants (SN) showed that NaCl concentration in IC31® had no influence on the binding capacity for the nine HCV-derived peptides. This was true for all of the nine peptides and is shown exemplarily for two peptides, Ipep 87, solubilized in water and 1:2 diluted in the respective IC31® formulation and Ipep 89, solubilized in 10 % DMSO and mixed to equal parts with the respective IC31® formulation in Figure 31 and Table 14 below.

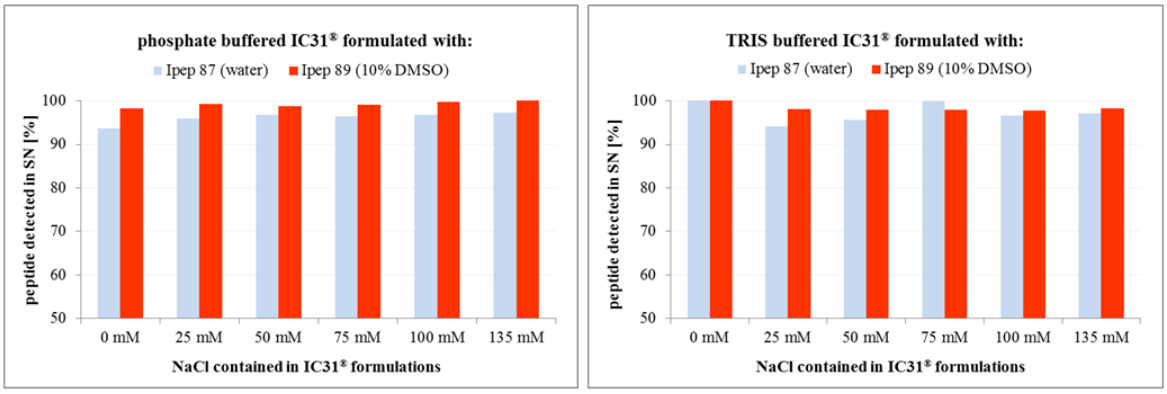


Figure 31: Binding capacity of IC31® with varying NaCl concentrations. A: Phosphate buffered IC31® formulated with Ipep 87 and Ipep 89. **B:** TRIS buffered IC31® formulated with Ipep 87 and Ipep 89.

IC31 formulation®		Ipep 87 in water			Ipep 89 in 10 % DMSO		
		Ret. Time [min.]	Area [mAU*min]	peptide found in supernatant	Ret. Time [min.]	Area [mAU*min]	peptide found in supernatant
	without IC31®	10,546	80,846		9,624	125,506	
5mM PO_4	0 mM NaCl	10,555	75,691	93,62%	9,593	123,298	98,24%
	25 mM NaCl	10,547	77,645	96,04%	9,577	124,570	99,25%
	50 mM NaCl	10,620	78,323	96,88%	9,566	124,040	98,83%
	75 mM NaCl	10,557	77,984	96,46%	9,576	124,449	99,16%
	100 mM NaCl	10,551	78,194	96,72%	9,589	125,277	99,82%
	135 mM NaCl	10,535	78,657	97,29%	9,587	125,580	100,06%
10 mM TRIS	0 mM NaCl	10,578	81,090	100,30%	9,589	126,037	100,42%
	25 mM NaCl	10,531	76,125	94,16%	9,583	123,226	98,18%
	50 mM NaCl	10,573	77,365	95,69%	9,567	122,889	97,91%
	75 mM NaCl	10,556	80,784	99,92%	9,588	122,883	97,91%
	100 mM NaCl	10,533	78,081	96,58%	9,583	122,744	97,80%
	135 mM NaCl	10,522	78,477	97,07%	9,571	123,463	98,37%

Table 14: Retention times, peak areas and calculated percentage peptide detected in supernatants for Ipep 87 and Ipep 89 to Figure 31 after incubation with phosphate and TRIS buffered IC31® formulations containing varying amounts of NaCl.

As decreasing concentrations of NaCl showed no effect on enhancing the binding capacity of IC31®, we decided to use the IC31® formulations containing 2000 nmol/ml KLK, 80 nmol/ml ODN1a and 135 mM NaCl which were available at GMP grade and preferred from the beginning to be used in prototype formulations.

Figure 32 and Table 15 below show that three peptides, Ipep 1846, 1426 and 1798 (with the TRIS buffered IC31® formulation) were not detected in the supernatants after incubation with IC31®. Ipep 1624 and Ipep 1798 (with the phosphate buffered IC31® formulation) could be detected in the supernatants to a significantly reduced amount.

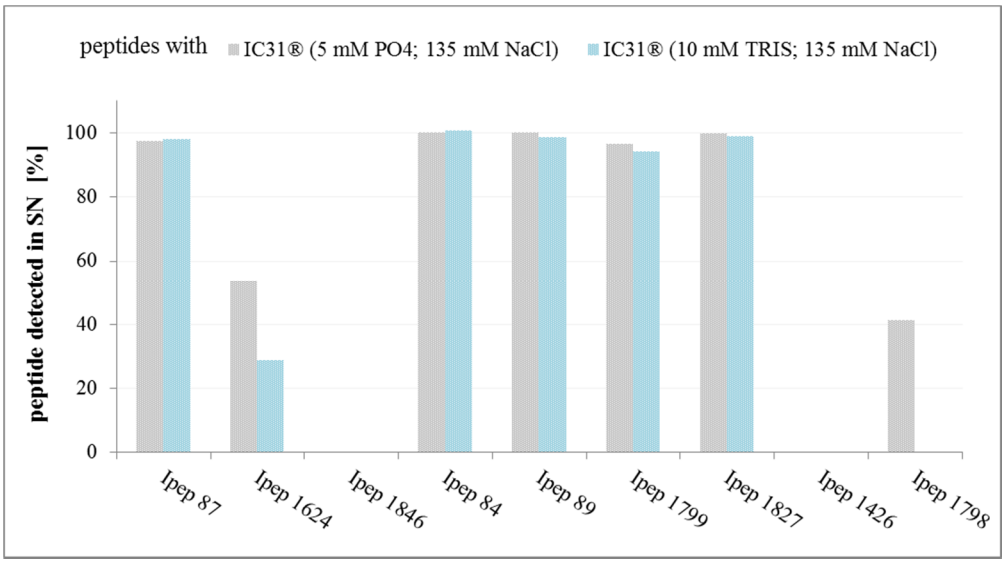


Figure 32: Binding capacity of phosphate and TRIS buffered IC31®. Recovery of all nine HCV-derived peptides in the supernatant (SN) is shown.

		IC31® (5 mM PO_4; 135 mM NaCl)			IC31® (10 mM TRIS; 135 mM NaCl)		
		Ret. Time [min.]	Area [mAU*min]	peptide found in supernatant	Ret. Time [min.]	Area [mAU*min]	peptide found in supernatant
Ipep 87	*reference*	10,546	80,846	97,29%	10,546	80,846	97,07%
	formulation	10,535	78,657		10,522	78,477	
Ipep 1624	*reference*	11,491	73,244	53,36%	11,491	73,244	28,46%
	formulation	11,558	39,083		11,487	20,848	
Ipep 1846	*reference*	12,719	119,418	0,00%	12,719	119,418	0,00%
	formulation						
Ipep 84	*reference*	8,393	59,571	100,47%	8,393	59,571	100,54%
	formulation	8,383	59,850		8,393	59,894	
Ipep 89	*reference*	9,624	125,506	100,06%	9,624	125,506	98,37%
	formulation	9,587	125,580		9,571	123,463	
Ipep 1799	*reference*	8,922	82,026	96,35%	8,922	82,026	94,05%
	formulation	8,939	79,035		8,946	77,145	
Ipep 1827	*reference*	7,233	43,442	99,61%	7,233	43,442	98,65%
	formulation	7,248	43,271		7,267	42,856	
Ipep 1426	*reference*	13,456	106,656	0,00%	13,456	106,656	0,00%
	formulation						
Ipep 1798	*reference*	14,441	43,649	40,86%	14,441	43,649	0,00%
	formulation	14,450	17,836				

Table 15: Retention times, peak areas and calculated percentage peptide detected in supernatants for IC45® peptides to Figure 32 after incubation with phosphate and TRIS buffered IC31® formulations.

Ipep 1846 and 1798 were shown to require 10 % DMSO for solubilization. By dilution with IC31® the solvent concentration is reduced to half this concentration, therefore

peptides precipitated and could not be found analyzing the supernatant. This is also true for Ipep 1426 which is only soluble in 100 % DMSO and precipitates when reducing DMSO concentration to 50 %. Ipep 1798 had been shown to be water soluble, however also this peptide just stimulated binding to IC31® in this assay and also precipitated after mixing with IC31®. We assumed that none of the HCV-derived peptides efficiently binds to IC31®.

As there could be seen no significant differences between phosphate buffered IC31® formulations and TRIS buffered IC31® formulations regarding their binding capacity, both were used in prototype formulations.

5.5 Preformulation studies

Considering our results from previous performed solubility studies (see Figure 20) we decided to divide the HCV-derived peptides into 2 groups for exploratory vaccine formulations containing IC31® as immunizer and designed a procedure for prototype formulation (see Figure 33).

We proposed that peptides which had shown to be water soluble should be combined to a stock containing Ipep 87, 1624, 84, 1799, 1827 and 1798. Water for Injection (WFI) will be used as solvent for these peptides at a concentration of 2.4 mg/ml each. The total amount of peptide in the water-stock will be 14.4 mg/ml to obtain a concentration of 1 mg/ml for each peptide in the final prototype formulation. As the addition of DMSO in prototype formulations is required for dissolution of Ipep 1846, 89 and 1426, we suggest that these three peptides will be combined in a stock using 100 % DMSO as solvent. This stock then contains 12 mg/ml of each peptide leading to a total amount of 36 mg/ml in the DMSO-stock and a final concentration of 1 mg/ml of each peptide in the prototype formulation. Peptides in respective stocks have to be completely dissolved by sonication for sterile filtration (0.22 µm) and will be formulated with both phosphate and TRIS buffered IC31®. Additionally, a non-adjuvanted control formulation containing 1 x TRIS or phosphate buffer instead of IC31® will have to be prepared. IC31® (5 mM PO_4, 135 mM NaCl or 10 mM TRIS, 135 mM NaCl) will be used at 2000 nmol/ml KLK and 80 nmol/ml ODN1a leading to contents of 500 nmol/ml KLK and 20 nmol/ml ODN1a in the final exploratory adjuvanted vaccine formulation.

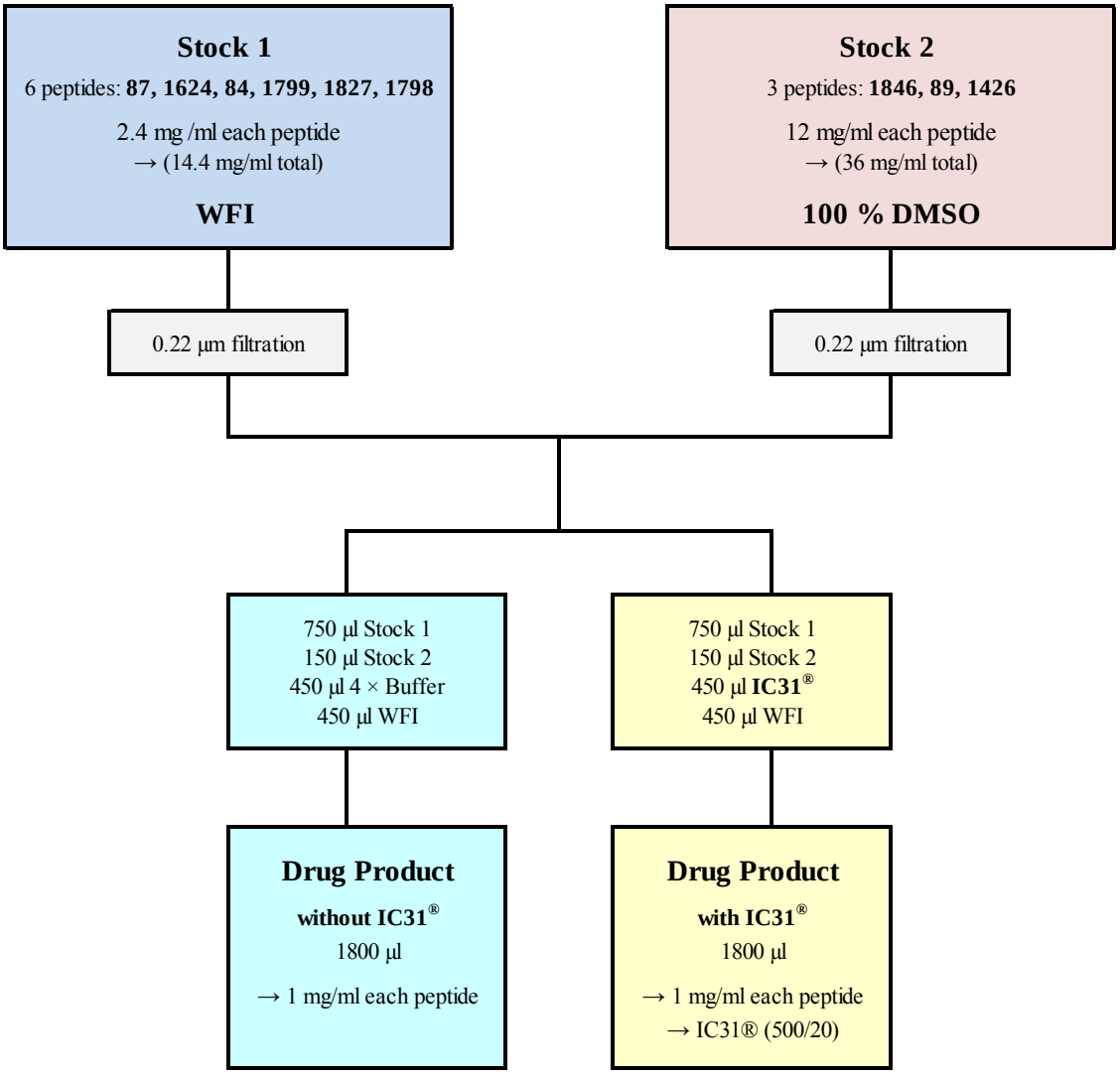


Figure 33: Designed procedure for IC45® prototype formulations. Ipep 87, 1624, 84, 1799, 1827 and 1798 are combined in Stock 1 and Water for Injection is used as solvent. Ipep 1846, 89 and 1426 are combined in Stock 2 and 100 % DMSO is used as solvent. Both the adjuvanted Drug Product and the Drug Product without IC31® contain 1 mg/ml of each peptide in the total volume of 1800 µl.

Prototype formulations for the therapeutic HCV vaccine IC45® consist of nine synthetic HCV-derived peptides (9-26 amino acids) being the active substance and Valneva's licensed adjuvant IC31®. The final formulation will be a suspension containing 1 mg/ml of each peptide, phosphate or TRIS buffered IC31® and approximately 8 % DMSO.

6 Discussion

With an estimated 170 million individuals chronically infected with Hepatitis C Virus and a current standard treatment showing severe side effects and relatively low response rates there is no doubt about the tremendous need for novel therapy approaches fighting Hepatitis C.

The exploratory therapeutic HCV vaccine (project name IC45®) from Valneva GmbH has the potential to induce a cellular immune response clearing HCV infection. IC45® is being developed containing 9 synthetic HCV-derived peptides as active component and Valneva's licensed adjuvant IC31® as a potent immunizer.

For IC45® vaccine, as for any other vaccine, a major challenge is to develop a stable formulation and to consider the simple fact that the formulation composition (solvent, buffer, pH, etc.) has to be applicable for human use. To enable appropriate conditions, activities referred here as preformulation studies, are necessary as a start of the process of formulation development. Preformulation studies for IC45® performed during this thesis focused on analytical method development, physicochemical characteristics and solubility screening of HCV-derived peptides. In addition, the interaction of peptides with the adjuvant IC31® was investigated.

The availability of an analytical method used in formulation work is essential as peptide recovery has to be monitored in each step and any degradation reactions should be monitored during exploratory stability testing. To meet this demand, the set-up and optimization of an analytical reversed-phase HPLC method was a major goal for subsequent preformulation studies. The analytical RP-HPLC developed method allowed efficient separation and identification of the nine HCV-derived peptides in a single run applying combination of linear gradient elution conditions. Peptides were separated on a C18 column primarily on the basis of hydrophobicity in the presence of an organic modifier (acetonitrile) and TFA (ion-pairing reagent). Retention times of eluted peaks allowed for peptide identification in total run time of only 25 minutes and allowed high-throughput analysis of samples.

As in RP-HPLC peptides are separated on the basis of hydrophobicity the most polar peptide elutes first while the most hydrophobic peptide elutes last. The resulting order of elution allows a grading of peptides respective their hydrophobicity and thereby was thought to also allow a prediction of their solubility characteristics.

Two more parameters, the grand average of hydropathicity index (GRAVY) and the isoelectric point (pI), were calculated for each peptide since both are commonly used to indicate the solubility characteristics of proteins and therefore could be useful also to define peptide properties. The GRAVY value is obtained by summing up hydropathy values for each amino acid residue and dividing by the length of the sequence. A positive GRAVY tags peptides as hydrophobic and a negative GRAVY as hydrophilic, thereby indicating solubility in aqueous solution. Another indicator for the solubility characteristics of proteins is the isoelectric point (pI). Primarily dependent on the charged amino acids the pI is the pH at which the net charge of a protein is zero. Therefore, solubility of proteins will be minimal in solvents at the pH that correspond to their pI.

The order in which GRAVY would list the peptides regarding their hydrophobicity and the order of chromatographic elution did not match. Additionally there was no obvious correlation between calculated pIs and the tendency towards solubilization in water. Most important, the sequence-based prediction and experimental solubility data did not match. Although a sequence-based prediction of protein solubility is possible [53], we assert that this is not true for the classification of peptides into 'soluble' and 'insoluble' in aqueous solution. Respective calculated isoelectric points, factors unrelated to sequence, such as buffer composition in later generated formulations, most probably also will have an influence on peptide solubility. Phosphate and TRIS buffered IC31®, both investigated in prototype formulations, slightly differ in their pH which could lead to marginal increase or decrease of peptide solubility.

However, characterization of solubility characteristics of each peptide was a central goal of preformulation work. Complete solubilization of the peptides is essential to ensure dosing accuracy and a final 0.22 µm sterile filtration step of peptide stocks. An extensive solubility screening was performed testing each peptide for its solubility in different solvent concenration, at different temperatures at different time points. We found that higher temperature up to 37°C had no influence on the solubility. In general, it seemed that a short incubation time of at least 15 minutes has a positive influence on the solubility. Finally the peptides were divided into three groups corresponding to their solubility properties. The six water soluble peptides (Ipep 87, 1624, 84, 1799, 1827 and 1798), two peptides had shown to be poorly water soluble and required at least 10 % DMSO for solubilization (Ipep 1846 and 89), and one which was only soluble in 100 % DMSO (Ipep 1426).

In conclusion we found that DMSO was required as excipient (inactive ingredient) used in the IC45® vaccine. DMSO is listed as a Class III solvent with low toxic potential to human. It has been approved by the FDA (Food and Drug Administration) for use in pharmaceutical formulations with a permitted daily exposure (PDE) of 50 mg per day. [54]

Referring to the interaction of the HCV-derived peptides with the adjuvant IC31®, Valneva's licensed adjuvant IC31® has been shown to form a depot at the site of injection. This is due to the properties of KLK that forms a stable complex with ODN1a via ionic and hydrophobic interactions in which not only the nuclease-sensitive ODN1a but also the antigens are thought to be protected against degradation. [47] However, the mechanism of adsorption of antigen molecules is not fully characterized and irrespective of a proposed mechanism has yet to be tested for individual antigens.

We investigated both phosphate and TRIS buffered IC31® formulations with varying NaCl concentrations. Increasing salt content and thereby increasing the ionic strength had no influence on the binding capacity of IC31®. As higher ionic strengths would diminish electrostatic interactions, we suggest that electrostatic adsorption is not responsible for a possible binding of peptide antigens to IC31®. At first glance, three peptides seemed to bind to IC31® because we could not detect them in the supernatants after centrifugation of IC31®. However, it was shown that absence of peptides in the supernatant was simply caused by precipitation of the respective peptides after mixing with IC31®. Summing up our results from the binding studies we virtually found none of the peptides stably binding to IC31®.

Although one goal of formulating a vaccine might be to form a stable and reproducible complex of the antigen to the adjuvant for eliciting an enhanced immune response [55], there is evidence that maximizing antigen binding is not always the best strategy for optimizing vaccine efficacy [56].

Considering our results we designed a procedure for a prototype formulation, consisting of the nine HCV-derived peptides and IC31® as potent immunizer, which provides a good basis for real formulation work to start with.

We assumed that high total amounts of peptides regarding solubility are not limiting in an appropriate solvent. Therefore we suggest combining the water soluble peptides and those requiring DMSO as solvent to stocks, respectively. Both stocks, as the active substance of the future vaccine formulation, then should be separately sterile filtrated. Finally both stocks will be mixed with IC31® under sterile conditions to give the final prototype formulation. The final prototype formulation will contain 1 mg/ml of each of the antigenic

peptides, IC31® (500 nmol/ml KLK and 20 nmol/ml ODN1a), water and DMSO as excipients.

We suspect that some peptides will not remain solubilized in this prototype formulation, primarily due to the reduction of DMSO concentration. Complete solubilization of the respective peptide stocks before adjuvantation is necessary for sterile filtration. Based on the properties of IC31®, the final vaccine will be a suspension; whether or not complete solubilization of peptides is required for the final formulation has to be further investigated.

The DMSO content in this prototype formulation will exceed the amount accepted by the FDA without justification. According to the dosing schedule of IC45® which will be weekly or monthly rather than daily however the approximate amount of 165 mg per vaccine dose (1800 µl) should also be acceptable.

One of the basic concerns of formulation development is the stability of the final product. However, during the course of this thesis stability was not investigated in detail. IC45® stability studies (Reinisch 2008, internal data) had identified the cysteine-containing peptides, Ipep 1846 and 89, not to be stable over time. This intra- or intermolecular modifications, possibly caused by the oxidation of free cysteins to disulphides, were not observed in short time studies. Ipep 1846 and 89 therefore were replaced by stable variants, Ipep 1846C and Ipep 89D, respectively. Additionally, Ipep 1624 and 1827 were shown to precipitate in the ‘water stock’. Therefore an alternative manufacturing procedure was tested. Ipep 1827 was included in the DMSO stock Ipep 1624 solubilized separately in PBS (Phosphate Buffered Saline) as indicated in Figure 34 below.

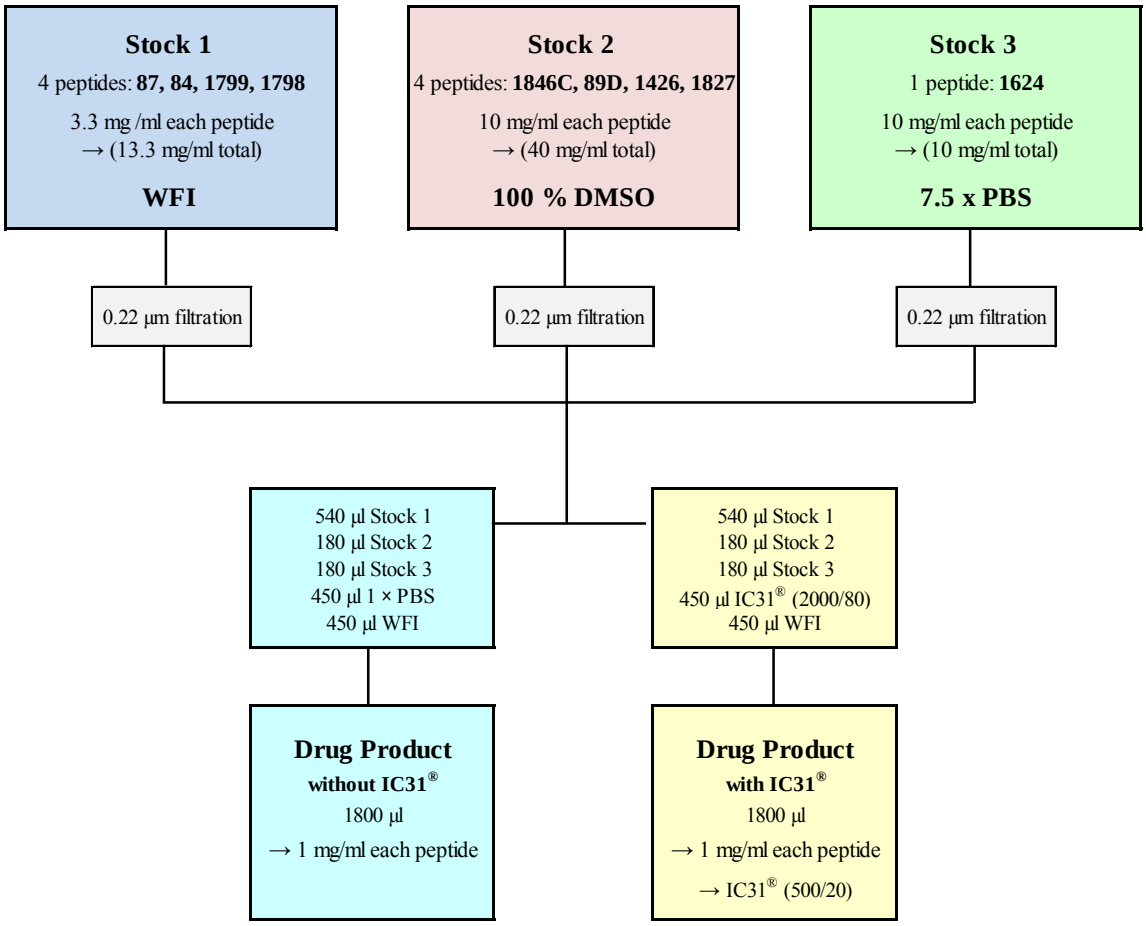


Figure 34: Alternative procedure for IC45® prototype formulations. Ipep 87, 84, 1799 and 1798 are combined in Stock 1 and Water for Injection is used as solvent. Ipep 1846, 89, 1426 and 1827 are combined in Stock 2 and 100 % DMSO is used as solvent. Ipep 1624 is separately solubilized in PBS in Stock 3.

It was proven that no unexpected losses occurred with this alternative formulation procedure compared to the previous one (Reinisch 2008, internal data). Solubilization of Ipep 1624 in PBS led to the introduction of an additional buffer system to provide physiological conditions. The final prototype formulation is a suspension containing 1 mg/ml of each HCV-derived peptide, Valneva's licensed adjuvant IC31® and approximately 10 % DMSO (v/v), ~ 200 mg per vaccine dose (1800 µl), respectively.

This final prototype formulation is a promising HCV vaccine candidate and as a next step needs to be further investigated for its immunogenicity. Due to the lack of suitable animal models for Hepatitis C this will be studied to a certain extent in mice expressing human HLA-molecules [57]. IC45® is based on synthetic materials and therefore minimal side effects are expected. Nevertheless, this also has to be investigated in a preclinical animal test.

7 References

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1994 – 1998	Secondary School, Gymnasium der Dominikanerinnen
1990 – 1994	Primary School Notre Dame de Sion

Professional Formation

02/2012 – present	Research Assistant at Medical University of Vienna; Clinical Institute of Pathology
03/2007 – 12/2008	Assistant at Intercell AG, Department of Analytical & Formulation Development Diploma Thesis: »Development of exploratory therapeutic Hepatitis C peptide vaccine formulations«
11/2006 – 02/2007	Support Staff at Boehringer Ingelheim Austria GmbH, Business Center Biopharmaceutical Manufacturing; Division Biopharmacy/Operations
03/2006 – 07/2006	Minor Employment at Histology, Cytology, Bacteriology and Serology Laboratory Dr. Kosak; Division Microbiology
07/2006 – 09/2006 and 08/2005 – 09/2005	Internship at Boehringer Ingelheim Austria GmbH, Business Center Biopharmaceutical Manufacturing; Division Biopharmacy/Operations

08/2001	Internship at Boehringer Ingelheim Austria GmbH, Quality Assurance, Division Manufacturing/Technology
08/2003 – 09/2003 and 06/2001 – 07/2001	Internship at NÖ Umweltschutzanstalt GmbH, Division Waste and Sewage Analysis