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Application Note

Improvements in Sensitivity for Quantification of Steroid Phosphate Drugs Using ACQUITY PREMIER and MaxPeak HPS Columns

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Abstract

Analytes with electron rich moieties such as phosphate and carboxylate groups are susceptible to chelation with metal surfaces across the chromatographic system and column. This often results in poor peak shape, reduced sensitivity, with poor robustness and reproducibility, all of which can adversely affect the development of a successful bioanalytical method. The ACQUITY PREMIER and MaxPeak High Performance Surface (HPS) columns mitigate this problem by providing a chemically inert hybrid organic-inorganic surfaces which prevents metal chelation without affecting chromatographic selectivity.

In this application note, we show the impact of using this novel surface for quantifying hydrocortisone phosphate and dexamethasone phosphate in extracts of human plasma. Using the ACQUITY PREMIER System and MaxPeak HPS Columns, we were able to achieve a 10-fold improvement in the lowest limit of quantification (LLOQ) for hydrocortisone phosphate and 7.5-fold increase in LLOQ for dexamethasone phosphate. Additionally, better overall chromatographic performance resulted in better peak shapes and robust and reproducible integration of peaks, especially at the low concentrations.

Benefits

Superior peak shape, simpler peak integration, and improved sensitivity

Introduction

Bioanalysis laboratories constantly strive for more sensitivity as they try to better define the pharmacokinetics (PK) of candidate drugs at low systemic concentrations or are required to use lower sample volumes to perform analysis in pre-clinical or pediatric studies. Over the years, continuous advancements in MS instrumentation and sample extraction procedures have allowed scientists to achieve lower limits of quantification enabling them to more deeply investigate their drug of interest. However, current instrumentation platforms still pose major challenges for certain analyte classes where metal chelation can be problematic. Examples of this are certain compounds containing phosphate groups (e.g., ATP), uncharged amines, and deprotonated carboxylate groups (citrate, lactate, etc.,) that are electron rich and are easily adsorbed onto metal surfaces such as the stainless steel used in LC systems. This can have a deleterious effect on bioanalytical methods resulting in complicated method development, time consuming data analysis, insufficient assay sensitivity, and poor reproducibility. In attempting to address these issues bioanalyst's have employed chelating reagents as mobile phase additives, PEEK connection tubing and system passivation.[1] However, these solutions are not ideal as they are either non-permanent, incompatible with solvents such as DMSO or reduce ionization efficiency for the analytes causing reduced sensitivity.[1]

To address these challenges, Waters has developed a class of new technologies, known as MaxPeak High Performance Surfaces (HPS). This MaxPeak HPS LC surface is comprised of a resilient, highly crosslinked layer that is chemically similar to bridged-ethyl hybrid silica.[1] The MaxPeak HPS provides a highly effective surface barrier that mitigates against undesired interactions with metal surfaces. Here, we highlight the benefits of the MaxPeak HPS for bioanalysis using hydrocortisone phosphate and dexamethasone sodium phosphate as exemplars. Dexamethasone phosphate and hydrocortisone phosphate are anti-inflammatory corticosteroids used in the treatment of endocrine disorders as well as immune and allergic conditions such as arthritis, psoriasis, lupus, and ulcerative colitis.[2] Dexamethasone phosphate was also recommended for COVID-19 patients with severe respiratory symptoms.[3] Therefore, there is exceptional interest in improving the sensitivity of detection for these compounds.

Experimental

Sample Preparation

Lyophilized hydrocortisone phosphate and dexamethasone phosphate were dissolved in DMSO to make stock solutions at 1 mg/mL. These stocks were further diluted in 5% methanol in water to make working standards at 100 µg/mL and 10 µg/mL. These working standards were then spiked into human plasma to generate a calibration curve from 5–1000 ng/mL for hydrocortisone phosphate and 1–1000 ng/mL for dexamethasone phosphate. 100 uL of each sample were transferred to a micro-centrifuge tube and extracted using 300 µL of methanol. The samples were vortexed and centrifuged at 13000 rcf for 10 mins. 200 µL of the supernatant was transferred to LC-MS vials and used for analysis.

Method Conditions

MRM methods for both analytes were developed using QuanOptimize. A short LC method with a generic gradient was used for analysis. The method details are listed below.

LC Conditions

LC system:	ACQUITY PREMIER or ACQUITY UPLC I-Class
Detection:	Xevo TQ-XS
Column:	2.1 x 50 mm ACQUITY MaxPeak HSS T3, 1.8 µm or 2.1 x 50 mm ACQUITY HSS T3, 1.8 µm
Column temp.:	60 °C
Sample temp.:	5 °C
Injection volume:	10 µL
Flow rate:	600 µL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient:	5–75% B over 2.5 minutes

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	Positive ion electrospray
Acquisition range:	MRM
Capillary voltage:	3 KV

MRM Transitions

Hydrocortisone phosphate:	443.19>327.15
Dexamethasone phosphate:	473.32>435.16

Data Management

MS software:	MassLynx v4.2
Informatics:	TargetLynx XS

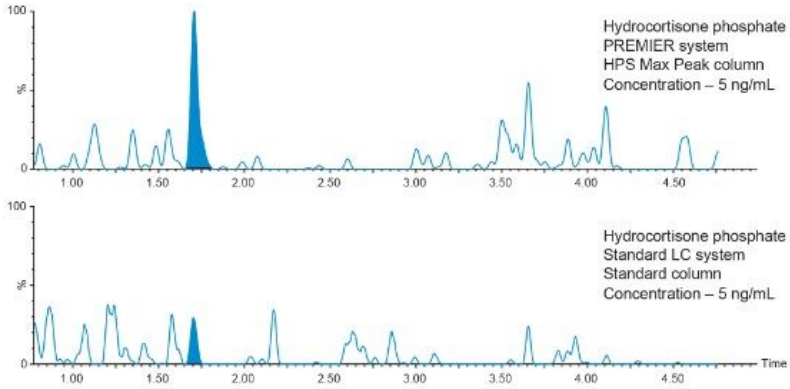
Results and Discussion

Due to the physico-chemical characteristics of hydrocortisone phosphate and dexamethasone phosphate, these analytes are susceptible to adsorption on stainless steel surfaces. This phenomenon is exacerbated at low concentrations as most or all the analyte can be lost to the needle, transfer tubing, pre-heaters, and column hardware. Mitigating these interactions through the use of chemically inert surfaces along the flow path has the potential of increasing the amount of analyte that reaches the detector, thereby improving the lower limit of quantification (LLOQ) that can be achieved, as well as increasing the precision of the assay.

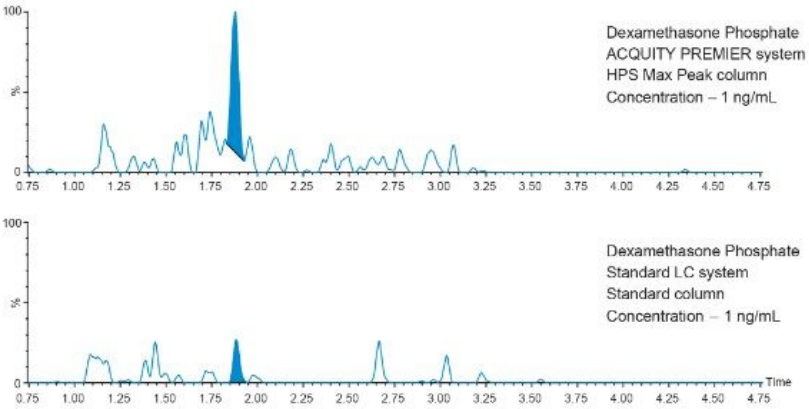
To test this hypothesis, calibration curve and QC samples were extracted using the procedure described above and injected on the ACQUITY PREMIER System with a Max Peak HPS Column as well as a conventional ACQUITY system with a standard ACQUITY UPLC column.

As seen in the top chromatogram in Figure 1a, using the ACQUITY PREMIER System with MaxPeak HPS Column, we achieved a LLOQ of 5ng/mL with signal-to-noise of >15 for hydrocortisone phosphate. The lower chromatogram of figure 1a shows the same sample injected on a conventional ACQUITY LC system and UPLC column. The peak in the lower chromatogram has a much lower intensity with a signal-to-noise <3 and may have much higher inter- and intra-day CV's which can affect the reproducibility, especially for low concentration samples. Similarly, as shown in the top chromatogram of Figure 1b, we achieved a LLOQ of 1 ng/mL with a signal-to-noise >10 for dexamethasone phosphate. However, the chromatogram of the same sample injected on the conventional LC system shows a peak with a significantly lower intensity as shown in the lower chromatogram of Figure 1b with a signal-to-noise of <4. Figure 1c compares the response obtained on the two systems at 50 ng/mL for hydrocortisone phosphate, which is the lowest concentration that could be accurately quantified on the standard ACQUITY LC system with standard ACQUITY columns. In addition to having a significantly higher peak area response, the signal observed on the Premier system/column is more symmetrical in shape and therefore more reproducibly integrated, compared to the peak observed on the standard hardware using the same integration parameters. Figure 1d compares the chromatograms for dexamethasone phosphate at 7.5 ng/mL, which is the observed lower limit on the standard system and column combination. The peak area responses and peak shapes for both systems are similar. However, concentrations below 7.5 ng/mL injected on the standard system/column either showed no peaks at all or provided irreproducible peaks which cannot be accurately integrated. This lack of reproducibility may be attributed to the analyte being adsorbed on the system. On the ACQUITY PREMIER System with MaxPeak HPS Columns, all concentrations from 7.5 ng/mL down to 1 ng/mL showed well defined chromatographic peaks which could be accurately integrated and showed a linear increase in response with increase in concentration.

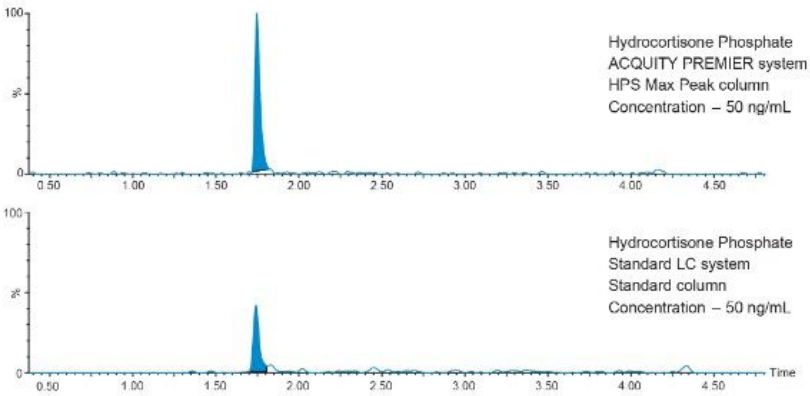
1A


Hydrocortisone phosphate
PREMIER system
HPS Max Peak column
Concentration – 5 ng/mL
Hydrocortisone phosphate
Standard LC system
Standard column
Concentration – 5 ng/mL
Time

1B


Dexamethasone Phosphate
ACQUITY PREMIER system
HPS Max Peak column
Concentration – 1 ng/mL
Dexamethasone Phosphate
Standard LC system
Standard column
Concentration – 1 ng/mL
Time

1C


Hydrocortisone Phosphate
ACQUITY PREMIER system
HPS Max Peak column
Concentration – 50 ng/mL
Hydrocortisone Phosphate
Standard LC system
Standard column
Concentration – 50 ng/mL
Time

1D

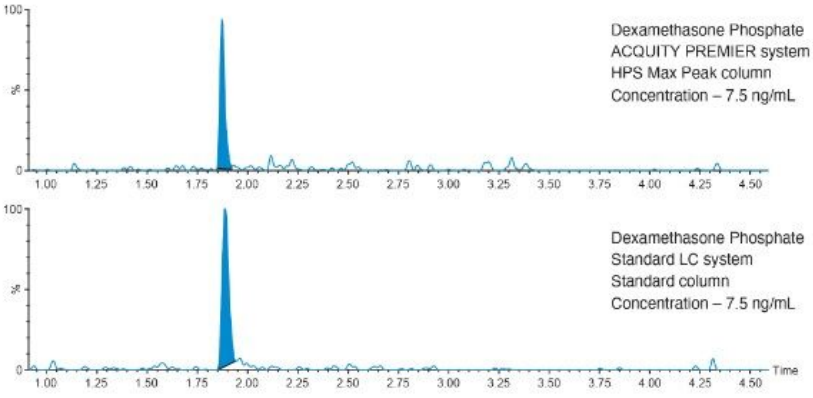

Dexamethasone Phosphate
ACQUITY PREMIER system
HPS Max Peak column
Concentration – 7.5 ng/mL
Dexamethasone Phosphate
Standard LC system
Standard column
Concentration – 7.5 ng/mL
Time

Figure 1a. Hydrocortisone phosphate – LLOQ comparison between ACQUITY PREMIER System with MaxPeak HPS Column vs standard ACQUITY LC system with standard column.
1b. Dexamethasone phosphate – LLOQ comparison between ACQUITY PREMIER System with MaxPeak HPS Column vs standard ACQUITY LC system with standard column.
1c. Hydrocortisone phosphate – LLOQ comparison between ACQUITY PREMIER System with MaxPeak HPS Column vs standard ACQUITY LC system with standard column.
1d. Dexamethasone phosphate – LLOQ comparison between ACQUITY PREMIER System with MaxPeak HPS Column vs standard ACQUITY LC system with standard column.

Using the ACQUITY PREMIER and MaxPeak HPS Columns, we achieved a linear calibration curve for hydrocortisone phosphate from 5–1000 ng/mL with r^2 >0.993, and for dexamethasone phosphate from 1–1000 ng/mL with r^2 >0.992 using a weighting factor of 1/x (Table 1a). The slope and intercept for hydrocortisone phosphate were 0.043 and 0.054 respectively, and for dexamethasone phosphate were 0.119 and 0.129 respectively. The closeness of these values to 0 implies negligible bias of the calibration lines and therefore more accurate quantification of unknown samples when using these calibration curves. This is confirmed by the accuracy and precision statistics calculated for both analytes at the LLOQ and the low, mid and high QC levels. For hydrocortisone phosphate, accuracies of between 98–102% and precision of <11% were observed (Table 1b). For dexamethasone phosphate, accuracy & precision values were between 92–112% and <8% respectively (1c).

	Linear range (ng/mL)	Weighting	r^2	Slope	Intecrept
Hydrocortisone phosphate	5–1000	1/x	0.993	0.043	0.054
Dexamethasone phosphate	1–1000	1/x	0.992	0.119	0.129

Table 1a. Calibration curve statistics for hydrocortisone phosphate and dexamethasone phosphate.

	Expected concentration (ng/mL)	Mean observed concentration (ng/mL)	Precision (%)	Accuracy (%)
LLOQ	5	5.03	9.39	100.67
LQC	10	10.20	6.86	102.00
MQC	75	73.90	10.53	98.53
HQC	750	743.63	7.13	99.15

Table 1b. Precision and accuracy statistics for hydrocortisone phosphate.

	Expected concentration (ng/mL)	Mean observed concentration (ng/mL)	Precision (%)	Accuracy (%)
LLOQ	1	0.97	5.97	96.67
LQC	10	11.10	4.68	111.00
MQC	75	80.17	4.39	106.89
HQC	750	694.73	7.49	92.63

Table 1c. Precision and accuracy statistics for dexamethasone phosphate.

Representative chromatograms at the low, mid, and high QC levels for hydrocortisone phosphate (Figure 2a) and dexamethasone phosphate (Figure 2b) show a linear, concentration dependent, increase in area counts for both analytes.

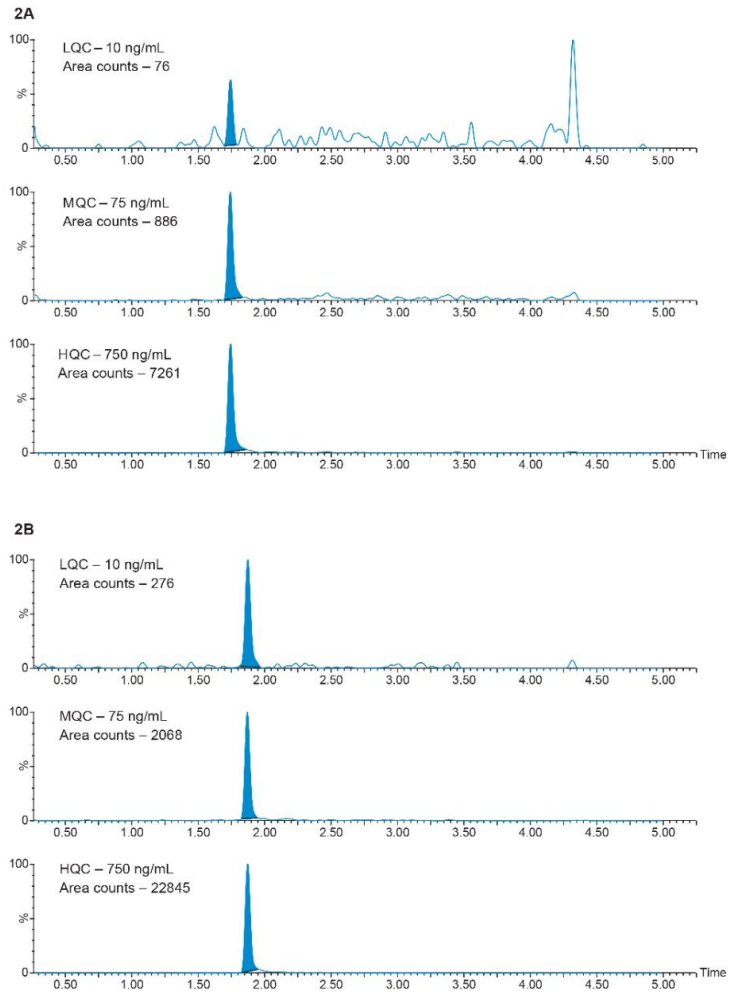


Figure 2a. Representative QC chromatograms for hydrocortisone phosphate on ACQUITY PREMIER System with MaxPeak HPS Column.

2b. Representative QC chromatograms for dexamethasone phosphate on ACQUITY PREMIER System with MaxPeak HPS Column.

Conclusion

Quantifying metal sensitive compounds on standard LC and column hardware suffers from poor peak shape and lack of reproducibility, resulting in an inability to achieve low LLOQ's.

Using ACQUITY PREMIER with MaxPeak HPS Columns for hydrocortisone phosphate and dexamethasone phosphate we achieved:

- Sensitivity improvements of 10x (hydrocortisone phosphate) and 7.5x (dexamethasone phosphate)
- Narrower peak widths and more symmetrical peak shapes allowing for more reproducible integration, especially at the lower concentration levels
- Assay precision of <11% (hydrocortisone phosphate) and <8% (dexamethasone phosphate) across LLOQ, LQC, MQC, and HQC levels

References

1. Dexamethasone in Severe COVID-19 Infection: A Case Series, M. E. Hassan ***et. al***., Respiratory Medicine Case Reports, Volume 31, 2020, 101205.

2. Gregor Cevc and Gabriele Blume, Hydrocortisone and Dexamethasone in very Deformable Drug Carriers Have Increased the Biological Potency, Prolonged Effect, and Reduced Therapeutic Dosage, ***Biochimica et Biophysica Acta (BBA) – Biomembranes*** Volume 1663, Issues 1–2, 27 May 2004, pages 61–73.

3. M. Lauber ***et al.***, Low Adsorption HPLC Columns Based on MaxPeak High Performance Surfaces, Waters Corporation, 720006930EN.

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