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Technical Report

Systematic Method Development:

The Selective Extraction and HPLC Analysis of Corticosteroids from Urine Using Discovery DSC-CN SPE

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C18 has become the most commonly used phase chemistry for reversed-phase SPE due to its broad affinity for a wide range of compounds in aqueous solutions. However, when dealing with contaminant rich samples, their broad selectivity can lead to the co-retention and elution of endogenous matrix interferences. As a result, high background, misleading responses, and system failure (due to high backpressure) can result during chromatographic analysis. One solution is to use a less hydrophobic and more selective SPE phase chemistry such as a Discovery DSC-CN .

DSC-CN contains a monomerically bonded cyanopropyl chain with approximately half the carbon content of most C18 phases. Its less retentive nature allows for the rapid release of hydrophobic molecules and can often improve selectivity by discriminating between the analytes of interest and endogenous sample interferences. Using the systematic method development approach illustrated in this report, extraction methods for both conventional C18 and CN SPE phases were developed for the recovery and HPLC analysis of four steroidal compounds from urine. Initial experimentation using standards revealed that the steroidal compounds were retained more strongly on the C18 phase when compared to the CN phase; however, retention was not great enough to employ stronger wash solvents without premature analyte elution. HPLC analysis of the C18 urine samples leads to HPLC system failure (due to high back pressure) early in the run sequence. In contrast, the CN SPE phase provided significantly cleaner chromatograms with recovery and RSD values averaging at 100.6 ± 1.9% for the 0.5 & 1.0 µg/mL spike levels tested.

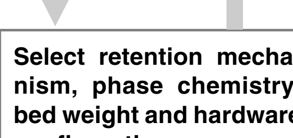
SPE Method Development Process Overview

Determine Sample Prep Objectives:

- What level of recovery is required?
- What level of interference removal is required for analysis?
- Is concentration required for optimal analysis?
- In what solvent should the analyte(s) be in for optimal analysis?
- What resources are available for method development and routine analysis?

Consider Sample Characteristics (Matrix & Analytes):

- What is the sample matrix and is it more polar or non-polar?
- What is the sample volume?
- What interferences are endogenous to the sample?
- What functional groups may influence the analytes' solubility, polarity, ionization state (pKa), etc.?



Select retention mechanism, phase chemistry, bed weight and hardware configuration:

- What phase chemistry, bed weight and hardware configuration best meets the predefined sample prep objectives for the sample characteristics?

Develop Analytical Method- Load Optimization & Wash/Elution Profile:

Experimentation:

- Develop analysis method (LC-UV, GC-MS, etc.)
- Using standards and buffered/organically modified solutions, identify and determine the effects of key variables (pH, organic strength, etc.) that influence analyte retention and elution.

Evaluation:

- Perform mass-balance analysis on collected eluates for each step of the extraction procedure
- Determine analyte behavior on sorbent in response to changing extraction conditions

Incorporate Sample Matrix:

- Determine preliminary, optimized method and incorporate sample matrix.
- Make determinations of sample matrix effect on sample recovery, cleanliness, reproducibility, and analytical (LC or GC) resolution
- Refine optimum conditions to account for sample matrix effects

Validate Method

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Determine Sample Prep Objectives

The objective of this study was to develop a simple extraction protocol that reproducibly achieves ≥ 90% recovery for four cortcosteroids (hydrocortisone, prednisilone, prednisone, and corticosterone) from human urine for HPLC-UV quantitation. Endogenous urine interferences should be substantially removed to simplify subsequent HPLC resolution, reduce analytical runtime (≤ 12 min.), prolong HPLC column life, minimize misleading background responses, and achieve detection/quantitation limits of ≤ 0.5µg/mL serum. The final sample matrix should also be a buffered solvent compatible with the HPLC mobile phase.

Consider the Sample Matrix

The sample matrix used in this study consists of 0.5mL samples of human urine. Human urine fluctuates in its composition daily and contains many contaminants that may co-retain with the analytes of interest under the reversed-phase retention mechanism. Endogenous contaminants that should be considered include nitrogenous metabolic end products (urea, uric acid, ammonia, and creatinine), sugars, ions, and electrolytes.

Because of the aqueous nature of the sample matrix, reversed-phase or ion-exchange are potential retention mechanism choices.

Consider the Analyses of Interest

Corticosteroids are widely used to treat a variety of inflammatory and non-inflammatory conditions including septic shock, adrenal insufficiency, congenital adrenal hyperplasia, and allergic reactions (Fig. 1). These moderately polar compounds contain a basic glucocorticoid structure that is chemically modified to produce different therapeutic agents. Each of these compounds contain a steroid nucleus consisting of four fused rings, three with six carbons and one with five. This planar and relatively rigid configuration offers an excellent hydrophobic footprint for potential reversed phase interaction (Fig. 2).

Figure 1: Structures of Four Corticosteroids

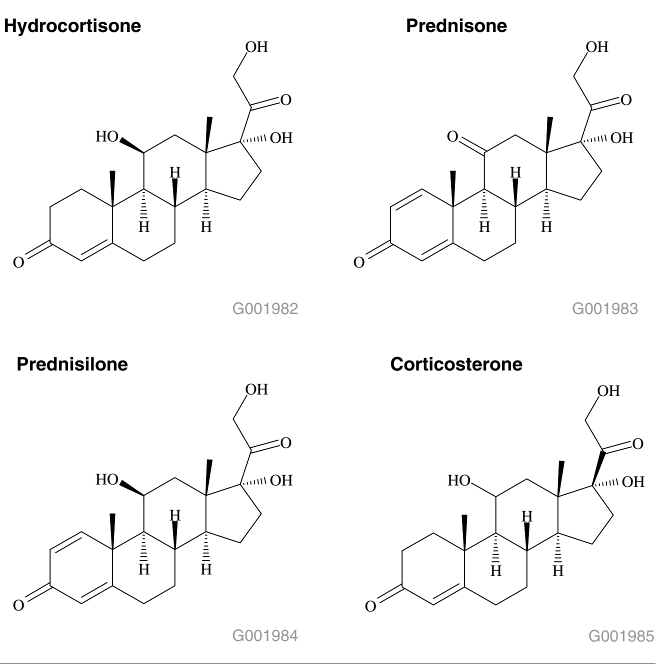
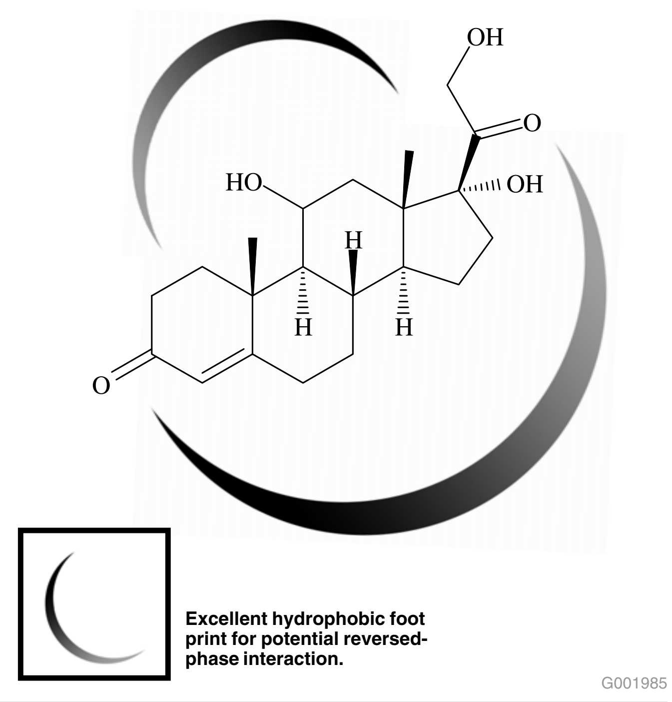


Figure 2: Corticosteroid Functional Groups that Influence Retention and Selectivity



Sample Prep Worksheet for Personal Use:

Determine Sample Prep Objectives:

What is your analytical technique (e.g., LC-UV, LC-MS, etc.)? ______________________________

____

What is your optimal analytical run time (e.g., 2-5min.)? ______________________________

____

What level of recovery is required to meet LOD/LOQ? ______________________________

____

How do you plan to quantitate (against external standards and/or internal standards)? ______________________________

____

What is your desired RSD (Inter- and Intra-day accuracy and precision)? ______________________________

____

What level of interference removal is required for analysis? ______________________________

____

What sample pretreatment steps may be required (dilution, clarification, pH adjustment, etc.?) ______________________________

____

Is concentration required for optimal analysis? If yes, what is the desired elution and/or reconstitution volume? ______________________________

____

In what solvent should the analyte(s) be in for optimal analysis? ______________________________

____

What resources are available for method development and routine analysis? ______________________________

____

Consider Sample Characteristics (matrix and analytes):

What is the sample matrix? ______________________________

____

Is the sample matrix more polar or non-polar? ______________________________

____

What is the sample volume? ______________________________

____

What key interferences are endogenous to the sample? ______________________________

____

What are the analyte(s) of interest? ______________________________

____

What functional groups may influence the analytes' solubility, polarity, ionization states (pKa), etc.? ______________________________

____

SPE Phase and Hardware Selection

Because the sample volume is 0.5mL, either a 1mL cartridge or a 96-well platform would be suitable for this application. The smaller bed weights (50-100mg for 1mL tubes; 25-100mg for 96-well) allow for small elution volumes. Smaller elution volumes provide higher analyte concentrations for increased sensitivity during analysis. Smaller elution volumes also allow for easier evaporation and buffer reconstitution prior to analysis.

The aqueous nature of the sample matrix and hydrophobic character of the analytes offers an excellent opportunity for reversed-phase retention. There are quite a number of reversed-phase sorbents available. Each of which offers varying degrees of hydrophobicity and selectivity that could be employed for this application. As stated earlier, urine contains many interfering components that can be potentially co-extracted with the analytes of interest during SPE sample preparation. Therefore, one of the main sample prep objectives for this application was to develop a procedure that selectively retains, elutes, and differentiates analytes of interest from urine contaminants. Such determinations were made by simultaneously evaluating two different phases of varying selectivity.

In this study, SPE method development was conducted on a conventional C18 SPE 96-Well Plate (100mg/well) and DSC-CN SPE 96-Well Plate (100mg/well) (Fig. 3).

Figure 3: Supelco Discovery DSC-CN SPE 96-Well Plate and PlatePrep Vacuum Manifold

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Experimentation

In SPE, selectivity is defined as the ability of the sorbent and extraction method to discriminate between the analyte(s) of interest and endogenous interferences within the sample matrix. In reversed-phase SPE, selectivity is typically governed by two main variables: pH and percent organic modifier. pH manipulation is typically used to control the ionization states of both analyte and sorbent functional groups. However, in this study, pH variation was not tested due to the lack of ionizable functional groups inherent with corticosteroids tested in this report.

By employing two or three experiments using standard solutions without the sample matrix, the researcher can systematically vary percent organic modifier conditions for the two phases tested. Through the use of standards, one can track the location of the analytes through the SPE process. By understanding how the analyte(s) interact with the sorbents under specific conditions, it allows for a systematic approach to finding the optimal sample prep conditions with greater efficiency and a higher degree of confidence.

Load Optimization

Standards containing 5.0µg/mL of each of the four corticosteroids were prepared in DI H_2O. 1mL of each of the standard test mix was loaded on to both conventional C18 and DSC-CN wells previously conditioned and equilibrated with 1mL methanol and 1mL DI H_2O. The load eluent was collected and analyzed via HPLC-UV.

Note that although target detection levels for the analysis was 0.5-1.0 ug/mL urine, the load concentration was increased to 5.0 ug/mL. This was to provide adequate signal response for detecting small analyte breakthough percentages. Also note that other pH load conditions were avoided due to the neutral (no ionizable functional groups) nature of the analytes.

Load Optimization Evaluation

A lack of analyte presence in the load eluate was found for both phases tested indicating adequate retention for both conventional C18 and DSC-CN phase chemistries.

Wash/Elution Profile

21 of the remaining conventional C18 and DSC-CN wells were conditioned and equilibrated with 1mL methanol and DI H_2O, and loaded with 1mL of the 5.0µg/mL cortcosteroid test mix. The samples were drawn through under vacuum, and the respective wells were washed/eluted with 1mL of a test solvent ranging from 0, 5, 10, 20, 40, 60, 80, and 100% methanol in DI H_2O. The wash/elution eluant was collected and analyzed via HPLC-UV.

The purpose of this experiment was to systematically track the retention and elution of the analytes under various organic modifier conditions.

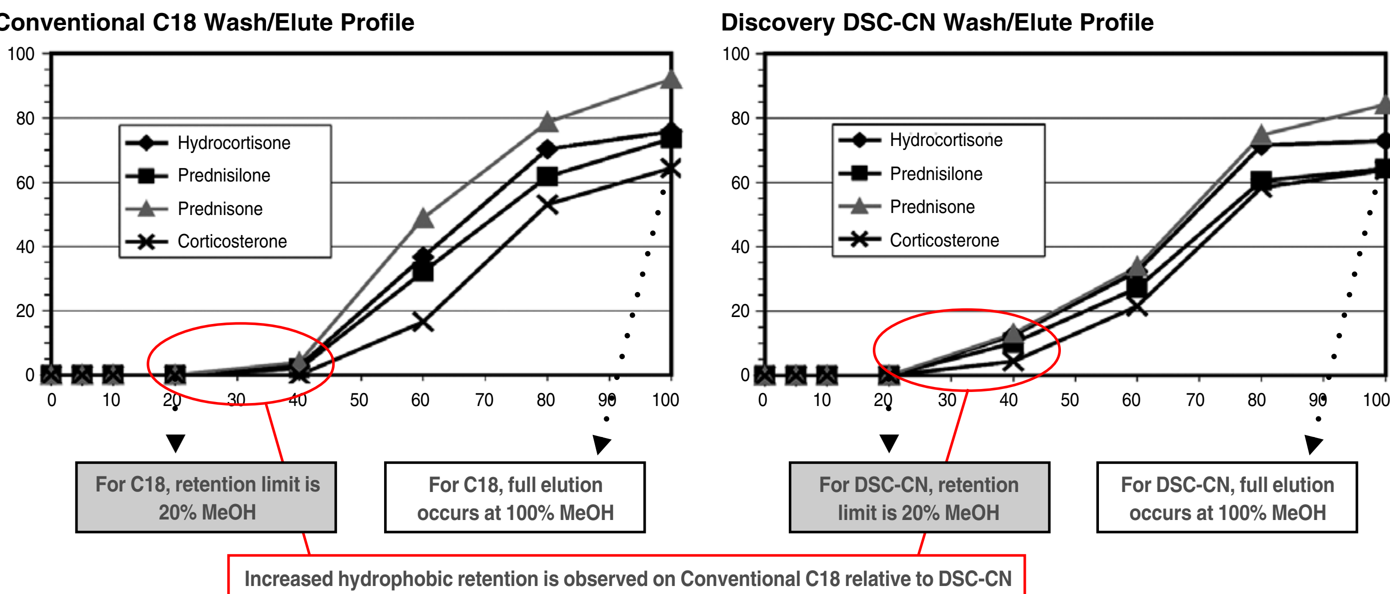
Evaluation

A graphic representation was used to measure organic strength vs. peak area response for each of the phase chemistries tested providing a wash/elution profile (Fig. 4).

Although the corticosteroids behaved similarly on both of the phases tested, differences in retention between the Conventional C18 and DSC-CN phase chemistries were particularly evident in the 20-40% methanol range of the wash/elution profile. Conventional C18, with its greater carbon load and alkyl chain length, offered increased retention for moderately polar hydrophobic molecules relative to DSC-CN.

Using the parameters suggested by the wash/elute profile, up to 20% methanol can be employed as a potential wash solvent before premature analyte elution is observed. 100% methanol is required to completely elute the analytes from both phase chemistries.

Figure 4: Results of the Wash/Elution Profile for 4 Corticosteroids on Conventional C18 and DSC-CN SPE Phases



Incorporation of Sample Matrix and Comparison Against Conventional C18

By profiling the major parameters affecting analyte retention and elution, application specific guidelines have been established for defining, optimizing, and troubleshooting an extraction method. In this experiment, human urine sample matrix was incorporated into a method defined from data generated from the precursory load optimization and wash/elute profile studies (Table 1). The method was tested on both the DSC-CN and conventional C18 phase chemistries and compared via HPLC analysis. For most applications, recovery values observed for real-matrix based solutions will parallel values obtained with standard solutions.

Table 1. Systematically Developed Method for Extracting Corticosteroids From Human Urine Using Conventional C18 and DSC-CN SPE 96-Well Plate

SPE: Conventional C18 96-Well Plate (100mg/well)
Discovery DSC-CN SPE 96-Well Plate (100mg/well)

1. Condition and equilibrate each well with 1mL methanol and 1mL DI H_2O
2. Load 0.5 & 1.0µg/mL corticosteroids spiked in human urine diluted in DI H_2O (1:1, v/v); n=3
3. Wash with 1mL 20% methanol in DI H_2O
4. Elute with 1mL 100% methanol
5. Evaporate eluate with nitrogen purge (30°C; ~10 min), and reconstitute in 200µL HPLC mobile phase

Results and Discussion

Four corticosteroids were extracted from human urine using both Conventional C18 and DSC-CN SPE 96-well plates. The method employed for each phase chemistry was defined from results obtained from preliminary load optimization and wash/elute profile studies. The resulting eluates were analyzed via HPLC-UV, and absolute recoveries and relative standard deviations were calculated against external standards (mobile phase sample matrix) not subjected to sample preparation.

Cleaner Extracts on DSC-CN SPE Phase through Improved Selectivity

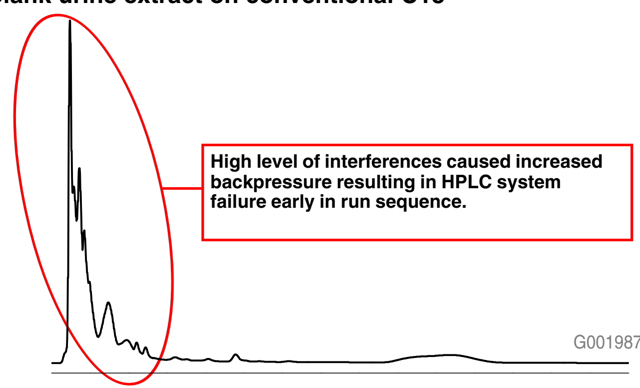
Prior experimentation revealed that although retention profiles of the corticosteroid standards were similar between the conventional C18 and DSC-CN phases, the steroidal compounds were retained more strongly on the conventional C18 than on DSC-CN. Retention on conventional C18, however, was not great enough to employ stronger wash solvents (> 20% methanol) without premature analyte elution. Upon incorporating spiked urine samples into the extraction protocols, the final conventional SPE eluate carried a yellow tint signifying insufficient removal of endogenous urine components. Subsequent eluate analysis of the C18 SPE urine extracts resulted in HPLC system (due to high back pressure and column fouling) early in the run sequence.

In contrast, eluate extracts generated on the DSC-CN SPE phase using the same extraction protocol were significantly cleaner (Fig. 5). As a result, subsequent HPLC analysis was feasible resulting in chromatograms free of interfering components that can result in column fouling (high back pressure), high background, and misleading peak responses (Fig. 6).

Figure 5. Example chromatograms of Blank Urine Extracts Generated from Systematically Developed Method Using Both Conventional C18 & Discovery DSC-CN SPE 96-Well Plate

Column: Discovery HS F5, 5cm x 4.6mm ID, 3µm particles
Mobile Phase: MeOH: DI H_2O (40:60)
Flow Rate: 1.5mL/min
Temp.: 35°C
Det.: UV, 240nm
Inj.: 5µL

Blank urine extract on conventional C18



Blank urine extract on Discovery DSC-CN

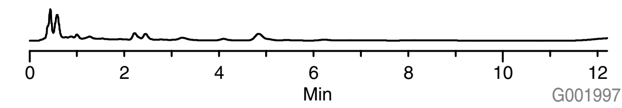
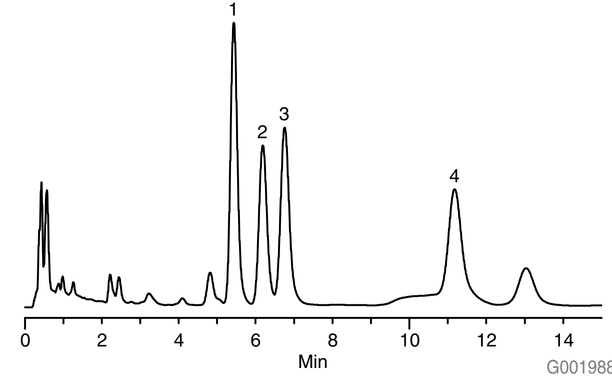
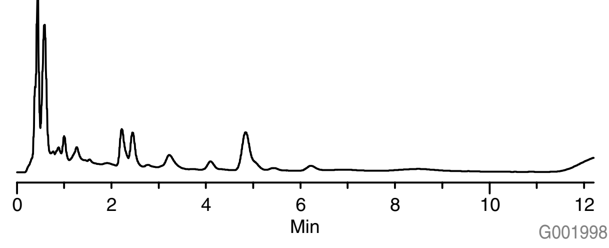


Figure 6. Example Chromatograms of Blank and Spiked Urine Extracts Generated from Systematically Developed Method Using Discovery DSC-CN SPE 96-Well Plate

1µg/mL spiked urine extract on Discovery DSC-CN



Blank urine extract on Discovery DSC-CN



Excellent Recovery and Reproducibility on DSC-CN

On the Discovery DSC-CN SPE 96-Well Plate using the systematically developed method, average absolute recovery (0.5 & 1.0µg/mL spike levels) and RSDs (Table 2) for the four corticosteroids, hydrocortisone, prednisilone, prednisone, and corticosterone, were 109.6 ± 1.6%, 99.6 ± 1.1%, 95.8 ± 1.4%, and 97.6 ± 3.4, respectively. On the conventional C18 SPE phase, recovery values were not obtained due to repeated HPLC system failure. System failure was caused by increased column back pressure resulting from insufficient clean-up of the spiked urine samples.

Table 2. Efficiency of Absolute Recovery of 0.5 and 1.0µg/mL Corticosteroids in Human Urine on Systematically Develop Method Using Discovery DSC-CN SPE 96-Well Plate

	%Recovery ± RSD (n=3)	
Compound	**0.5µg/mL spike level**	**1.0µg/mL spike level**
1. Hydrocortisone	123.3±1.4%	95.9±1.7%
2. Prednisilone	107.2±1.1%	91.9±1.1%
3. Prednisone	103.2±1.0%	88.4±1.8%
4. Corticosterone	102.0±1.2%	93.1±5.6%

In SPE, selectivity is defined as the sorbent/extraction method's ability to discriminate between the analytes of interest and endogenous interferences within the original sample. By evaluating phases of varying selectivity and using standards to test key variables influencing analyte retention and elution, one can strategically manipulate and make quick adjustments to the extraction method to meet the sample prep objectives during the life of the assay

Notes:

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