Extraction of Corticosteroids from Urine:- Method Optimization using EVOLUTE ABN prior to LC-MS/MS Analysis

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Introduction

Naturally occurring corticosteroids are steroid hormones produced in the adrenal cortex and perform many functions within the body. Due to their widespread action many synthetic corticosteroids based on the hydrocortisone (cortisol) structure have been produced. Some of their uses involve treatment of various forms of arthritis, dermatitis, hepatitis and ulcerative colitis. They can be administered orally, injected into veins or muscle, nasal sprays and drops or used as topical creams. Reliable screening methods for these drugs are important as they are classed as 'banned substances' by many anti-doping agencies and regulated for use in meat producing animals.

This poster will demonstrate the use of EVOLUTE ABN™ for the extraction of corticosteroids from urine, specifically investigating the role of pH during extraction and the effect of optimized wash protocols on analyte recovery. The first experiment investigated the role of pH on analyte extraction by modifying a generic method to four different pH values between pH 2.7 and pH 7. The second experiment then investigated the effect of interference wash solvent composition on analyte recoveries.

Experimental

Reagents

All analytes and chemicals were purchased from Sigma Chemical Co. (Poole, UK). Blank human urine was obtained from a pre-screened healthy human volunteer. All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

Experiment 1:- SPE pH Recovery Investigation.
Solid Phase Extraction Procedure
SPE was performed on blank human urine spiked at 20 ng/mL concentrations using the 50 mg/3 mL column configuration.

Sample Pre-treatment: Human urine (1 mL) was pre-treated (1:1, v/v) with various buffers. The buffers investigated in this study were: 1% (v/v) aqueous formic acid, 0.1% (v/v) aqueous formic acid, 20 mM ammonium acetate buffer at pH 5 and H_2O.

Column Conditioning: Methanol (3 mL)
Column Equilibration: Buffers used in pre-treatment (3 mL). The equilibration buffer varied with the sample loading conditions. For the 1% and 0.1% formic acid pre-treated urine the equilibration buffer was 0.1% formic acid.
Sample Application: Pre-treated sample (2 mL)
Interference Elution: Water (3 mL)
Analyte elution: Methanol (3 mL)

Experiment 2:- Interference Wash Investigation.
Blank human urine was extracted as per the 20 mM ammonium acetate pH 5 method. The interference wash step was modified from 100% H_2O with up to 20% MeOH and the effect on analyte recovery observed.

Post Extraction: Extracts were evaporated to dryness and reconstituted in 80:20 (v/v) H_2O/MeOH (500 µL) for subsequent LC-MS/MS analysis.

HPLC Conditons
Instrument: Waters 2795 Liquid Handling System (Waters Assoc., Milford, MA, USA).
Column: Zorbax Eclipse XDB C18 3.5 µm analytical column (100 x 2.1 mm id, 3.5 µm) (Agilent Technologies, Berkshire, UK).
Guard Column: C8 guard column (Agilent Technologies, Berkshire, UK).
Mobile Phase: 0.1% formic acid aq and MeCN (acetonitrile) at a flow rate of 0.25 mL/min.
Gradient: See **Table 1** below.

Table 1. *HPLC Gradient Conditions*

Time	0.1% Formic acid aq (%)	MeCN (%)
0	80	20
15	60	40
15.1	10	90
19	10	90
19.1	80	20

Injection Volume: 10 µL
Temperature: Ambient

Mass Spectrometry
Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the multiple reaction monitoring mode (MRM).

Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.3 x 10^{-3} mbar

The base peak in each compound spectrum was attributed to the protonated molecular ion $[M+H]^+$ and were subsequently used as the precursor ions in the resulting MRM transitions.

Results

pH control of the urine was achieved using 1:1 (v/v) either: 1% formic acid aq, 0.1% formic acid aq, 20 mM NH_4OAc pH5 buffer or H_2O. The pH values obtained when the buffers were mixed with urine are shown in **Table 2**.

Table 2. *pH conditions obtained when urine/buffer mixed 1:1 (v/v)*

Urine /Buffer 1:1 (v/v)	pH Value
1% Formic acid: Urine	2.69
0.1% Formic acid: Urine	4.01
20mM NH_4OAc, pH5: Urine	6.02
H_2O: Urine	6.99

Figure 1. shows the bar chart of recoveries comparing the various pH generic methods. Recoveries greater than 90% and RSD's below 10% were seen for the majority of analytes with all extraction protocols.

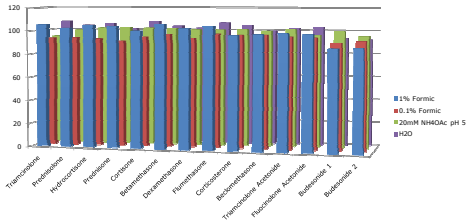

Figure 1. *Corticosteroid recoveries comparing various pH generic methods*

The 20mM ammonium acetate pH 5 method was selected as the method to proceed for the interference wash investigation. **Figure 2**. shows the recoveries observed when modifying the interference wash from 100% H_2O with up to 20% MeOH. The results show that increasing the MeOH content up to 20% in the wash step does not affect analyte recoveries. Full recoveries and corresponding RSD's are also shown in **Table 2**.

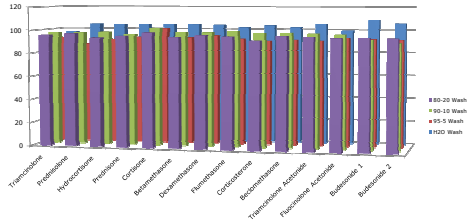

Figure 2. *Corticosteroid recoveries using a 20mM ammonium acetate pH 5 method with various interference wash solvent compositions*

Table 2. *Corticosteroid recoveries (% RSD) using a 20mM ammonium acetate pH 5 method and various interference wash solvent compositions*

Analyte	% Recovery (%RSD)			
	H_2O Wash	95/5 H_2O/MeOH Wash	90/10 H_2O/MeOH Wash	80/20 H_2O/MeOH Wash
Triamcinolone	97 (7)	92 (7)	97 (6)	95 (6)
Prednisolone	103 (9)	86 (3)	97 (6)	97 (4)
Hydrocortisone	103 (6)	91 (5)	98 (3)	93 (6)
Prednisone	103 (3)	93 (5)	96 (7)	95 (6)
Cortisone	103 (6)	101 (4)	102 (5)	98 (6)
Betamethasone	103 (7)	92 (8)	98 (6)	94 (6)
Dexamethasone	103 (7)	94 (4)	98 (4)	96 (7)
Flumethasone	101 (3)	92 (6)	99 (5)	95 (5)
Corticosterone	103 (6)	90 (4)	98 (7)	92 (7)
Beclomethasone	102 (7)	91 (5)	98 (5)	96 (5)
Triamcinolone Acetonide	104 (8)	91 (5)	97 (6)	95 (8)
Fluocinolone Acetonide	99 (8)	93 (4)	97 (7)	95 (5)
Budesonide 1	108 (11)	92 (7)	92 (13)	95 (8)
Budesonide 2	105 (11)	92 (6)	90 (13)	95 (8)

Conclusions

- High reproducible recoveries were observed using the four different pH extraction methods.
- The wash solvent investigation showed that it was possible to increase the MeOH content to 20% without observing any adverse effects on recoveries.
- This poster shows the application of EVOLUTE ABN to the extraction of a wide set of corticosteroids from urine delivering recoveries in excess of 90% with corresponding RSD's below 10% for a variety of method combinations.

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