Extraction of Corticosteroids using 96-well Supported Liquid Extraction (SLE) and 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.

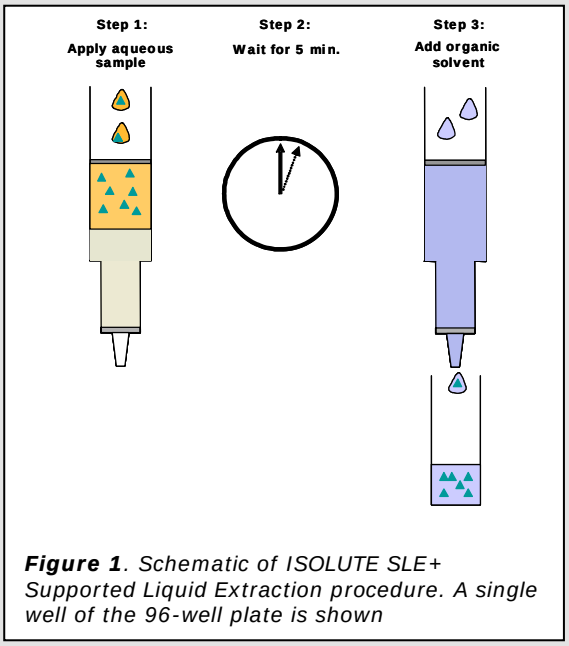


***Figure 1**. Schematic of ISOLUTE SLE+ Supported Liquid Extraction procedure. A single well of the 96-well plate is shown*

Supported Liquid Extraction (SLE) is a 96-well based technique that is analogous to traditional liquid-liquid extraction (LLE). The extraction interface occurs between the buffered sample absorbed onto an inert solid support and a water immiscible solvent. This provides excellent extraction efficiency while alleviating many of the liquid handling and emulsion formation issues associated with traditional LLE.

This poster will show the application of Supported Liquid Extraction using ISOLUTE SLE+ Plates for corticosteroids in human plasma using a variety of extraction solvents and loading pHs.

Experimental Procedure

Reagents

All analytes (see **Table 3**, **page 3**) and formic acid were purchased from Sigma Chemical Co. (Poole, UK). Blank human plasma was obtained through the Welsh Blood Service (Pontyclun, UK). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

Supported Liquid Extraction Procedure

Plate: ISOLUTE SLE+ Supported Liquid Extraction Plate 200 mg, part number 820-0200-P01
Sample: Blank human plasma (100 µL) was spiked with the corticosteroids at 200 ng/mL. The plasma was then diluted 1:1 v/v with either 0.1% (v/v) formic acid aq or H_2O prior to loading. This sample dilution results in approximate loading pH's of 6.1 and 8.0, respectively.
Sample Application: The pre-treated plasma was loaded onto the plate, a pulse of vacuum applied to initiate flow and the samples left to absorb for 5 minutes.

Analyte Extraction: Addition of 1 mL of various water immiscible extraction solvents. The extraction solvents evaluated were 90:10 (v/v) DCM/IPA, EtOAc, DCM and MTBE.
Post Extraction: The extract was evaporated to dryness and the analytes reconstituted in 500 µL of 80:20 (v/v) H_2O/MeOH prior to 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.
Injection Volume: 10 µL
Temperature: Ambient

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

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

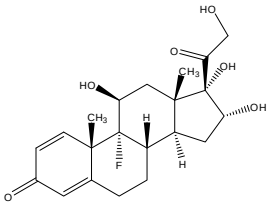
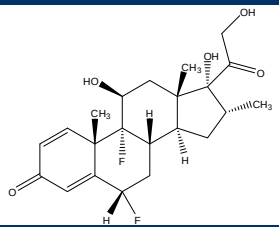
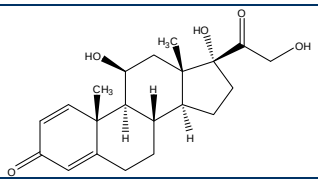
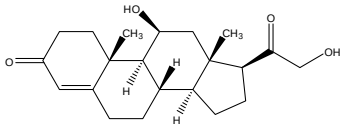
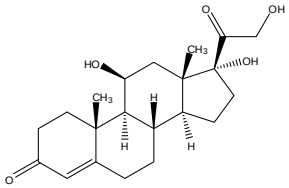
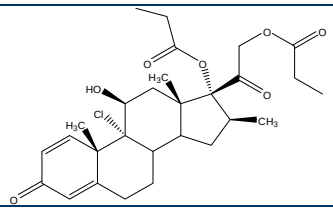
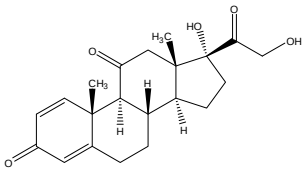
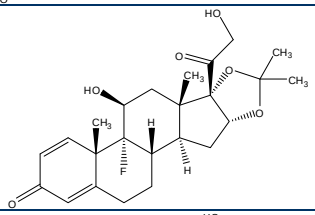
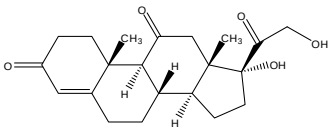
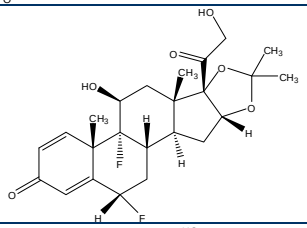
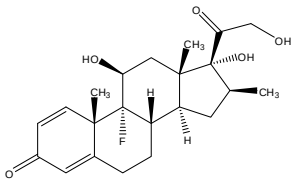
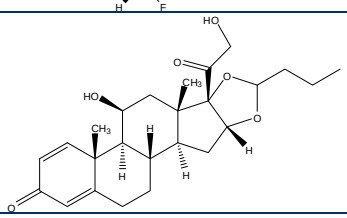
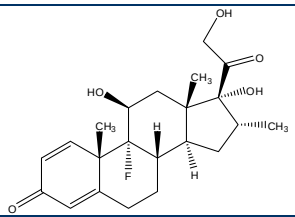
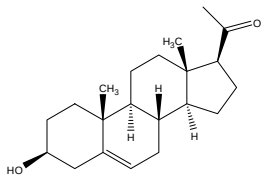
The base peak in each compound spectrum apart from 5-pregnen-3β-ol-20-one was attributed to the protonated molecular ion $[M+H]^+$ and were subsequently used as the precursor ions in the resulting MRM transitions. 5-pregnen-3β-ol-20-one showed acidic/thermal degradation of the protonated molecular ion to $[M+H-H_2O]^+$, as a result this ion was used as the precursor for the MRM transition. Full MRM transitions and ionization conditions are given in **Table 2** below.

Table 2*. Quattro Ultima Pt mass spectrometer parameters.*

Scan Function	Analyte	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
1	Triamcinolone	395.3 > 375.3	35	9
	Prednisolone	361.3 > 343.3	35	9
	Hydrocortisone	363.3 > 121.2	35	20
	Prednisone	359.3 > 341.3	35	9
	Cortisone	361.3 > 163.2	35	22
2	Betamethasone	393.3 > 373.3	35	8
	Dexamethasone	393.3 > 373.3	35	8
	Flumethasone	411.3 > 253.2	35	14
	Corticosterone	347.3 > 329.3	35	14
	Beclomethasone	409.3 > 391.3	35	11
	Triamcinolone Acetonide	435.3 > 415.3	35	9

	Fluocinolone Acetonide	453.3 > 413.3	35	9
3	Budesonide Structural Isomer 1	431.4 > 413.4	35	10
	Budesonide Structural Isomer 2	431.4 > 413.4	35	10
	5-pregnen-3β-ol-20-one	299.4 > 281.4	35	11

***Table 3**. Corticosteroid structures.*

Analyte	Structure	Analyte	Structure
Triamcinolone		Flumethasone	
Prednisolone		Corticosterone	
Hydrocortisone		Beclomethasone	
Prednisone		Triamcinolone acetonide	
Cortisone		Fluocinolone Acetonide	
Betamethasone		Budesonide*	
Dexamethasone		5-Pregnen-3β-ol-20-one	

** Two structural isomers observed.*

Results

A comprehensive range of corticosteroids was selected to show the applicability of Supported Liquid Extraction (SLE) for this class of steroidal hormones. These small steroids are neutral molecules so two simple plasma pre-treatment strategies were selected to give a loading pH around neutrality.

The first plasma pre-treatment involved 1:1 (v/v) plasma/0.1% formic acid aq to give an approximate loading pH of 6.1; the second was 1:1 (v/v) plasma/H_2O providing a loading pH of 8.0. Four extraction solvents were selected possessing different extraction characteristics and polarities.

Figure 2 shows the recoveries of all analytes using the various extraction solvents with a loading pH of 6.1. **Figure 3, page5** shows the recoveries using a loading pH of 8.0. **Table 4**, **page 5** provides a summary of all recoveries and RSDs. Recoveries greater than 80% and RSD's below 10% were seen for the majority of analytes with all extraction protocols.

Triamcinolone showed low recoveries when extracting with DCM, however, modifying the extraction solvent with IPA increased the polarity and recoveries were substantially improved. Recoveries for all analytes were very similar whether the loading pH was at 6.1 or 8.0 with equivalent extraction solvents.

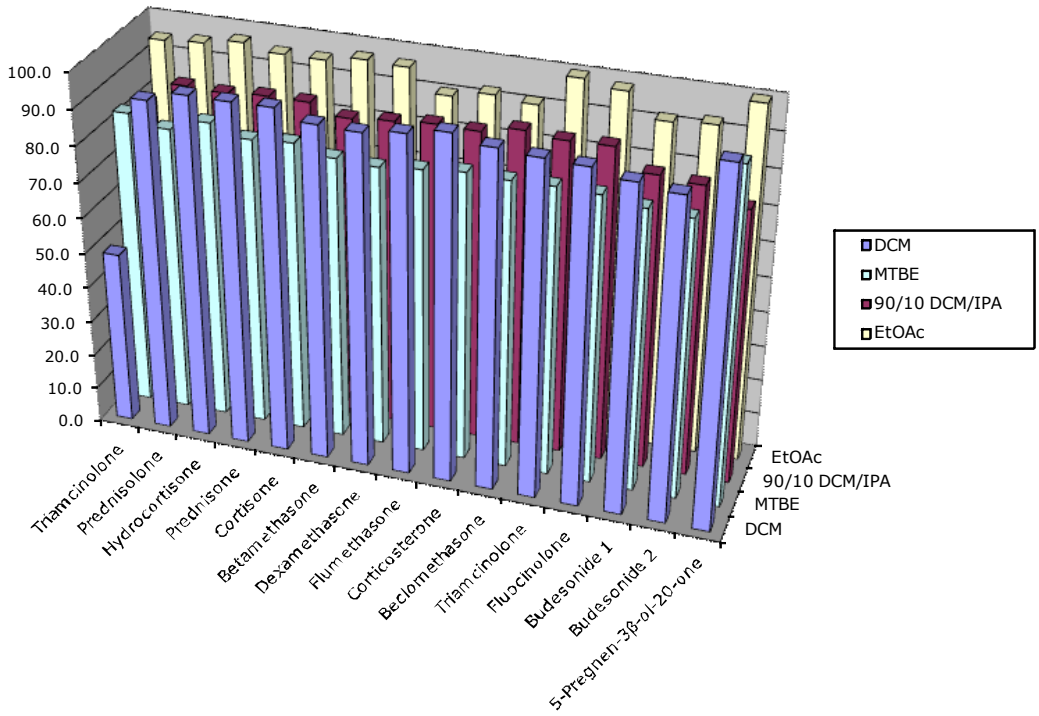


***Figure 2**. Corticosteroid recoveries using a 1:1 (v/v) plasma/0.1% formic acid aq load with various extraction solvents.*

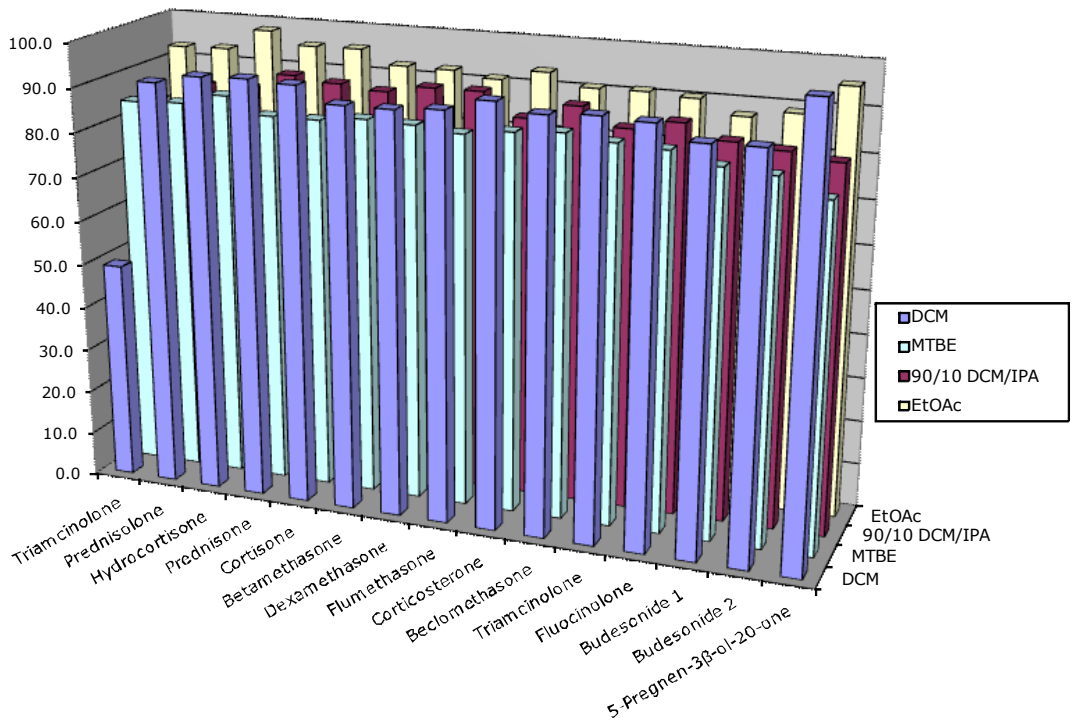


Figure 3. *Corticosteroid recoveries using a 1:1 (v/v) plasma/H_2O load with various extraction solvents.*

Table 4. *Summary of recoveries (n=7, with RSD's in parenthesis) for all extraction conditions.*

	1:1 Plasma/0.1% formic acid pH 6.1 % Recovery (RSD)				1:1 Plasma/H_2O pH 8.0 % Recovery (RSD)			
Analyte	**DCM**	**MTBE**	**90:10**	**EtOAc**	**DCM**	**MTBE**	**90:10**	**EtOAc**
Triamcinolone	49 (5)	84 (4)	82 (3)	95 (2)	50 (6)	85 (3)	81 (2)	93 (4)
Prednisolone	94 (3)	81 (3)	88 (3)	96 (3)	92 (1)	85 (2)	87 (2)	93 (1)
Hydrocortisone	97 (2)	85 (3)	88 (3)	97 (3)	94 (2)	88 (3)	87 (2)	98 (2)
Prednisone	97 (2)	82 (4)	89 (3)	96 (3)	94 (1)	84 (2)	91 (3)	95 (1)
Cortisone	97 (3)	83 (3)	89 (3)	96 (3)	94 (2)	84 (2)	90 (3)	96 (2)
Betamethasone	94 (3)	80 (2)	86 (2)	97 (4)	90 (2)	85 (2)	89 (2)	92 (2)
Dexamethasone	94 (3)	80 (2)	87 (2)	97 (3)	90 (2)	85 (2)	91 (3)	92 (2)
Flumethasone	95 (2)	81 (3)	88 (2)	91 (4)	91 (1)	84 (1)	91 (2)	91 (2)
Corticosterone	97 (3)	82 (3)	88 (2)	93 (4)	94 (2)	85 (2)	86 (3)	94 (2)
Beclomethasone	95 (2)	81 (2)	90 (2)	91 (2)	92 (2)	86 (2)	89 (2)	91 (2)
Triamcinolone Acetonide	94 (1)	82 (2)	88 (5)	106 (16)	93 (1)	85 (2)	85 (2)	91 (3)
Fluocinolone Acetonide	94 (3)	81 (2)	89 (3)	98 (3)	92 (2)	84 (4)	88 (1)	90 (3)
Budesonide Structural Isomer 1	92 (3)	80 (3)	83 (2)	92 (1)	89 (1)	82 (2)	84 (2)	87 (3)
Budesonide Structural Isomer 2	90 (2)	79 (3)	82 (2)	93 (1)	89 (2)	81 (2)	83 (3)	89 (2)
5-pregnen-3β-ol-20-one	101 (4)	95 (4)	78 (6)	104 (34)	100 (5)	77 (5)	82 (7)	95 (4)

Conclusions

- Very little difference was observed between loading pHs using the four extraction solvents.
- DCM only gave recoveries around 50% for triamcinolone. This is probably due to solvent polarity and analyte solubility. Once the DCM polarity was increased by adding 10% (v/v) IPA the recoveries increased to over 80%.
- Overall excellent recoveries and RSDs were obtained for the majority of analytes using any of the extraction solvents with either loading pH.
- ISOLUTE SLE+ shows excellent application to the extraction of corticosteroids from plasma using a variety of extraction solvents and loading pHs.

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