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APPLICATION NOTE 21686

Simplified, high-throughput separation of glucocorticoids

Author
Derek Hillbeck, Thermo Fisher Scientific, Runcorn, UK

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Application benefits

- Improved separation of closely related glucocorticoids with reduced method complexity
- High-throughput analysis possible through a reduced complexity, rapid, two-minute isocratic method
- Associated reduction in cost per sample through reduced mobile phase consumption and waste generation

Goal

To demonstrate how the use of alternate UHPLC stationary phase selectivities can simplify the separation of structurally similar analytes and facilitate method speed up

Introduction

Glucocorticoids are a group of hormones, both naturally occurring and synthetic. Structurally similar (Figure 1), they can be challenging to separate.

Previous application notes[1] have shown that a rapid separation, in under 4 minutes, can be achieved using a ternary mobile phase and a C18 column chemistry. This application note extends that work to a solid core C30 column.

ThermoFisher SCIENTIFIC

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[2]

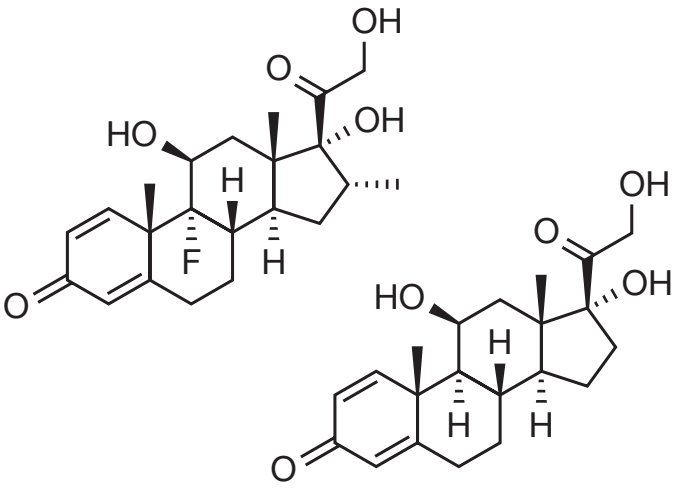

Figure 1. Structure of dexamethasone and prednisone.

The Vanquish Flex Quaternary UHPLC system has the benefit of SmartInject technology, and improvements in injection system hardware synchronization. This results in excellent retention time precision providing the user with greater data confidence during method development. The Vanquish Flex Quaternary system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with superior sensitivity and low peak dispersion due to small internal volume.

Experimental

Consumables and apparatus

- Accucore C30, 150 × 2.1 mm, 2.6 µm column (P/N 27826-152130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade methanol (P/N A456-212)
- Fisher Scientific LC-MS grade tetrahydrofuran (P/N 268290025)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The compounds used were representative of this class and were purchased from a reputable supplier: prednisone (1), cortisone (2), prednisolone (3), hydrocortisone (4), corticosterone (5), betamethasone (6), and dexamethasone (7). The number relates to their elution order and peak labelling in the subsequent chromatograms.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)
- Thermo Scientific Virtuoso Vial Identification System (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in water/acetonitrile (20:80, v/v) to produce 1 mg/mL primary solutions. A mixed working standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (80:20, v/v) at a concentration of 0.1 mg/mL.

Sample handling

Vial labeling was supported by the Virtuoso Vial Identification System.

UHPLC conditions (final method)

UHPLC column:	Accucore C30, 2.6 µm, 150 mm × 2.1 mm
Mobile phase A:	Water
Mobile phase B:	Methanol
Mobile phase C:	Tetrahydrofuran
On-pump mixing:	73% A/8% B/19% C
Flow rate:	0.6 mL/min
Column temperature:	60 °C, still air with eluent pre heating
Injection volume:	1 µL
Mixer:	50 µL capillary + 350 µL static in combination
UV detection:	240 nm

Note that prolonged use of significant levels of THF as a mobile phase component may require replacement of UHMW polyethylene piston seals with those more tolerant of this solvent, please refer to pump technical manual for further guidance.

Results and discussion

Using the method outlined in the previous application note[1], the C30 column was configured and the mixed standard analyzed with a flow rate of 0.4 mL/min and a column temperature of 50 °C (Figure 2a). This resulted in all the standards eluting within four minutes and presented a good starting point for further development. This experiment was also repeated with a column oven temperature of 60 °C (Figure 2b).

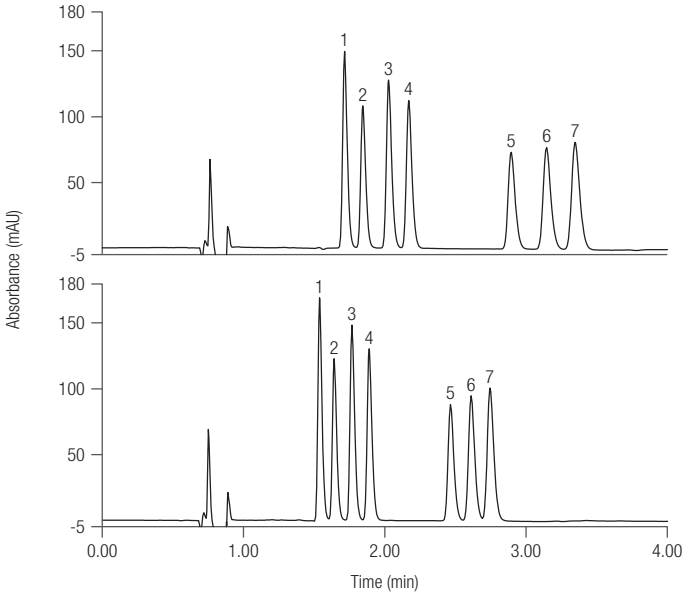

Figure 2. Chromatogram showing mixed standards analyzed at two different column temperatures a) 50 °C and b) 60 °C. (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) corticosterone, (6) betamethasone and (7) dexamethasone.

The elevated temperature shows the expected reduction in retention time and peak width. There were no overt selectivity changes; however, the peak resolution dropped below the usual USP guidance of greater than 2 but was still greater than the 1.5 value usually considered as baseline resolution, and well within the consistent integration capabilities of the Chromeleon software (Figure 3).

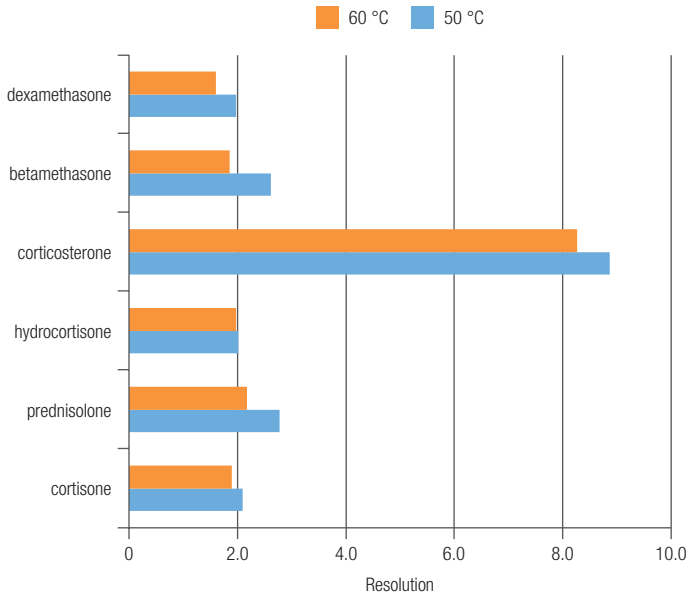

Figure 3. Comparison of resolution values at two column temperatures. Compounds are listed in reverse elution order.

Solid core HPLC columns are capable of equivalent efficiencies to much smaller fully porous particles. This allows high efficiency separations at a fraction of the cost in pressure. This, coupled with the solid-core columns ability to run at optimum (highest) efficiency over a much larger linear velocity range[1], enables chromatographers to significantly speed up their methods.

Maintaining the column temperature at 60 °C, the effect of flow rate was investigated by injecting the standards mixture at flow rates from 0.4 to 0.7 mL/min.

Figure 4 shows the efficiency of the separation normalized to that achieved with a 0.4 mL/min flow rate. There is a gradual reduction in efficiency as the flow rate departs from the optimal flow, but even at 0.6 mL/min it is still within 80% of the lower flow efficiency. Table 1 shows the effect of flow rate on resolution.

A flow rate of 0.6 mL/min allows the separation to maintain a minimum resolution value, between the last two peaks, of 1.5 with the column temperature at 60 °C. Twenty-four replicate injections were made under these conditions (Figure 5 and Table 2).

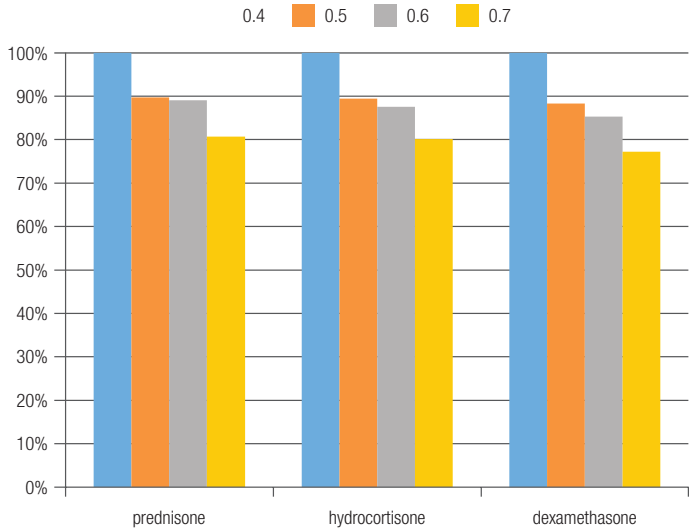

Figure 4. Relative efficiencies for three compounds at four different flow rates normalized to their values at 0.4 mL/min.

Table 1. Resolution values between peaks at different flow rates using USP criteria.

Flow rate (mL/min)	Cortisone	Prednisolone	Hydro-cortisone	Corti-costerone	Beta-methasone	Dexa-methasone
0.4	1.9	2.2	2.0	8.3	1.9	1.6
0.5	1.8	2.1	1.9	7.8	1.7	1.5
0.6	1.8	2.0	1.9	7.8	1.6	1.5
0.7	1.7	1.9	1.8	7.3	1.5	1.4

These results show that the method is very stable with differences between maximum and minimum retention times of less than 0.5 seconds across the replicate injections. This is much better than the 2% RSD criteria usually associated with legacy USP-type methods. The separation of all seven components is achieved within 2 minutes.

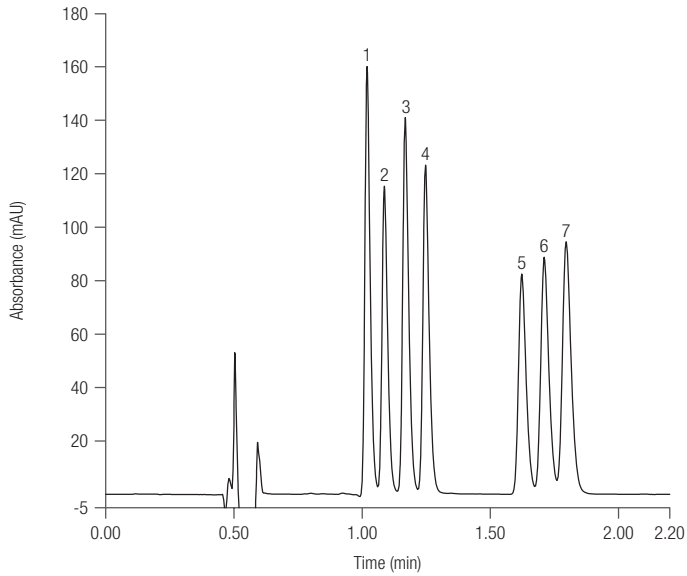

Figure 5. Overlay of 24 replicate injections of standards mixture at 60 °C and a flow rate of 0.6 mL/min.

Table 2. Peak summary data for 24 replicate injections of standards mixture at 60 °C and a flow rate of 0.6 mL/min.

	Parameter	Pred-nisone	Cort-isone	Pred-nisolone	Hydro-cortisone	Corti-costerone	Beta-methasone	Dexa-methasone
Minutes	Maximum RT	1.020	1.087	1.170	1.249	1.626	1.714	1.800
	Average RT	1.018	1.085	1.167	1.247	1.623	1.711	1.797
	Minimum RT	1.016	1.083	1.164	1.243	1.619	1.706	1.792
	Standard Deviation	0.001	0.001	0.002	0.002	0.002	0.002	0.002
	RSD%	0.10%	0.11%	0.13%	0.14%	0.11%	0.11%	0.10%

When compared to typical legacy methods (20 min, 1.2 mL/min), this method development has provided a 10-fold increase in sample throughput and a 20-fold reduction in mobile phase consumption / waste generation (Figure 6).

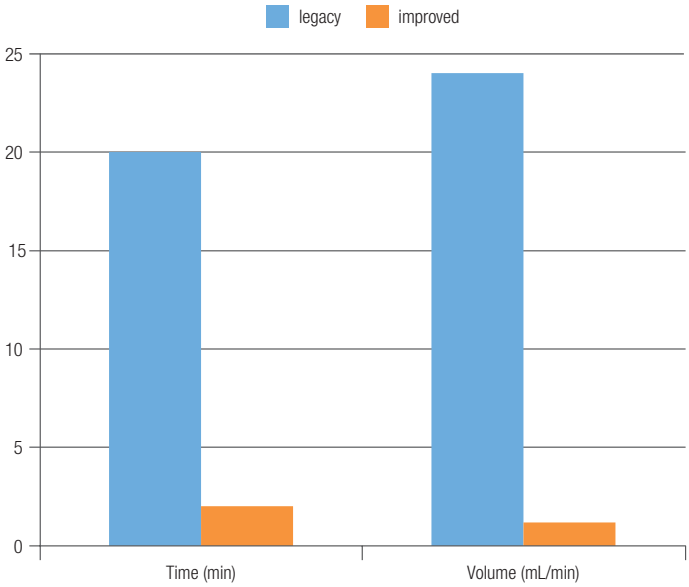

Figure 6. Relative differences in method time and mobile phase consumption between typical legacy methods and this improved method.

Conclusions

A high-throughput application has been demonstrated showing the separation of seven closely related glucocorticoids in under two minutes with a simplified UHPLC method that demonstrates the following:

- Critical pair resolution maintained in under two minutes with a simple isocratic method
- Ten-fold increase in method throughput when compared to typical legacy methods on 250 × 4.6 mm columns
- Associated reduction in cost per sample through reduced mobile phase consumption and waste generation

References

1. Rapid analysis of nine corticosteroids using a Thermo Scientific Acclaim RSLC C18 column https://appslab.thermofisher.com/App/205/rapid-analysis-nine-corticosteroids-using-a-thermo-scientific-acclaim-rslc-c18-column
2. Accucore HPLC Columns Technical Guide https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf

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