Application of a New Resin-based SPE Column for the Extraction of Diuretics from Urine and Analysis by LC-MS/MS

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Introduction

Diuretics are prescribed to treat various conditions such as heart, liver, kidney or lung diseases. However, their mode of action has been misused in sport and has led to the International Olympic Committee (IOC) and various horse racing authorities banning their use in competition. From this standpoint it is important to be able to screen for these drugs in various biological fluid samples, e.g. urine.

This poster describes the use of a new resin-based non-polar SPE sorbent for the extraction of diuretics from urine. It describes a generic approach to the extraction of diuretics to which additional compounds can be included with minimal method development time. The analyte suite includes thiazides, carbonic anhydrase inhibitors, loop and potassium sparring diuretics, with wide ranging pK_a and logP values. Analyte structures and properties are shown in **Table 2** on **page 3-4**.

The particle size of the sorbent and packing of the SPE column have been optimized to ensure excellent flow characteristics and reliable processing for the extraction of a wide range of analytes from biological fluids, such as urine.

Experimental Procedure

Reagents

All analytes (see **Table 2**, **page 3**) 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).

Solid Phase Extraction Procedure

SPE was performed on blank human urine spiked at 50 ng/mL concentrations using the 100 mg/10 mL XL column configuration.
Sample Pre-treatment: Human urine (1 – 2 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 (2–4 mL)
Interference Elution: Water (3 mL)
Analyte elution: Methanol (3 mL)
Post Extraction: Extracts were evaporated to dryness and reconstituted in 50:50 (v/v) H_2O/MeOH (1 mL) for subsequent LC-MS/MS analysis.

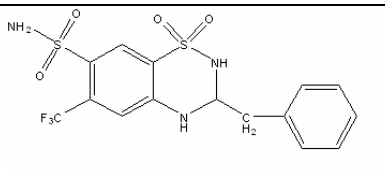
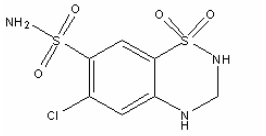
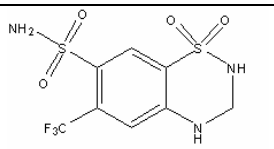
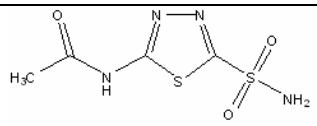
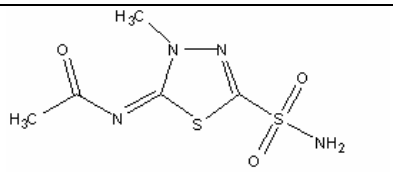
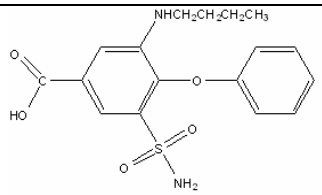
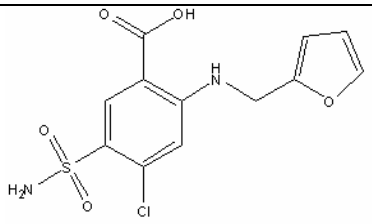
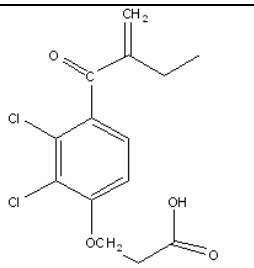
HPLC Conditons
Instrument: Waters 2795 Liquid Handling System (Waters Assoc., Milford, MA, USA)
Column: Zorbax Eclipse XDB C18 3.5 µm analytical column (100 x 2.1 mm ID) (Agilent Technologies, Berkshire, UK)
Guard Column: C8 guard column (Agilent Technologies, Berkshire, UK)
Mobile Phase: 0.1% aqueous formic acid and MeCN at a flow rate of 0.25 mL/min.
Gradient: 90%, 0.1% aqueous formic acid and 10% (v/v) MeCN at a flow rate of 0.25 mL/min increasing to 90% (v/v) MeCN over 7 minutes. The high concentration organic mobile phase was held for 1 minute then returned to the initial starting conditions.
Injection Volume: 10 µL
Temperature: Ambient temperature

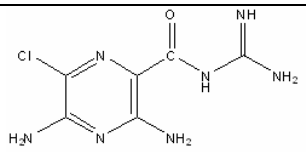
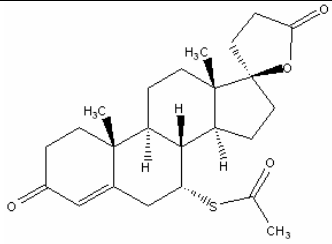
Mass Spectrometry
Instrument: Ultima Pt triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the multiple reaction monitoring mode (MRM)
Desolvation Temperature: 350 °C
Ion Source Temperature: 100 °C
Collision Gas Pressure: 2.9 x 10^{-3} mbar
The base peak in each compound spectrum was attributed to the protonated, $[M+H]^+$ or deprotonated molecular ions $[M-H]^-$ and were subsequently used as the precursor ions in the resulting MRM transitions. Positive/negative ion switching was utilized to analyze all the diuretics in this suite. Full MRM transitions and ionization conditions are shown in **Table 1**.

Table 1. Quattro Ultima Pt mass spectrometer parameters

Scan Function	Analyte	MRM Transition	Ionization Polarity	Cone Voltage (V)	Collision Energy (eV)
1	Amiloride	223.1 > 181.1	+	35	12
	Acetazolamide	230.1 > 189.1	+	35	15
2	Hydrochlorothiazide	296.1 > 269.0	-	100	19
3	Methazolamide	237.1 > 195.1	+	35	12
4	Hydroflumethiazide	330.1 > 303.0	-	100	19
5	Furosemide	329.1 > 285.0	-	35	15
6	Bendoflumethiazide	420.1 > 289.1	-	100	22
7	Bumetanide	365.2 > 240.2	+	35	15
8	Spironolactone	417.2 > 341.2	+	35	14
9	Ethacrynic acid	301.1 > 243.1	-	35	12

Table 2. Diuretic properties and structures.

Analyte	logP*	pK_a*	Structure
Thiazides			
Bendroflumethiazide	2.09	8.5	
Hydrochlorothiazide	-0.27	7.9, 9.2	
Hydroflumethiazide	0.11	8.9, 10.7	
Carbonic Anhydrase Inhibitors			
Acetazolamide	0.25	7.2, 9.0	
Methazolamide	0.23	7.3	
Loop Diuretics			
Bumetanide	3.26	4.0, 10.0	
Furosemide	1.51	3.52, 3.04, 0.48	
Ethacrynic Acid	3.41	3.5	

Potassium Sparring Diuretics			
Analyte	**logP**	**pK**	**Structure**
Amiloride	-1.25	8.7	
Spironolactone	4.31	N/A	

* logP and pKa values were taken from literature or calculated values if not available

Results

Figure 1, **page 5** shows the chromatographic separation obtained for the diuretic suite. A number of the analytes used have functionalities amenable to both positive and negative ionization. The choice of ionization mode and the transitions used in this study were selected based on the best signal-to-noise ratio for each analyte.

pH control of the urine was achieved using 1:1 (v/v) either:
(a) 1% formic acid aq,
(b) 0.1% formic acid aq,
(c) 20 mM NH_4OAc pH5 buffer or
(d) H_2O.

The pH values obtained when the buffers are mixed with urine are shown in **Table 1** below. Good recoveries and RSD's were obtained for all analytes with all four extraction methods. Spironolactone showed slightly lower recoveries (79%) and higher RSD's (10%) using the 1% (v/v) aqueous formic acid method.

Table 3. pH conditions when mixing urine/buffer 1:1 (v/v)

Urine /Buffer 1:1 (v/v)	**pH Value**
1% Formic acid: Urine	2.55
0.1% Formic acid: Urine	3.47
20mM NH_4OAc, pH5: Urine	6.13
H_20: Urine	6.97

Figure 2, **page 5** shows the recovery of each analyte using the various methods. The methods using 1% and 0.1% aqueous formic acid showed some interfering matrix components (approximately 10% of fortified peak area) in the acetazolamide trace at the appropriate retention time. These matrix components were not, however, present when the 20 mM NH_4OAc pH 5 buffer or H_2O methods were used.

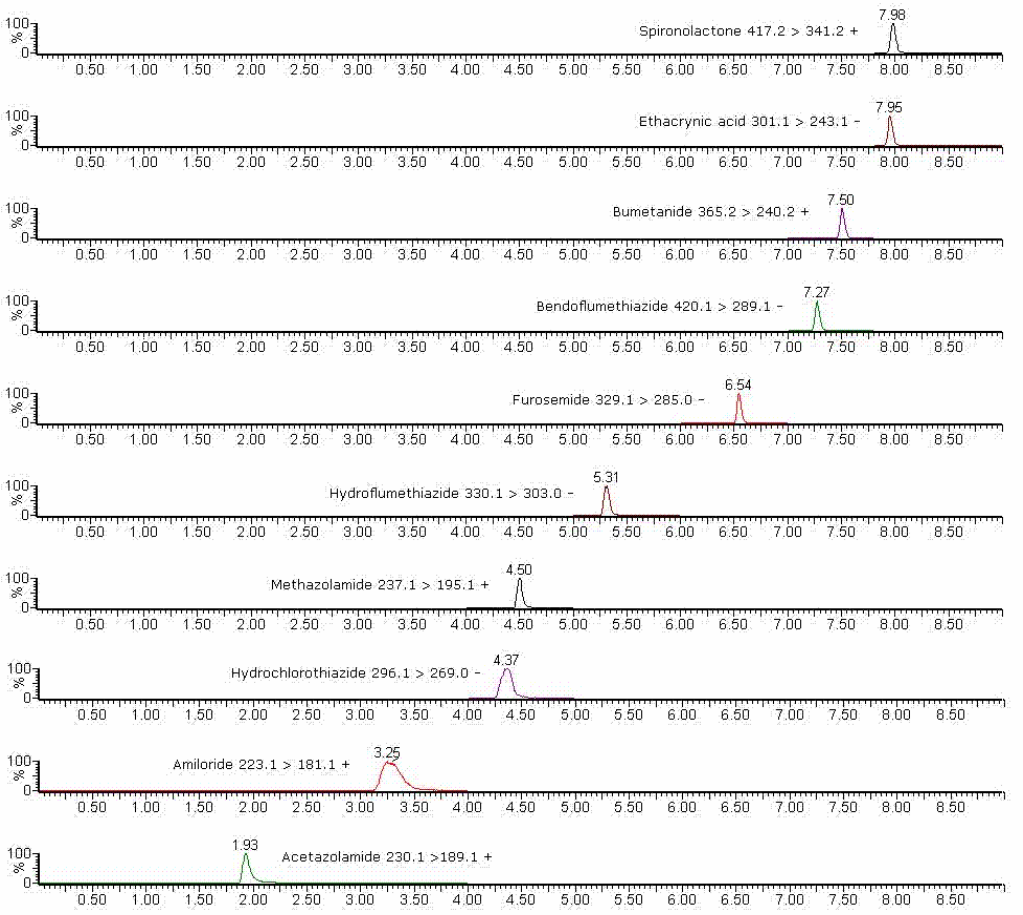


Figure 1. Chromatographic separation of the selected diuretics from a 10 µL standard injection at a concentration of 50 pg/µL

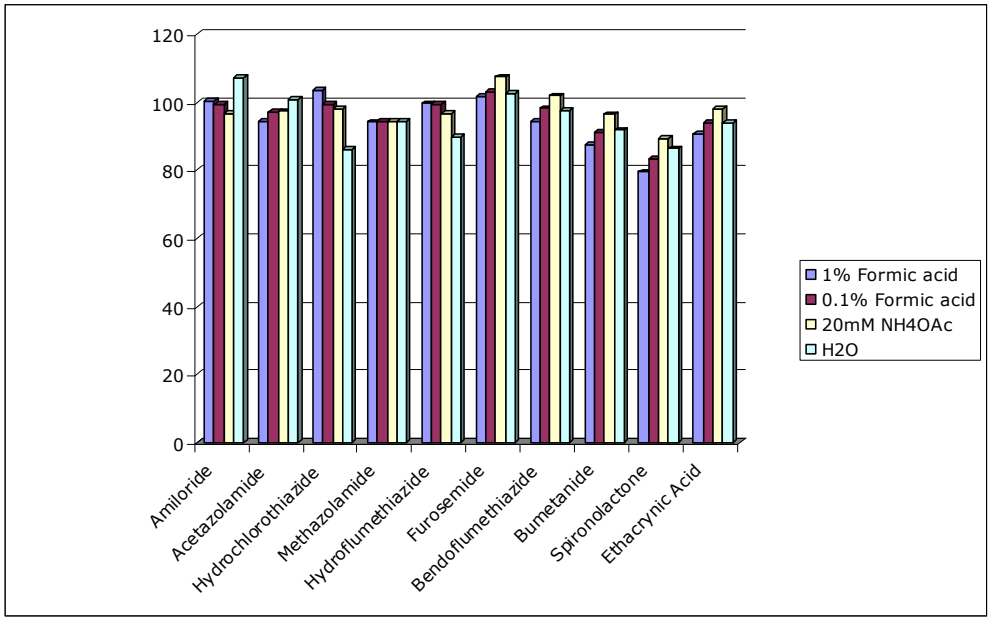


Figure 2. Analyte recovery chart comparing the effect of loading conditons on analyte recovery (n=5)

The best performing method taking into account analyte recoveries and extract cleanliness was the 20 mM NH_4OAc pH 5 buffer (see **Figure 3** for full details). **Table 4** shows analyte recoveries and relative RSD's using this approach.

Table 4. Analyte recovery and relative RSD's (n=5) using the 20 mM NH_4OAc pH 5 method

Analyte	% Recovery	% RSD
Amiloride	96.8	2.5
Acetazolamide	97.3	3.2
Hydrochlorothiazide	97.9	6.8
Methazolamide	94.3	3.4
Hydroflumethiazide	96.7	4.9
Furosemide	107.4	3.9
Bendoflumethiazide	101.9	4.1
Bumetanide	96.5	3.5
Spironolactone	89.4	2.2
Ethacrynic acid	98.1	4.6

Recommended SPE Procedure

Column Configuration: 100 mg/10 mL
Sample Pre-treatment: Dilute urine sample (1-2 mL, 1:1 v/v) with 20 mM ammonium acetate buffer, pH 5
Column Conditioning: Methanol (3 mL)
Column Equilibration: 20mM ammonium acetate buffer, pH 5 (3 mL)
Sample Application: Apply diluted sample
Interference Elution: Rinse with water (3 mL)
Analyte Elution: Methanol (3 mL)

Figure 3. Recommended SPE procedure, giving best overall recoveries and extract cleanliness

Conclusions

- Good recoveries and RSD's were obtained for all analytes using the four different methods outlined.
- Higher RSD's and slightly lower recoveries were observed for spironolactone under the acidic conditions.
- The highly acidic loading conditions show more interfering matrix components in the early part of the chromatogram.
- The 20 mM ammonium acetate, pH 5 buffer gave optimal results in terms of analyte recovery and extract cleanliness.

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