

Stability of Multi-Peptide Vaccines in Conditions Enabling Accessibility in Limited Resource Settings

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Abstract

We have previously shown that lyophilized mixtures of six- and twelve-melanoma peptide vaccines retain stability, purity, and amino acid sequence identity for up to five years when stored at -80°C. However, this temperature requirement presents a significant obstacle to storage and distribution of vaccines for low-resource environments. Thus, we examined the stability of these peptides over a range of temperatures for varying durations of time. When stored at + 4°C or at room temperature for up to one month, 17 of the 18 peptides remained stable. The only change for the remaining peptide was an increase in an oxidized methionine residue. The results presented in this report support broadening the accessibility of these and other peptide-based vaccines to resources-limited communities.

1. Introduction

Many vaccines have a limited range of thermostability, requiring temperature regulation during transport and storage (Sun et al. 2022), (Dadari et al. 2021). Peptide-based vaccines, however, have established themselves as promising candidates in improving vaccine shelf-life and accessibility: manufacturers report long-term stability of lyophilized peptides when stored at -80°C (Sigma-Aldrich, 2024). Peptide vaccines may consist of one or multiple amino-acid sequences derived from proteins specific to the vaccine's intended target. The integrity of each peptide is essential for vaccine safety and potency. Therefore, factors influencing the chemical stability of these peptides are a concern regarding accessibility. The limited thermal tolerance of vaccines represents a major obstacle for their design, production, distribution, and storage. Cold chain systems made up of interconnected networks of refrigeration equipment alleviate this problem and broaden distribution. Still, storage burdens rural or impoverished areas lacking the infrastructure required to store temperature-labile vaccines.

Peptide molecules are inherently sensitive to degradation in solution. Lyophilization of peptide solutions can significantly improve their stability and shelf-life (Dalvi et al. 2021). This supports peptide-based vaccines as advantageous over other vaccine types, as mRNA vaccines are less easily lyophilized under standard practices (Zeng et al. 2005), (Purcell et al. 2007). Still, many lyophilized peptide-based vaccines require sub-ambient temperatures for long-term storage (Rexroad et al. 2002). Though lyophilization enhances stability, the potential for degradation remains, especially for the final peptide mixtures. Stability at room temperature, or even at 4°C, would be pivotal for global vaccination efforts, allowing vaccines to be handled without ultra-cold conditions.

For this study, we evaluated the stability of two lyophilized, clinical-grade multi-peptide vaccines: six-melanoma helper peptide (6MHP) and twelve-melanoma peptide (12MP). These 18 peptides vary in length and contain susceptible residues: methionine and cysteine, and aspartic acid-proline bonds. We have previously shown that both vaccines retain their integrity for up to 5 years when stored at -80°C (Chianese-Bullock et al. 2009). It remains to be tested whether these peptides remain stable at temperatures amenable to underserved communities over time intervals meaningful for clinical use. We

hypothesized that peptides in the 6MHP and 12MP preparations would retain stability, identity, and purity at 4°C and room temperature for up to one month.

2. Material and Methods

The 6MHP (Lot AC0626) (Table 1), and 12MP (Lot AC0558) (Table 1) vaccines were synthesized, purified, and vialled under GMP conditions by Clinalfa-Merck as previously described (Chianese-Bullock et al. 2009). These lyophilized samples were stored in a light-protected, -80°C environment until moved to their assigned testing condition. Conditions included storage at -80°C, + 4°C, and + 20°C, light-protected, for one day, one week, or one month. Samples were then assessed using high-performance liquid chromatography (HPLC) and mass spectrometry by the University of Virginia Biomolecular Facility Core Laboratory.

Table 1
Twelve melanoma peptide (12MP) and Six melanoma helper peptide (6MHP) vaccines

Vaccine	Allele	Sequence	Abbreviation	Epitope
12MP	HLA-A1	DAEKSDICTDEY	DAE	Tyrosinase ₂₄₀₋₂₅₁ ^a
		SSDYVIPIGTY	SSD	Tyrosinase ₁₄₆₋₁₅₆
		EADPTGHSY	EAD	MAGE-A1 ₁₆₁₋₁₆₉
		EVDPIGHLY	EVD	MAGE-A3 ₁₆₈₋₁₇₆
	HLA-A2	YMDGTMSQV	YMD	Tyrosinase ₃₆₉₋₃₇₇ ^b
		IMDQVPFSV	IMD	gp100 ₂₀₉₋₂₁₇ ^c
		YLEPGPVTA	YLE	gp100 ₂₈₀₋₂₈₈
		GLYDGMEHL	GLY	MAGE-A10 ₂₅₄₋₂₆₂
	HLA-A3	ALLAVGATK	ALLA	gp100 ₁₇₋₂₅
		LIYRRRLMK	LIY	gp100 ₆₁₄₋₆₂₂
		SLFRAVITK	SLF	MAGE-A1 ₉₆₋₁₀₄
		ASGPGGGAPR	ASG	NY-ESO-1 ₅₃₋₆₂
6MHP	HLA-DR4	AQNILLSNAPLGPQFP	AQN	Tyrosinase ₅₆₋₇₀ ^d
	HLA-DR15	FLLHHAFVDSIFEQWLQRHRP	FLL	Tyrosinase ₃₈₆₋₄₀₆
	HLA-DR4	RNGYRALMDKSLHVGTCALTRR	RNG	Melan-A/MART-1 ₅₁₋₇₃
	HLA-DR11	TSYVKVLHHMVKISG	TSY	MAGE-3 ₂₈₁₋₂₉₅
	HLA-DR13	LLKYRAREPVTKAE	LLK	MAGE-1,2,3,6 ₁₂₁₋₁₃₄
	HLA-DR1 & HLA-DR4	WNRQLYPEWTEAQRLD	WNR	gp100 ₄₄₋₅₉

^a Substitution of S for C at residue 244.

^b Post-translational change of N to D at residue 371.

^c 209-2M, substitution of M for T at residue 210.

^d An alanine residue was added to the N-terminus to prevent cyclization.

2.1. High-Performance Liquid Chromatography

Each vial of lyophilized peptides was reconstituted with sterile water. A 3-mcg sample of each peptide was diluted to 200 µL with 0.1% trifluoroacetic acid (TFA) before injection. Reverse phase chromatography was performed in University of Virginia's Biomolecular Analysis Facility Core on a Phenomenex Jupiter C18 (catalog 00F-4053-B0), 2 mm × 150 mm. Solvent A was 0.1% TFA, solvent B was 0.09% TFA in acetonitrile. The column equilibrated in 5% B (95% A). The elution gradient was 5% B for 5 min, 5–29% B in 36 min, and 29–70% B in 9 min. The Agilent 1100 pump (Agilent Technologies, Inc.; Santa Clara, CA) was used. Flow rate was 200 µL/min, column temperature was 40°C, and the effluent was measured by absorbance at 215 nm. Fractions were collected manually.

2.2 Mass Spectrometry

The HPLC fractions were immediately vacuum-dried and stored at -80°C. These samples were reconstituted with 1 mL 0.1% formic acid (FA), and 100 µL of a 1:10 sample dilution in 0.1% FA was added to autosampler vials. The LC-MS system had a Thermo nanoEASY-LC 1200 coupled to a Thermo Orbitrap Exploris 480 mass spectrometer with an Easy Spray ion source. Samples were injected into the mass spectrometer through an analytical PepMap RSLC C-18 Easy Spray column (Thermo Scientific – 3 µm particle size, 100 Å pore size, 150 mm column length; 75 µm internal diameter. Catalog ES900) with a pre-column Acclaim PepMap 100 C-18 (Thermo Scientific – 3 µm particle size, 100 Å pore size, 20 mm column length; 75 µm internal diameter. Catalog 164946). 1 µL of each sample was injected. Peptides were eluted from the column using an acetonitrile gradient in 0.1% FA (5–60% B in 20 min, 60–95% B in 4 min, and held at 95% for 6 min. Mobile phases: Solvent A 0.1% FA, solvent B 0.1% FA in 80% acetonitrile) at a 0.3 µL/min flow rate. The mass spectrometer was operated in positive, data-dependent mode, in which one full MS scan was acquired in the m/z range of 375–1500 (repeat count = 3, exclusion duration = 20s, threshold = $1E + 06$) followed by MS/MS acquisition using higher energy collisional dissociation of the ten most intense ions from the MS scan using window width of 2.0 m/z . The MS scans were manually examined for non-background ions. Observed ions were manually sequenced. For each fraction, the following data were presented for each species detected: selected ion chromatogram (SIC), MS spectrum to determine the $(M + H)^+$, MS/MS spectrum to confirm the sequence. For fractions that contained more than one species, the approximate percentage of each species was calculated using the area under the SIC curves.

3. Results

To examine stability of all 18 peptides (Table 1), samples of the 6MHP and 12MP lyophilized peptide preparations were stored at -80°C, + 4°C, and + 20°C for periods of one day, one week, and one month. Following storage, we used HPLC to assess for alterations to the integrity of their constituent peptides by ensuring that their elution peaks match those from stability tests performed at the time of vialing: 2005 for 12MP and 2006 for 6MHP. The HPLC analyses showed that peptides in the 6MHP vaccine

mixture consistently elute as 6 principal peaks for each testing condition: the chromatograms are comparable for samples stored at -80°C and at + 20°C for 1 month (Fig. 1A). The elution time for each peak is consistent over time and temperature (Fig. 1B). We have previously shown that HPLC peaks from samples kept at -80°C correspond to the expected peptides in the vaccine (Chianese-Bullock et al. 2009).

HPLC data for 12MP were also comparable, with 11 dominant peaks for each. HPLC curves for the samples stored at -80°C and for 1 month at + 20°C are largely superimposed in Fig. 2A. No new peaks were identified. Elution times for each peak were consistent across all experimental samples (Fig. 2B). We have previously found that some peptides co-eluted in the same peak. Also, peptide degradation could be missed for peptides eluting close to others. Thus, we determined the identity of peptide species in each of the HPLC peaks for the - 80°C sample and the sample stored for 1 month at + 20°C (Table 2). The peaks are numbered from left to right: peaks 1, 2, 3, 7, and 10 contained one peptide each (ASGPGGGAPR, EADPTGHSY, DAEKSDICTDEY, ALLAVGATK, and SSDYVIPIGTY, respectively). Peak 9 contained two peptides that co-eluted equivalently (SLFRAVITK, EVDPIGHLY). Peaks 4, 8, and 11 contained one peptide each (YMDGTMSQV, GLYDGM EHL, IMDQVPFSV, respectively), but each contained a proportion including an oxidized methionine residue, an expected modification (Chianese-Bullock et al. 2009). The percentage of oxidized methionine residues was low for each. The only exception was the oxidized methionine in IMDQVPFSV in peak 11, which increased from 3% of the total at -80°C to 48% when stored at + 20°C for a month (Table 2). Others were similar across conditions. Peaks 5 and 6 were adjacent/overlapping and contained 2–3 of the 12 peptides each, and small proportions of LIYRRRLMK containing an oxidized methionine residue. Peak 5 contained LIYRRRLMK and included a subdominant peak (Fig. 2A) that was previously found to be purely LIYRRRLMK; peak 5 also had YLEPGPVTA. Peak 6 contained LIYRRRLMK, ALLAVGATK, and YLEPGPVTA. Other anticipated modifications were not observed: there was no evidence of dimerization or other modification at cysteine residues, and there was no degradation of peptides containing adjacent aspartic acid and proline, which can be susceptible to hydrolysis (D-P popping).

Table 2
Mass spectrometry results for 12MP vaccine

Peak	Peptide	Percentage	
		-80°C	RT
1	ASGPGGGAPR	100	100
2	EADPTGHSY	100	100
3	DAEKSDICTDEY	100	100
4	YMDGTMSQV	87	85
	YM(o) ^a DGTMSQV	6	10
	YMDGTM(o)SQV	7	6
5	LIYRRRLMK	73	62
	LIYRRRLM(o)K	4	3
	YLEPGPVTA	23	34
6	LIYRRRLM(o)K	12	12
	LIYRRRLMK	71	16
	ALLAVGATK	16	56
	YLEPGPVTA	1	16
7	ALLAVGATK	100	100
8	GLYDGMEHL	89	84
	GLYDGM(o)EHL	11	16
9	SLFRAVITK	55	50
	EVDPIGHLY	45	40
10	SSDYVIPIGTY	100	100
11	IMDQVPFSV	97	52
	IM(o)DQVPFSV	3	48
^a Oxidized methionine			

4. Discussion

Peptides degrade with fluctuations in temperature. Consideration of the thermostability of vaccines' constituent peptides must be taken to maintain their stability and efficacy. We have previously shown that the peptides that constitute the 6MHP and 12MP vaccines retain purity for at least five years when stored at -80°C. We now show that the lyophilized peptide mixtures are stable for at least 17 years. Most significantly, we find that 17 of the 18 peptides are stable at room temperature for one month, evidenced by stable HPLC elution times and profiles (Figs. 1A, 2A) and by confirmation of peptide identity and purity by mass spectrometry for the 12 peptides in 12MP, except for increased oxidized methionine for one peptide (Table 2).

The peptides in 6MHP represent epitopes for CD4 T cells and are presented by Class II major histocompatibility complex molecules. They vary in length and include two peptides with methionine residues and one with a cysteine residue (Table 1). The HPLC profiles of these 6MHP samples stored at -80°C, +4°C, and +20°C for one day, one week, and one month were comparable to those obtained soon after vaccine production. Importantly, none of our samples showed new peaks, significant shifts in height, width, or elution time. The lack of changes indicates a lack of contaminants, peptide breakdown products, dimers, or other variants of their peptide species. Subtle differences in peak height and elution time are presumably due to minor variations in the HPLC gradients. These findings suggest that the peptide species of the 6MHP vaccine are stable at room temperature for at least one month.

The HPLC profiles of all 12MP samples showed strong similarity to our previously published results. These samples displayed only minor peak elution time shifts and heights, which is likely attributable to minor variation in HPLC gradients. Mass spectrometric analysis of individual peaks confirmed that all 12 peptides are present after 1 month at room temperature. There were small proportions of oxidized methionine residues for up to about 15% of several peptides in the samples stored at -80°C for 17 years, and they did not change meaningfully after a month at +20°C, with one exception. Methionine-containing peptides can undergo spontaneous oxidation, generating chemical species possessing distinct physicochemical or biological properties. A recent study suggests that the methionine-oxidized variant of YMDGTMSQV augments CD8 + T cell activation compared to its native form (Chirițoiu et al. 2023). This warrants a discussion about whether methionine residues may be useful for optimizing peptides for vaccines. From this, one might hypothesize that the location of the methionine residue in this sequence could increase immunogenicity. However, further testing is required to fully understand the effect that oxidation has on peptide vaccines.

It is currently unclear why we observed a significant increase in IMDQVPFSV peptide oxidation following 1 month of storage at +20°C, while the other methionine-containing peptides in the 12MP vaccine were relatively unaffected. We previously reported sequence alterations such as dimerization and D-P popping, but these were primarily observed after long-term storage of peptides as aqueous solutions rather than as lyophilized mixtures (Chianese-Bullock et al. 2009), (Preston et al. 2021). The absence of either in the present study suggests that, when stored as lyophilized mixtures, alterations can be avoided for over a decade at -80°C and up to a month at +20°C.

5. Conclusion

The data presented demonstrate the stability of 18 peptides ranging in length, including peptides with cysteine and methionine residues and aspartic acid-proline bonds, all of which are susceptible to modification/degradation over time. However, we found that 94% of them were very stable at room temperature for at least a month and that even for the one with increased oxidized methionine at 1 month, most of its molecules were unchanged. These data may be useful for others utilizing peptides as vaccines or other therapies. These findings offer promise for providing access to lyophilized peptide vaccines for rural or impoverished areas lacking the infrastructure required to store temperature-labile vaccines. Some caution should be exercised, especially with peptides containing methionine residues, but testing by HPLC and/or mass spectrometry can assure stability for peptides other than those tested here.

Declarations

Statements and Declarations

Craig L. Slingluff, Jr. has the following disclosures: Research support to the University of Virginia from Celldex (funding, drug), Glaxo-Smith Kline (funding), Merck (funding, drug), 3M (drug), Theraclion (device staff support); Funding to the University of Virginia from Polynoma for PI role on the MAVIS Clinical Trial; Funding to the University of Virginia for roles on the Scientific Advisory Board for CureVac. Also, Craig L. Slingluff, Jr. receives licensing fee payments through the UVA Licensing and Ventures Group for patents for peptides used in cancer vaccines.

Competing Interests

Craig L. Slingluff, Jr. has the following disclosures: Research support to the University of Virginia from Celldex (funding, drug), Glaxo-Smith Kline (funding), Merck (funding, drug), 3M (drug), Theraclion (device staff support); Funding to the University of Virginia from Polynoma for PI role on the MAVIS Clinical Trial; Funding to the University of Virginia for roles on the Scientific Advisory Board for CureVac. Also, Craig L. Slingluff, Jr. receives licensing fee payments through the UVA Licensing and Ventures Group for patents for peptides used in cancer vaccines.

Author Contribution

C.S., E.A., and B.M. contributed to the study conception and design. Material preparation, data collection and analysis were performed by J.B., D.S., N.S., E.A., and C.S. The first draft of the manuscript was written by E.A. and B.M. and all authors commented on the manuscript. E.A. prepared figures 1 and 2 and tables 1 and 2. All authors read and approved the final manuscript.

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Figures

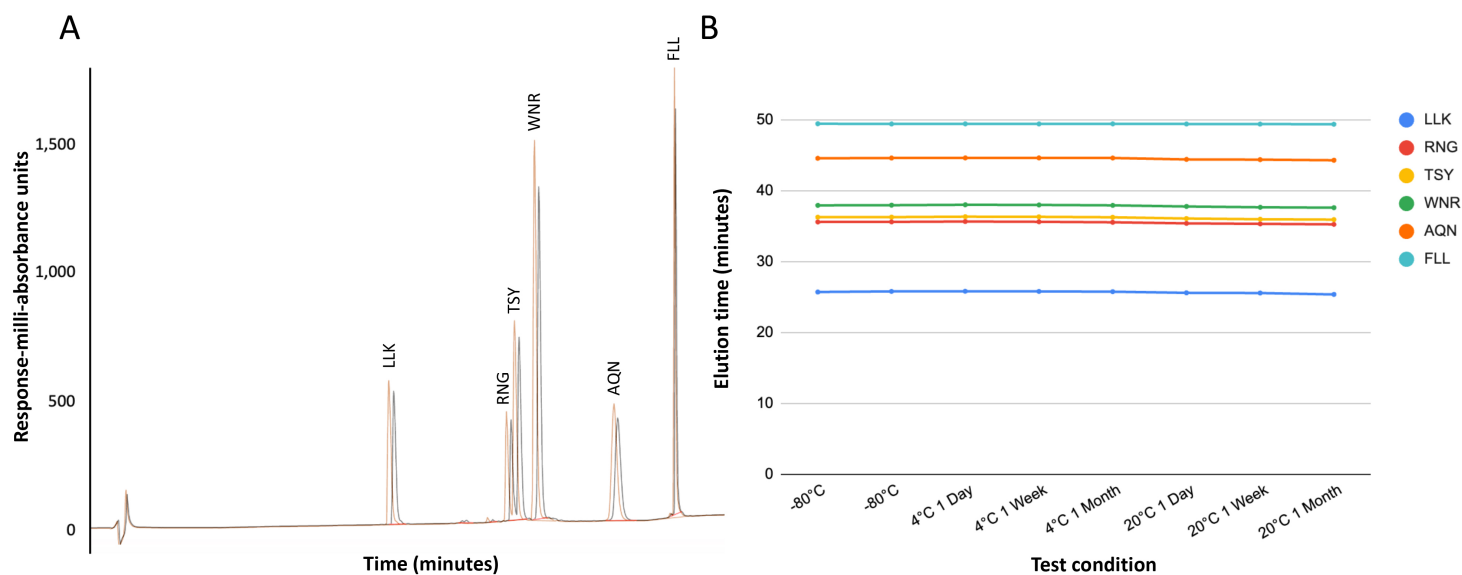


Figure 1

Overlay of HPLC analysis of the 6MHP vaccine maintained at -80°C for 1 month (black) and 20°C for 1 month (red) (A) and test condition (storage temperature and duration) vs. elution time of peptide species in the 6MHP vaccine (B)

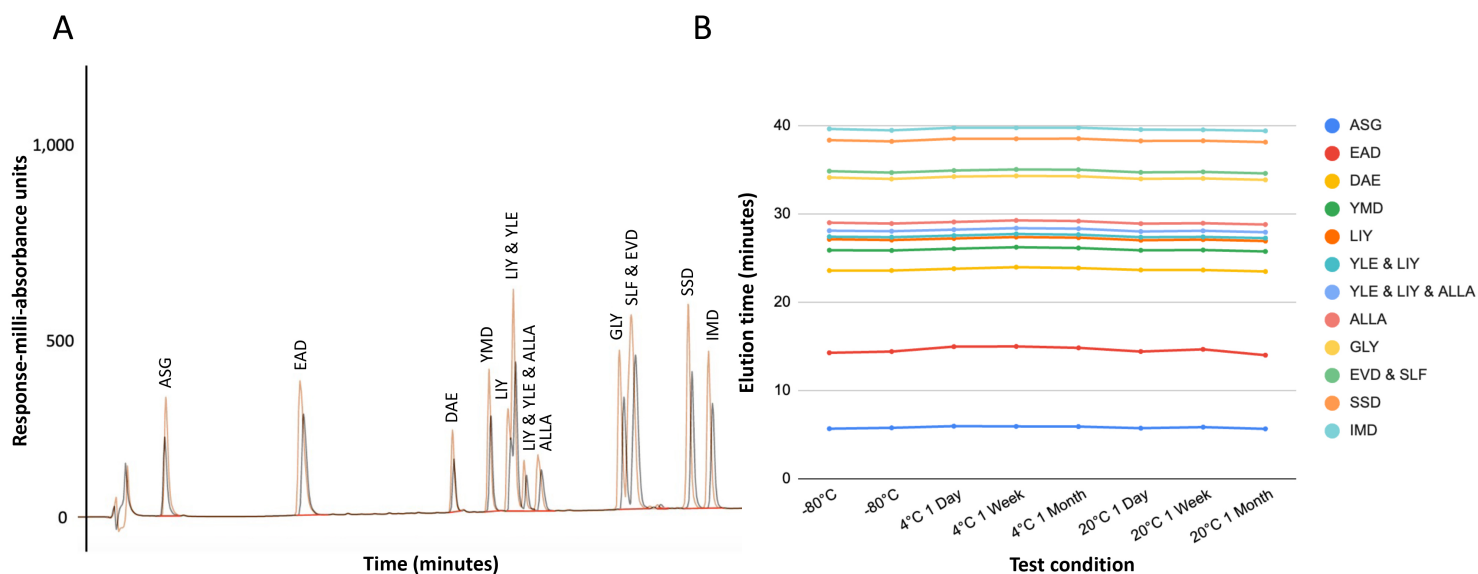


Figure 2

Overlay of HPLC results of the 12MP vaccine maintained at -80°C for 1 month (black) and 20°C for 1 month (red) (A) and test condition (storage temperature and duration) vs. elution time of peptide species in the 12MP vaccine (B)