

Measurement of Stability and Purity of Cell-Penetrating Peptides Used for siRNA Delivery

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ABSTRACT

Short, double-stranded RNA segments (21–25 oligonucleotides long) called small interference RNA (siRNA), have the ability to knock down genes of interest. Because disease processes are dependent on the activity of multiple genes, the expectation is that decreasing the activity of a specific gene with a siRNA may produce a therapeutic benefit. Research has rapidly advanced from the laboratory to clinical trials, but successful clinical application of siRNA strategies has been hampered by several complicating factors. These include stability of the siRNA complex, toxicity related to immune responses or possible disruption of cell membrane functionality, targeting a specific tissue/organ, and delivery of the complex through the cell's plasma membrane. Current delivery strategies include the use of cationic liposomes, polyethylene glycol (PEG)ylated nanocarrier complexes, polymeric systems, and cell penetrating peptides. As regulatory agencies begin to prepare guidelines for these new siRNA therapies, improved methods for quantitation must be considered.

Cell-penetrating peptides (CPP) are often enriched with cationic groups (e.g., arginine, lysine residues). These charged amino acids impart a positive charge to the carrier that helps form the peptide/siRNA complex and facilitates penetration of the cell membrane and delivery of siRNA molecules. Analysis of CPP and fragments can be complicated, as amino acids have variable responses using high-performance liquid chromatography (HPLC)-UV detection due to varying extinction coefficients. Charged aerosol detection is a universal mass-based technique capable of measuring any nonvolatile and many semivolatile compounds. It is sensitive (ng sensitivity), offers a wide dynamic range (>4 orders of magnitude), is gradient compatible and, most importantly, demonstrates a consistent response independent of chemical structure. As reported here, HPLC with charged aerosol detection was used to quantify a number of cationic peptides along with their stability and purity.

INTRODUCTION

RNA interference (RNAi) has rapidly advanced from the lab to clinical trials. siRNA are being evaluated to serve as potent agents against various diseases, e.g., cancer, heart disease, and Huntington's neurodegenerative processes. Small pieces of RNAi can influence endogenous cellular processes by blocking the translation of specific proteins. Successful clinical application of therapeutic strategies has been hampered by several complicating factors, including the stability of the siRNA complex, toxicity related to immune responses or possible disruption of cell membrane functionality, and delivery of the complex through plasma membranes to target location. Current delivery strategies under evaluation include cationic liposomes, PEGylated nanocarrier complexes, polymeric systems (dendrimers, polyplexes and natural polymers), and cell-penetrating peptides.

The siRNA complex must exhibit a positive net charge to effectively penetrate the cell membrane.¹ A peptide endowed with cationic charge characteristics makes it relatively simple to package a segment of RNA into a stable and protected particle. This approach has distinct advantages since the peptide complex can be further tailored to target specific cell surface receptors thus improving the specificity of the final product. Many cell penetrating peptides are prepared using synthetic sequences of strategic amino acids. However, some peptide-guided gene delivery systems have also been devised by using short peptide sequences from viral proteins.

As regulatory agencies begin to prepare guidelines for these new siRNA therapies, the study of impurities and degradants must be considered. Amino acids have varying extinction coefficients and resulting peptides and their fragments will have variable responses when using UV detection. HPLC with universal detection that exhibits consistent response factors provides better estimates of impurities. The Thermo Scientific ESA Charged Aerosol Detector (CAD[®]) is a sensitive, mass-based detector, especially well-suited for the determination of any nonvolatile analyte, independent of chemical characteristics. It has been used for many different types of assays, from low molecular weight ions to the analysis of larger polymers such as PEG. Charged aerosol detection, with response independent of chemical structure, is ideally suited for the measurement of peptides and impurities. Here, the authors explore the use of HPLC using a CAD detector for:

- A. Direct amino acid analysis
- B. Tryptic digest of a protein
- C. Cell-penetrating peptide analysis
- D. Forced degradation of cationic peptides

METHOD

A. Direct Amino Acid Analysis

Column: C18, 4.6 × 250 mm, 5 μm
 Mobile Phase: 0.1% TFA in A) water and B) CH₃CN
 Gradient: 5 min hold at 100% A, 0–40% B over 20 min
 Flow Rate: 0.6 mL/min
 Column Temperature: Ambient
 Injection Volume: 10 μL
 Detection: UV at 214 nm
 Sample: 1 mg/mL
 CAD: 35 psi nitrogen flow, 100 pA range, no filter

B. Tryptic Digest HPLC Parameters:

Mobile Phase: A) 0.1% TFA in water; B) 0.085% TFA in CH₃CN
 Gradient: 2% B for 5 min 2% to 42% over 55 min
 Flow Rate: 1 mL/min
 Column: Zorbax[®] Eclipse XDB-C8, 4.6 × 150 mm, 5 μm
 Column Temp: Ambient
 Injection Vol: 10 μL
 Sample Preparation: SeQuant Proteomic Calibration Kit – Bovine Serum Albumin, Cow, dissolved at 5 nmol/mL in 0.1% formic acid/CH₃CN 70:30

C. Cell-Penetrating Peptide Analysis

System: Thermo Scientific Dionex UltiMate[®] 3000 RSLC with Diode Array (DAD) and ESA Corona[®] *ultra*[™] CAD
 Column: Thermo Scientific Dionex ProSwift[®] RP-1S, 4.6 × 50 mm
 Mobile Phase A: DI water + 0.1% TFA
 Mobile Phase B: Acetonitrile + 0.1% TFA
 Gradient:

Time (min)	% Mobile Phase B
0	3
0.5	3
7.0	45
7.2	45
8.2	3
12.0	3

 Injection Volume: 3 μL
 Corona *ultra* Settings: Nitrogen 35 psi, Filter medium, Collection Rate: 60 Hz

D. Forced Degradation Sample Preparation

For each material, 50 μL aliquots of standard material were transferred to three autosampler vials containing glass inserts.

The following additions were then made:

- Vial 1: 50 μL Formic acid (98%)
- Vial 2: 50 μL Sodium hypochlorite
- Vial 3: 50 μL of Methanesulfonic acid (99.5%)

- All samples were vortexed then added to the autosampler tray set to 40 °C.
- The first point on the samples were analyzed between 1 and 3 h after acid addition.
- Subsequent injections were then made at 7 h increments.
- The control was not diluted by one-half and is, therefore, 2x the concentration of the acidified samples.
- 2 μL injections were made using the method described in Method C.

RESULTS AND DISCUSSION

The function and operation of the CAD detector is shown in Figure 1.

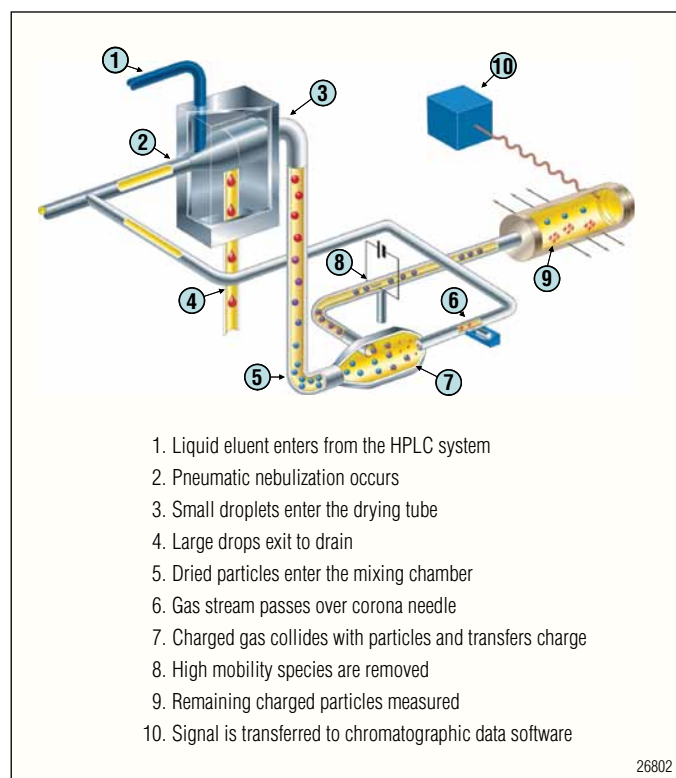


Figure 1. The Corona CAD—inside the engine.

A. Direct Amino Acid Analysis

Using the chromatographic conditions described in Method A, each of the amino acids that make up a test peptide were separated and detected directly by UV and the CAD detector as shown in Figure 2. Only some of the amino acids—those with UV chromophores—are detected by UV, while the CAD detector responds to all seven amino acids that comprise the test peptide. These response issues with UV preclude its use in examining individual amino acids or smaller peptide fragments that may result during a degradation study. Also, because amino acid analysis can be performed underivatized, the CAD detector can also be used for compositional analysis using a simple method.

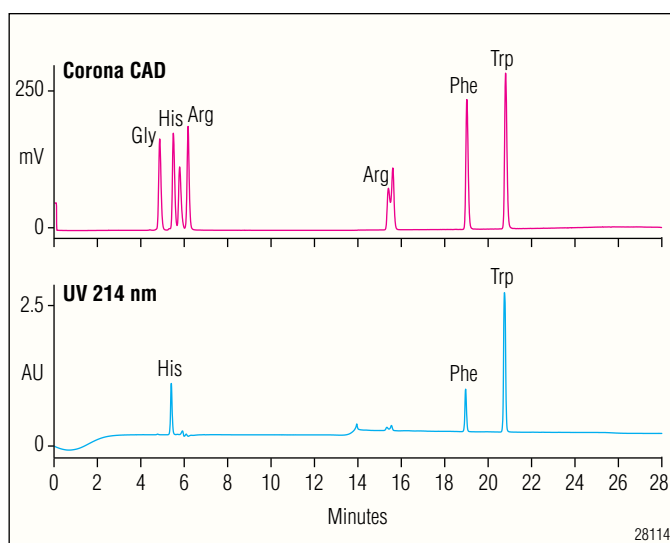


Figure 2. RP-HPLC AA separation: CAD/UV detection.

B. Tryptic Digest of a Protein

Tryptic enzyme degradation is a common analysis tool for proteins that can provide useful compositional information when followed by a separation of the resulting peptide fragments. Figure 3 represents a chromatogram of a bovine serum albumin (BSA) sample (5 nmol/mL) after tryptic digestion. These chromatograms show the response obtained for UV at 280 nm, 215 nm, and for the CAD detector. Note that only the CAD detector provides a significant response at this level, while diminished response is observed using the UV detector at 280 or 215 nm. These data illustrate that the CAD detector provides better sensitivity over UV detection for the analysis of individual peptide fragments. This is a good example of using the CAD detector in a forced degradation study.

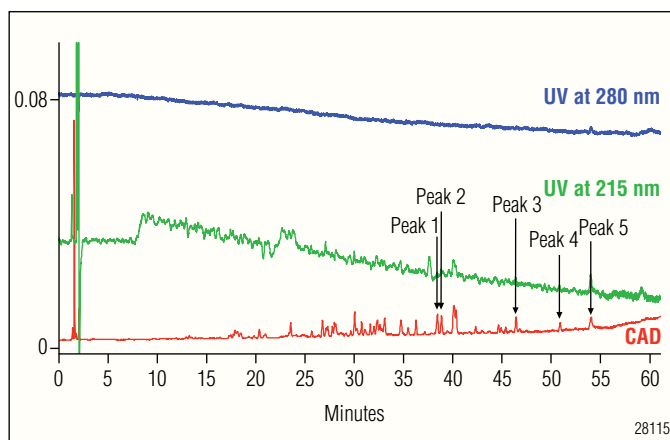


Figure 3. Chromatogram of a BSA tryptic digest using UV and the CAD detector.

Table 1 indicates the reproducibility of the CAD detector response for five peaks obtained by tryptic digest of the BSA material shown in Figure 3. The % RSD for these peaks ranged from 2.5% to 4.5% (n = 5) and indicates that suitable reproducibility can be obtained using the CAD detector.

Table 1. Reproducibility of Response to Five Peaks Obtained by Tryptic Digest of BSA					
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Mean Area (n = 5)	61739	39029	58678	51174	69147
STD	1579	1172	2629	1663	1701
RSD (%)	2.6	3.0	4.5	3.2	2.5

C. Cell-Penetrating Peptide Analysis

The use of siRNA as a therapeutic agent requires optimized delivery vehicles. The CAD detector enables measurement of the purity and stability of both lipids and peptides used in these delivery systems. Figure 4 shows the separation of three cell-penetrating peptides (SynB1, MPG, and transportan standards) using the CAD detector. Standard operating conditions using a ProSwift RP-IS column as indicated in Method C were used with the CAD detector. The universal, uniform, and sensitive response of the CAD detector is a distinct advantage for these types of separations as compared to evaporative light-scattering detection (ELSD) or UV detection.

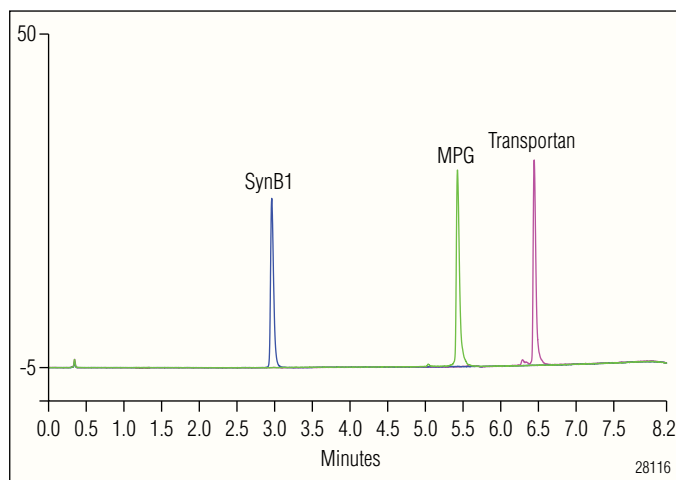


Figure 4. Separation and detection of cationic cell-penetrating peptides.

Peptide content and purity can be easily determined using the CAD detector, as illustrated in Figure 5. The purity of each peptide determined as area percent is 96.8% for SynB1; 97.1% for MPG and only 93.2% for transportan. The products used for the preparation of siRNA complexes require high purity peptides to maintain suitable quality standards. The CAD detector serves as a useful tool for the quality control analysis of these starting materials due to its good sensitivity and reproducibility.

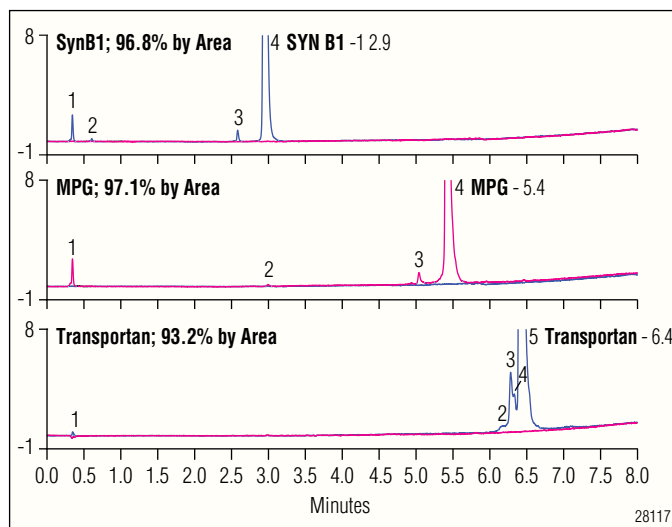


Figure 5. Peptide content and purity of cationic cell-penetrating peptides.

D. Forced Degradation

The investigation of forced degradation of the 27-residue cationic peptide transportan was performed using formic acid, sodium hypochlorite, and methanesulfonic acid. Results (Figure 6) obtained from these experiments helped determine the best acid to use for degradation of transportan. Formic was chosen for further study as MSA may have ion-paired with the peptide and fragments and resulted in poor response. In order to mimic possible immune responses (i.e., MPO production of hypochlorous acid), forced degradation using bleach was also studied. This did not provide sufficient degradation under the conditions used.

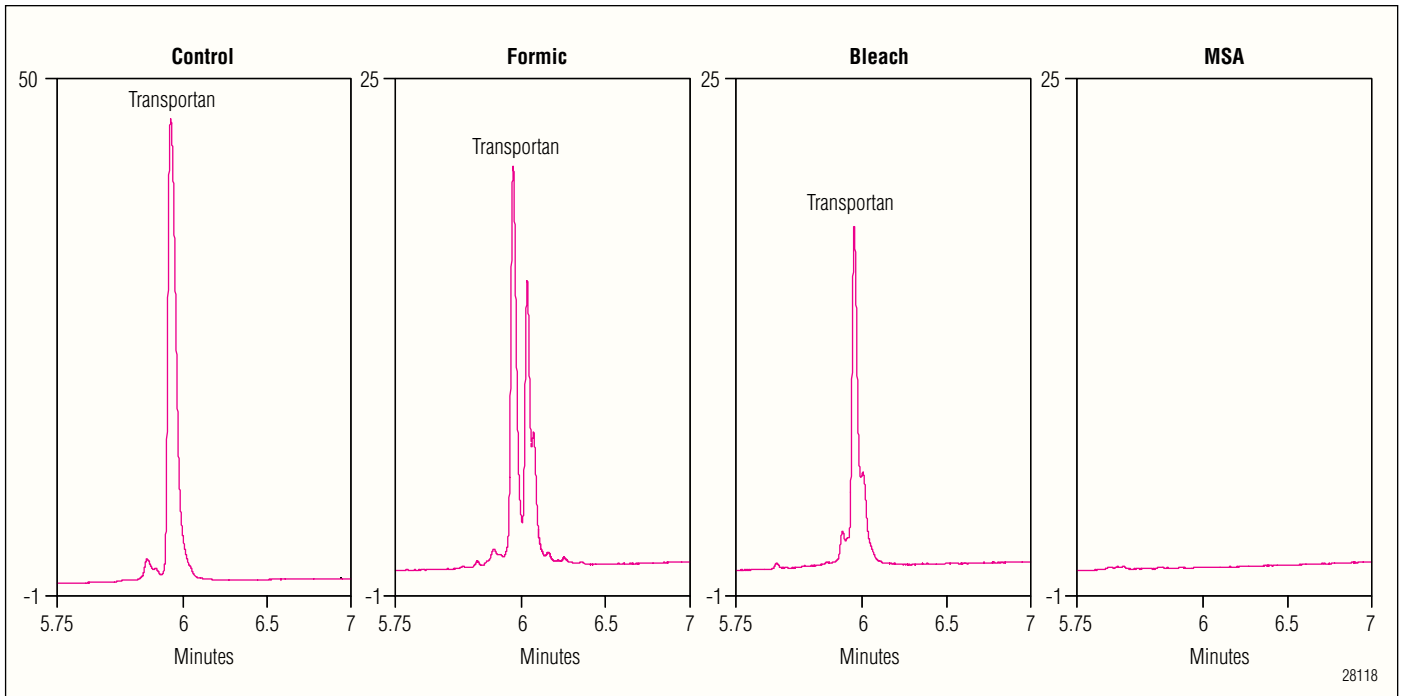


Figure 6. Transportan acid degradation comparison $T = 16 \text{ h}$ at $45 \text{ }^\circ\text{C}$.

One important goal for developing a stability assay is to find the conditions that provide ~10% loss in main component with possible mass balance. This provides suitable stability, indicating method conditions to be used for long-term stability studies. Results obtained for the time course of formic acid degradation using the peptide transportan are shown in Figure 7. Only a 3 h exposure time with formic acid was required to obtain adequate degradation of the peptide.

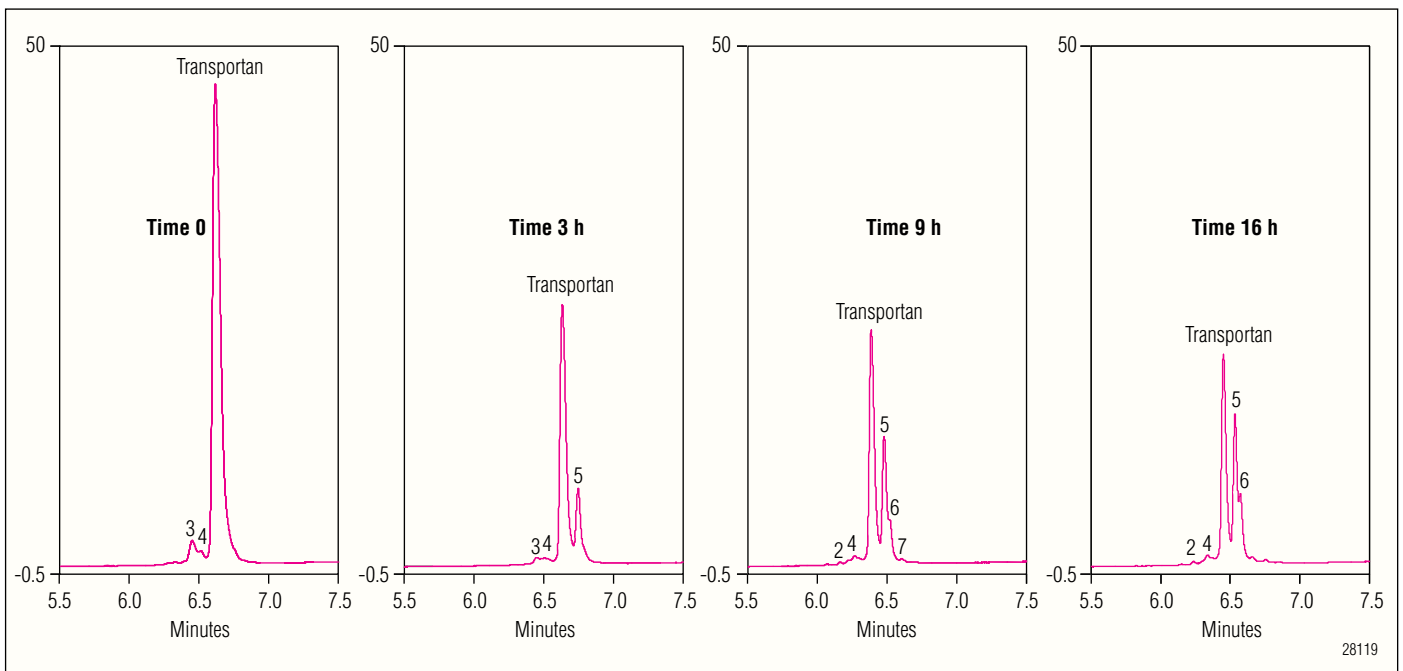


Figure 7. Time course for formic acid degradation of transportan.

CONCLUSIONS

- When compared to a UV detector, the CAD detector provides more uniform response for both amino acids (Figure 2) and peptides (Figures 3 and 4), and provides good reproducibility (Table 1). Even trace impurities are observed using the CAD detector (Figure 5), which provides valuable quality control information.
- Methods for the determination of the purity and stability of peptides used for the delivery of RNA complexes were developed. The CAD detector easily had sufficient sensitivity to detect levels of impurities in each of the commercially available peptides evaluated.
- A forced degradation study of the peptide transportan—using a simple HPLC method with the CAD detector—illustrates that treatment with formic acid provides suitable conditions for a stability-indicating method to help characterize possible fragments of cell-penetrating peptides

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