Capillary electrophoresis of heparin and related impurities using highly concentrated buffers in a 25 µm bubble cell capillary

Application Note

Drug Testing

Authors

Robert Weinberger
CE Technologies, Inc.
Chappaqua, NY 10514
USA
Todd Wielgos
Baxter Healthcare
Round Lake, IL 60073
USA

Abstract

The heparin contamination crises drove the development of emergency methods to ensure that contaminated material did not enter the world's supply. One of these methods employs capillary electrophoresis. In June 2008, an official USP method was approved. Now that the immediate problem was behind us it was necessary to improve the resolution and sensitivity of the original method. The official method used 36 mM phosphate buffer, pH 3.5. Partial resolution of oversulfated chondroitin sulfate (OSCS) was obtained and the limit of detection was about 1.5% of heparin by weight. By careful optimization of variables such as pH, buffer concentration, and counterion, complete resolution of OSCS and other glycosoaminoglycans (GAGS) such as dermatan sulfate was obtained in 10 min. The optimal buffer was 600 mM lithium phosphate, pH 2.5. Since such a high buffer concentration was required for the separation, a 25 µm capillary was required to the control the current. Since the capillary diameter defines the optical path length the sensitivity of the method often suffers. The Agilent bubble-factor capillary actually provided a longer optical path length compared to a 50 µm capillary. In conjunction with good sample stacking by the high concentration buffer, the limit of detection of OSCS was far below 0.1%.

This Application Note describes a high-speed, high-resolution, high-precision, and high-sensitivity method for the determination of impurities in heparin samples. The method is robust and requires no special capillary preparation. Sample preparation is simply dilute and filter.

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Background

Heparin is one of the oldest medications still in use. The drug is an important anticoagulant used for preventing thrombosis in dialysis patients, cardiac diagnostic and surgical patients, those undergoing angioplasty, and for many other indications. Lithium, sodium, or ammonium heparin is the anticoagulant used in green top blood collection tubes.

A brief history of the development of heparin can be found in a Connaught Laboratories newsletter.[1] Heparin was discovered at Johns Hopkins in 1916 but did not find much use until an inexpensive process was developed in the mid-1930s by Connaught Laboratories Ltd. Since its approval by the United States Food and Drug Administration (FDA) in the 1940s, heparin has been safely used in countless treatments.

In late December of 2007, an increase in the number of adverse events associated with heparin was identified by Baxter Healthcare's pharmacovigilance system. On January 17, 2008, Baxter issued a voluntary recall of nine lots of heparin multidose vials. Baxter expanded this recall on February 29, 2008, to include all heparin vial products, including heparin lock flush. From March through May 2008, the recall was extended to include products from Covidien, Braun, American Health Packaging, and Medtronic.[2] Adverse events were soon reported on a worldwide basis. These included allergic reactions and hypotension, both of which can be serious.

An intense effort at Baxter was initiated to identify an unknown contaminant in heparin. Chromatography failed to distinguish lots associated with adverse events from lots that were not associated with adverse events. They tried capillary electrophoresis (CE) as one of a battery of analytical procedures. In early February of 2008, an unusual peak was found for a heparin sample associated with adverse events (Figure 1). That peak was absent from samples that did not have adverse events associated with them. This was confirmed by high field NMR, the only other technique that detected the unknown contaminant. This CE separation along with NMR became emergency methods to ensure the world's supply of heparin was not contaminated. Both methods appeared on the FDA Web site.

By April of 2008, the contaminant was identified as OSCS.[3] Another compound, dermatan sulfate (DS) was also found in many heparin samples. A limit test for DS may be required by the USP, although this compound has not been associated with adverse events. The FDA and United States Pharmacopeia (USP) conducted Web based meetings in April and June of 2008, the purpose of which was to speed through the validation process and publish an official monograph.[4] That monograph appeared on June 18, 2008.

Method Improvements

The official CE method was very useful in identifying highly contaminated heparin in the world's supply. The resolution of the method was poor and the limit of detection (LOD) for OSCS was estimated at 1.5%. Ideally the LOD for a toxic impurity should be 0.1% or less.

Methods development by CE is actually a simple process provided the developer stays disciplined. Since heparin and its relatives are all negatively charged, a bare silica capillary was chosen. In fact heparin has the greatest negative charge density of all known biomolecules. That being said, it is unlikely that heparin would bind to the capillary wall. The most important experimental variables in CE are pH, buffer selection, and buffer concentration. Selection of the buffer counter-ion can be important as well. In some cases, it is necessary to use reagents such as surfactants or cyclodextrins to adjust selectivity; reagents to ensure solubility may also be required.

About 150 English language references concern CE of heparin and/or other GAGs. Most deal with disaccharide or

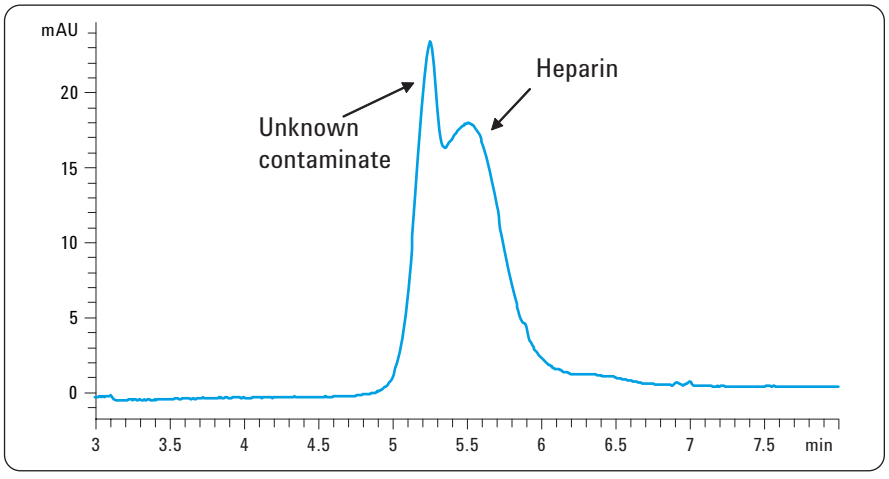

Figure 1
Heparin separation by the USP method.

monosaccharide separations from digestions of the intact GAG. A number of references describe separations of intact heparin in low pH dilute phosphate buffers.[5–7] At least one of these papers formed the basis for the emergency method developed earlier. All of these methods use countermigration capillary electrophoresis (CMCE). At low pH, the electroosmotic flow (EOF) is sufficiently low that the highly mobile GAGS migrate toward the positive electrode. The CE (Agilent G1600 Capillary Electrophoresis System) is operated in the reversed polarity mode such that the capillary inlet is near the negative electrode and reversed polarity (negative voltage) is used. Phosphate buffer is a good choice for CE since it buffers well in the low pH region and is optically transparent in the low UV portion of the spectrum.

Experimental design and evolution of the method

It is well known that increased buffer concentration reduces the EOF as well as the electrophoretic mobility of the solute. In Figure 2, there appeared to be no change in migration time when the buffer concentration was increased from 36 to 100 mM at pH 3.5. Coincidently, it appeared that the EOF and mobility of the solutes were equally decreased. Since they were in opposite directions, there was no net change in migration time. While the heparin peak was largely unchanged the OSCS peak was sharpened. This was due to increased sample stacking which resulted from the higher buffer concentration.

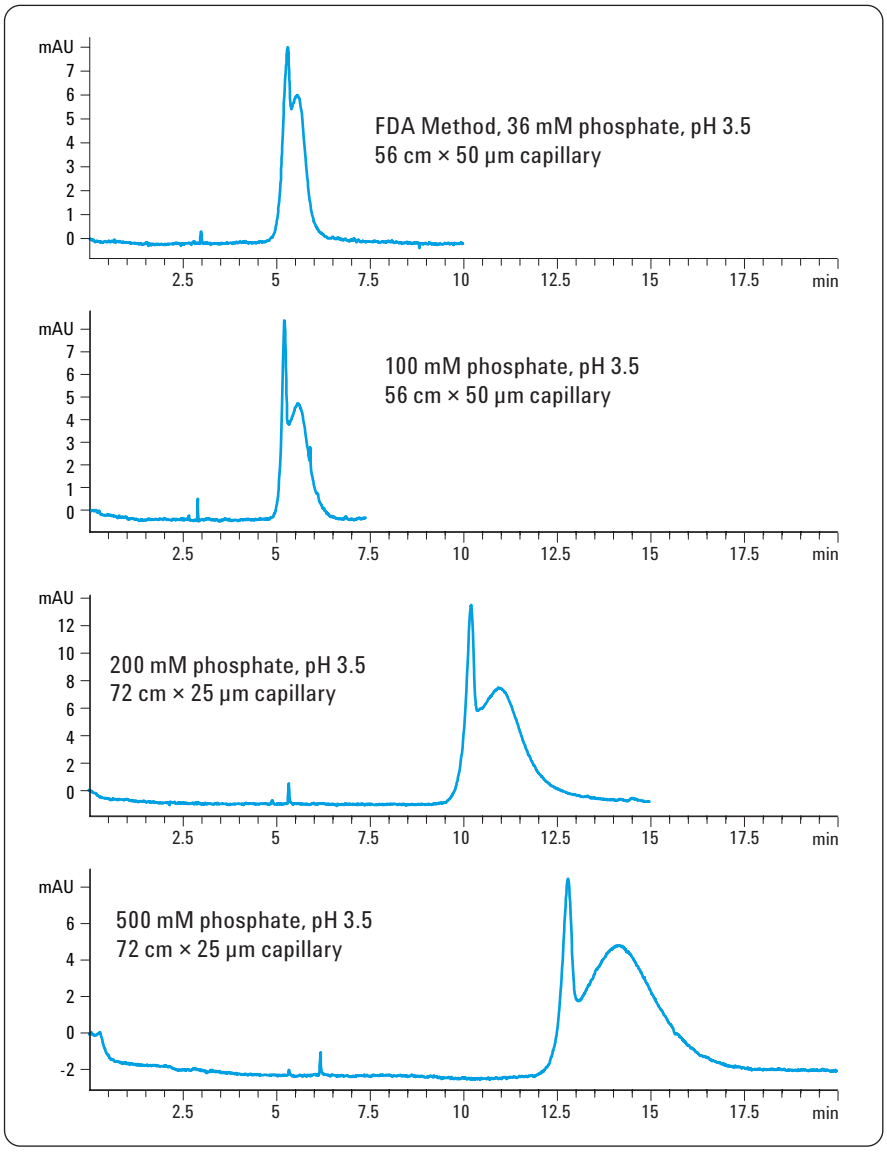

Figure 2
Effect of buffer concentration on the separation.

When the buffer concentration was increased to 200 mM, a 25 µm id capillary was required to reduce the current. A longer length was used as well.

When the buffer concentration was increased from 200 to 500 mM, the resolution between heparin and OSCS continued to improve. The broadness of the heparin peak was due to its extreme heterogeneity.

The effect of buffer pH is illustrated in Figure 3. As the pH was reduced, the resolution between heparin and OSCS continued to improve. While not shown in the figure, the optimal pH was 2.5. With the improvements in resolution, it now became possible to reduce the length of the capillary and thus shorten the time for separation.

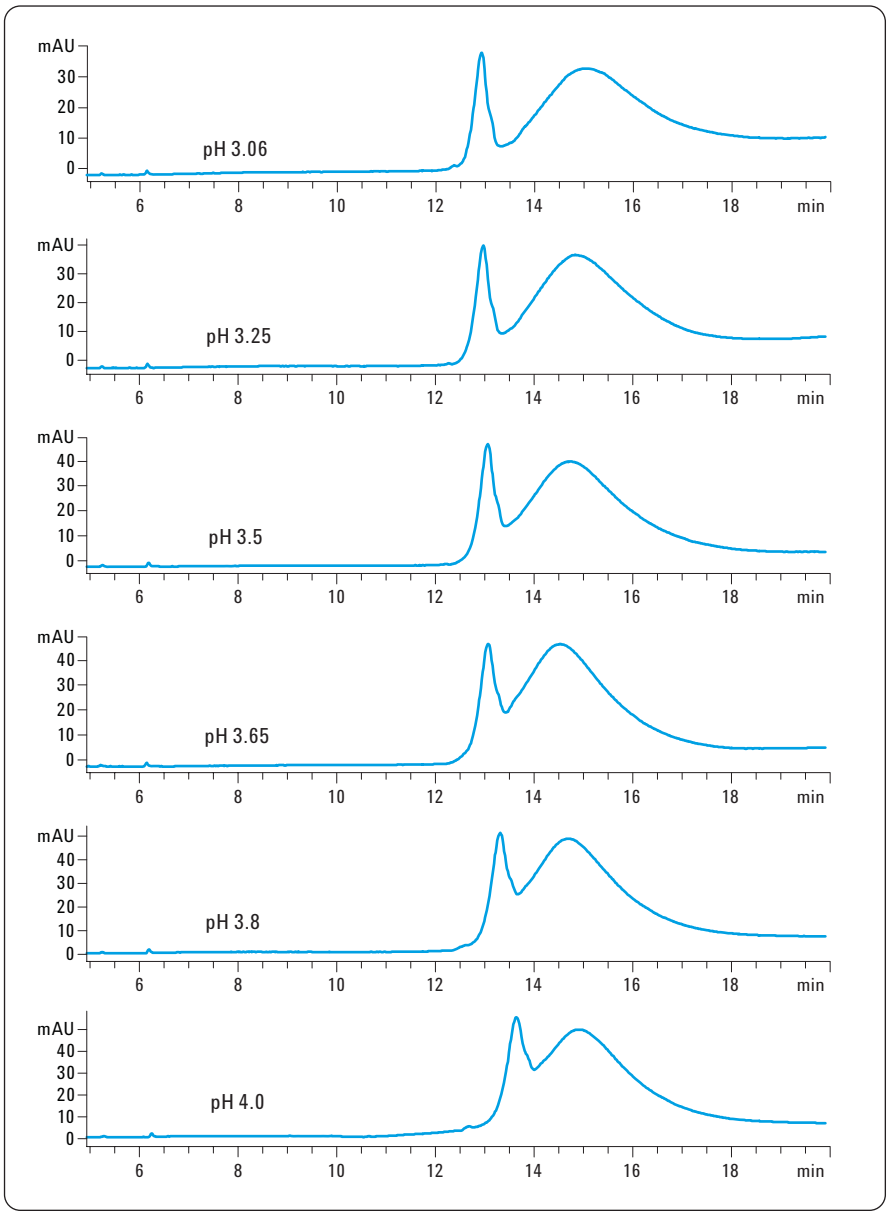

Figure 3
Effect of pH on the separation.

Figure 4 illustrates another buffer concentration study on the short end of the capillary which has a length of 8.5 cm. Such a short capillary gave very rapid separations but there was incomplete resolution between heparin and OSCS. Increasing the capillary length to 24 cm would help but the increase in resolution would only be proportional to the square root of the increase in migration time due to diffusion.

In order not to suffer from the square root problem, the field strength had to be increased; however the current was up to 50 µA. The solution was to change the counter-ion. Buffer conductivity is a function of both the mobility and concentration of both the co-ion and the counter-ion. Lithium ion was considered in lieu of sodium. While lithium is seemingly more mobile than sodium based on their charge to mass ratios, that did not consider the hydration sphere around each ion. In fact lithium has a much greater hydration sphere compared to sodium and thus is less mobile and less conductive. The voltage was increased from –10 to –14 kV when lithium was employed without increasing the current. A separation is shown in Figure 5.

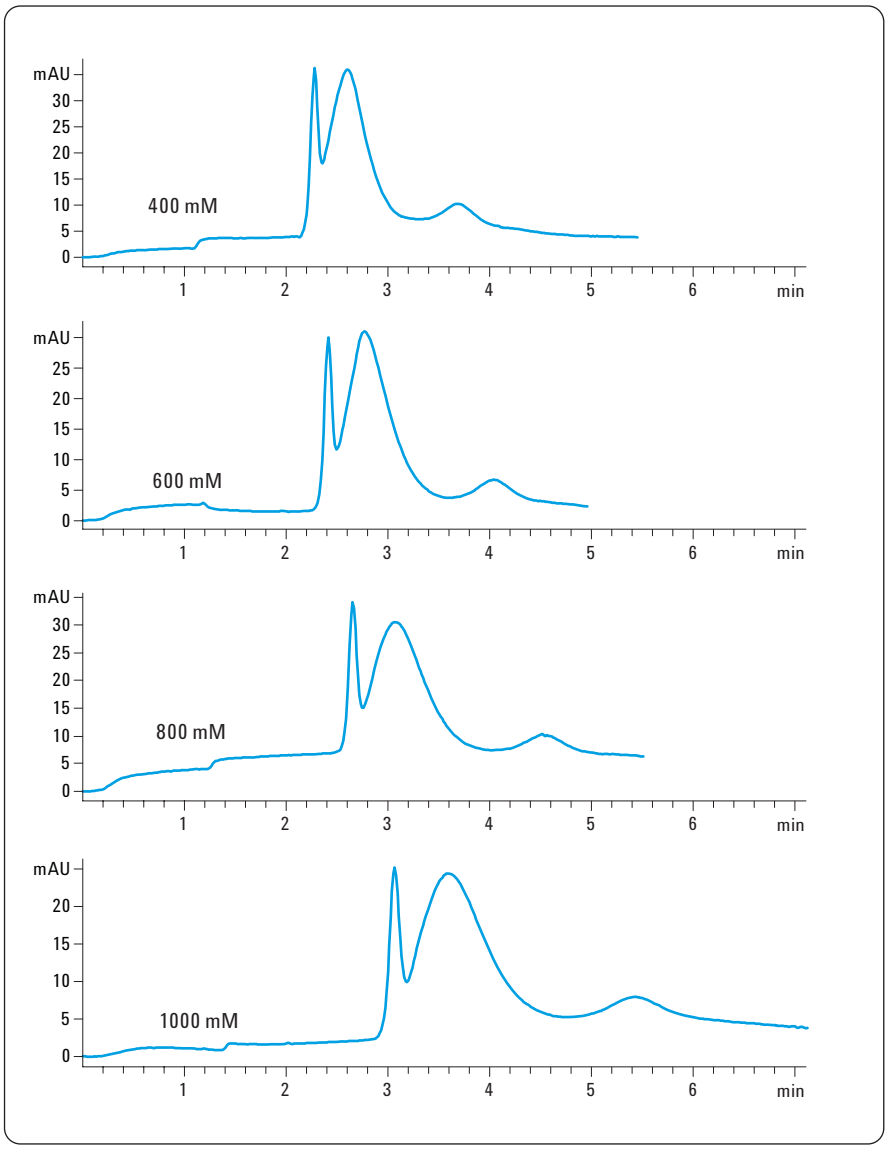

Figure 4
Effect of buffer concentration on the separation. Short-end injection (8.5 cm). Sodium phosphate buffers. voltage: 10 kV, temperature: 20 °C, injection: 300 mbs, sample concentration: 30 mg/mL.

Operating characteristics of the method

Operation with a 25 µm id capillary is quite simple. So long as buffers and samples are filtered through 0.2 µm filters, clogging is not a problem. Because of the favorable stacking characteristics of high concentration buffers, large injections of heparin at a concentration of 30 mg/mL can be made without appreciable bandbroadening. While a 100 millibar second injection is normal using a 50 µm id capillary, injections as large as 2000 mbs can be made without appreciable band broadening in the 25 µm id tube.

Table 1 contains the operational parameters of the high-speed high-resolution method. The method is very precise. Figure 6 shows an overlay of 11 runs of a heparin sample spiked with 0.1% OSCS and 2% DS. The peak area percent relative standard deviation (%RSD) is less than 2% for that sample. The migration time %RSD for OSCS is less than 0.1%. The limit of detection for OSCS is well below 0.1%. This is due to three factors: good sample stacking, high heparin concentration, and the 5X bubble cell. The 125 µm optical pathlength of the capillary removes perhaps the last disadvantage of 25 µm capillaries.

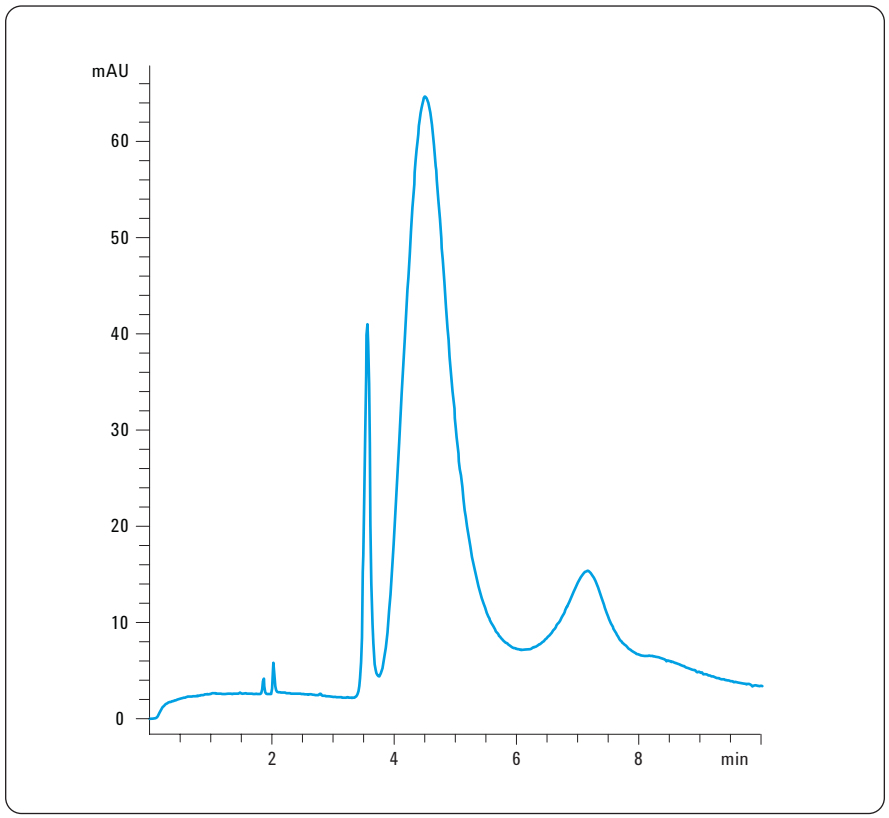

Figure 5
High-speed high-resolution separation. See Table 1 for separation conditions.

Instrument:	Agilent G1600 Capillary Electrophoresis System (p/n G1602BA)
Capillary:	Extended light path capillary 25 µm id, bubble factor 5 (Agilent p/n G1600-60132) cut to a total length of 33 cm
Buffer:	600 mM lithium phosphate, pH 2.5. Prepare by titrating 600 mM lithium phosphate with 600 mM phosphoric acid. Fill from the inlet buffer vial. The buffers are good for at least 10 runs.
Injection:	1000 mbs
Temperature:	20 °C
Voltage:	–14 kV, current ~53 µA
Detection:	UV, 200 or 195 nm; reference off
Run time:	10 min
Equilibration procedure:	1 min 0.1N sodium hydroxide, 1 min 600 mM phosphoric acid, 3 min buffer
Sample concentration:	30 mg/mL heparin
Sample diluent:	Water or 10 mM SDS
Filter:	Buffers and samples are filtered using 0.2 µm cellulose acetate filters.

Table 1
Operational parameters of the method.

Phosphate buffer can take several hours to fully equilibrate the capillary wall. It is best to store the capillary in buffer overnight although we have used new capillaries very soon after installation. The base and acid wash were necessary because some samples could interact with the capillary wall. A residual blank of 0.1% OSCS was sometimes observed if the wash step was omitted. In at least one case, a sample known to have 0.1% OSCS tested negative. Dilution of that sample with 5 to 10 mM sodium dodecyl sulfate (SDS) resolved the problem. The same heparin sample spiked with greater amounts of OSCS tested positive, even when diluted with water. The problem was probably due to wall adsorption of trace amounts of OSCS possibly due to the presence of proteins in the sample.

Conclusion

In this Application Note we present a robust, fast, high-resolution, high-precision, and high-sensitivity method for the determination of impurities in heparin samples. The method is robust and can be implemented on commercially available capillaries with very simple sample preparation steps. Agilent's extended light path capillaries provide a significant sensitivity advantage for the detection of heparin-related impurities.

The work was presented in part at CE Pharm 2008 on October 15, 2008, in San Francisco and is published in the *Journal of Pharmaceutical and Biomedical Analysis.*[8]

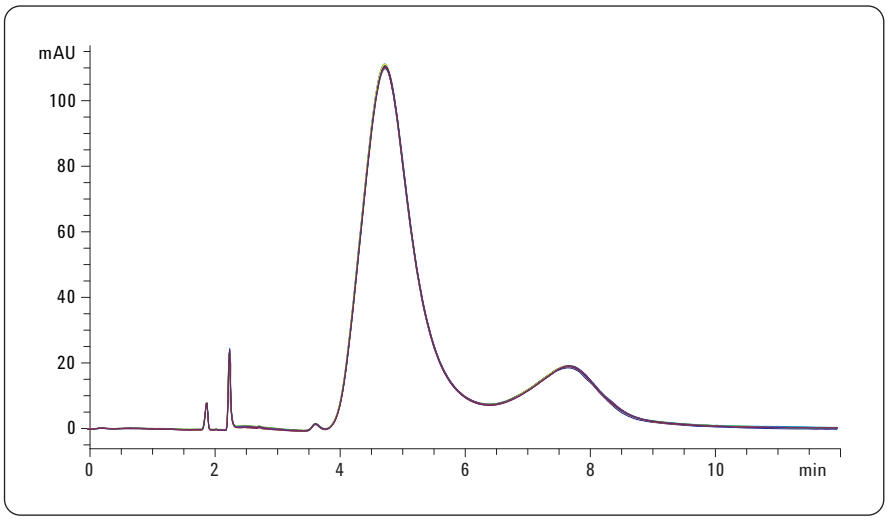

Figure 6
Overlay of 12 sequential runs. See Table I for experimental conditions.

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