Application of a New SPE Polymer, EVOLUTE® ABN 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 EVOLUTE® ABN, 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, but also deals with advanced wash protocols to decrease ion suppression/enhancement effects. 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 listed in **Table 2**, **pages 3-4**.

Experimental Procedure

Reagents

All analytes (see **Table 2**) 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: Recovery Investigation

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 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)

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 Conditions

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.

Desolvation Temperature: 350 °C

Ion Source Temperature: 100 °C

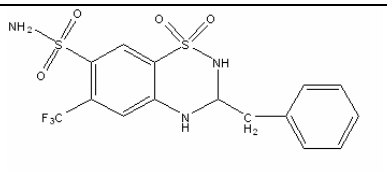
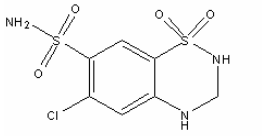
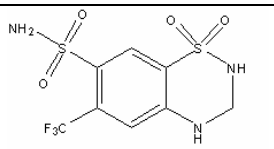
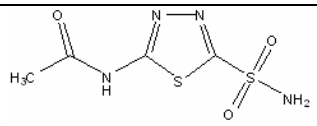
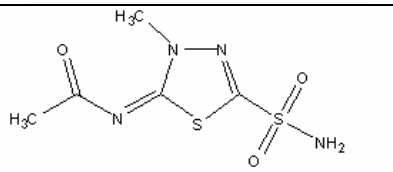
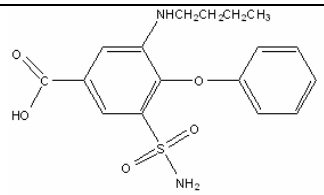
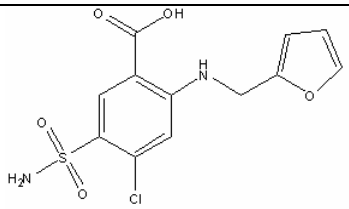
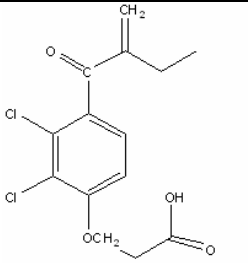
Collision Gas Pressure: 2.9 x 10^{-3} mbar

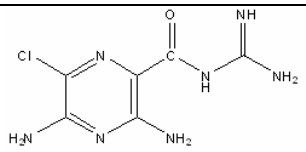
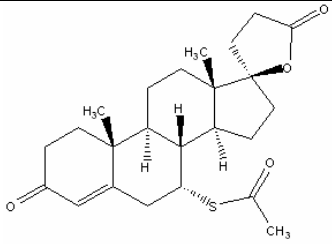
The base peak in each compound spectrum was attributed to the protonated, $[M+H]^+$ or deprotonated molecular ions $[M-H]^-$ and were subsequently used as the precursor ions in the resulting MRM transitions. Positive/negative ion switching was utilized to analyze all the diuretics in this suite. Full 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

Experiment 2: Ion suppression cleanliness optimization using FIA-MS/MS

Blank urine samples (1 mL) were extracted as in the 20mM ammonium acetate method described in **Experiment 1**. Interference wash steps were investigated as follows: 3 mL H_2O, 95:5, 90:10 or 80:20 H_2O/MeOH; or 1 mL H_2O followed by a successive 1 mL wash of H_2O, 95:5, 90:10, 80:20 or 70:30 (v/v) H_2O/MeOH. The MeOH eluates were collected, evaporated to dryness and reconstituted in 1 mL of 50:45:5:0.1% H_2O/ACN/MeOH/Formic acid (v/v) mobile phase spiked with 1 µg/mL caffeine concentration. The 2795 liquid handling unit delivered an isocratic mobile phase of 50:45:5:0.1% H_2O/ACN/MeOH/Formic acid (v/v) at 0.25 mL/min. Injection volumes were set to 5 µL and the MS set to monitor the MRM transition for caffeine (195>138, cone voltage 45 V and collision energy 16 eV).

Experiment 3: Analyte recovery investigation using advanced washing protocols

Using the modified washing protocols above, analyte recovery was monitored for the same analyte suite. All conditions were as described in **Experiment 1**.

Results

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. Full details of chromatographic separation are shown in **Figure 1**.

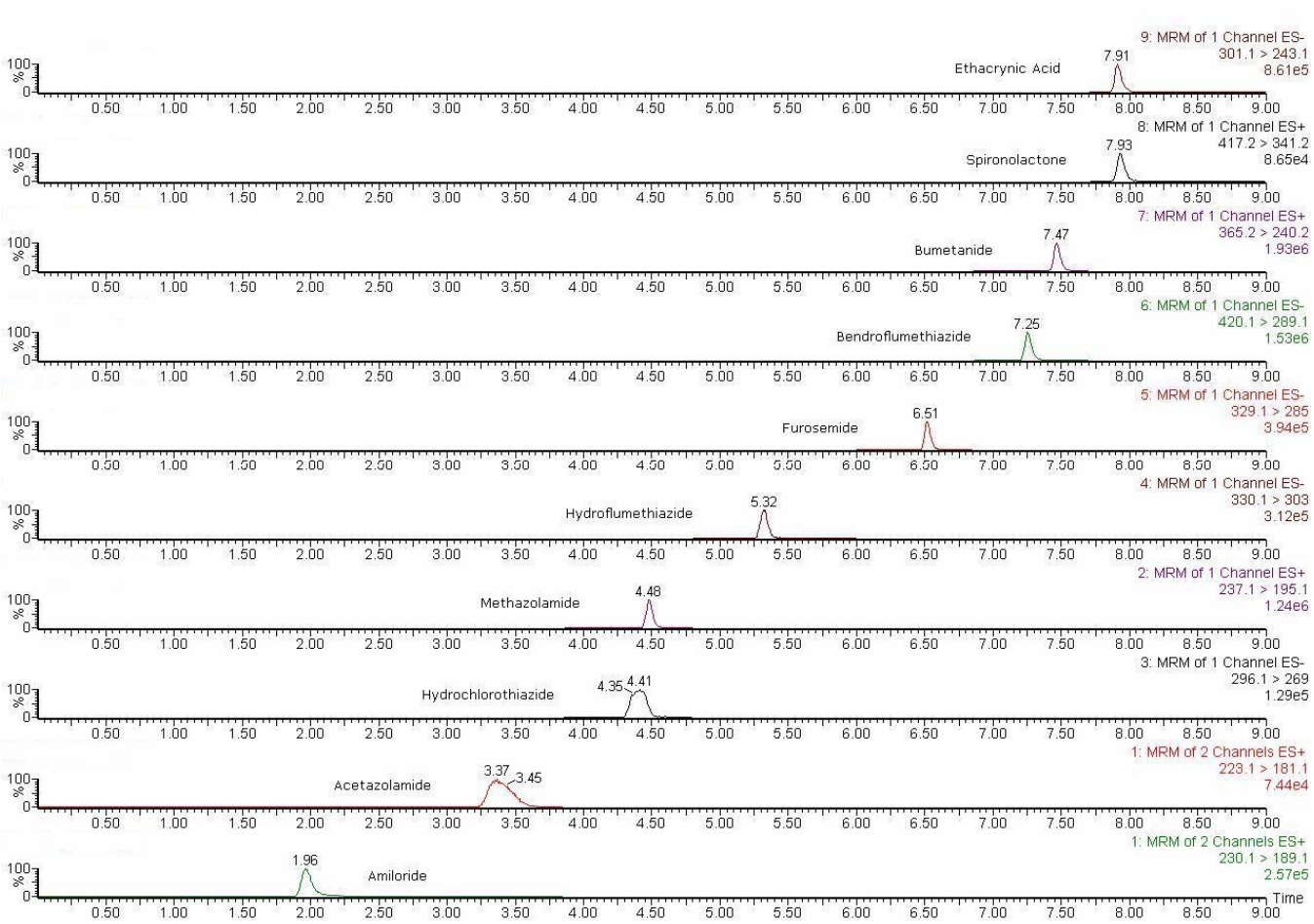


Figure 1. Chromatographic separation of 10 diuretics from urine

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 3**. Excellent recoveries and RSDs 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) formic acid aq 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 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.

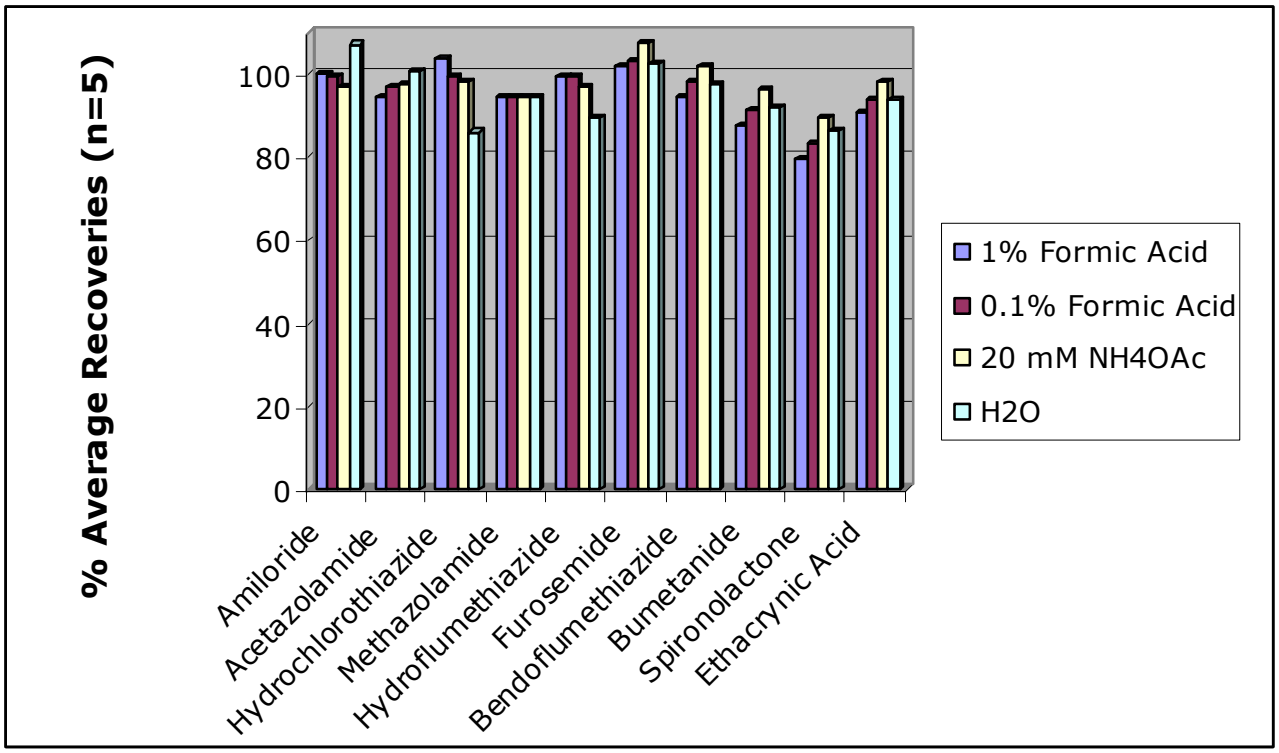


Figure 2. Analyte recovery chart comparing the effect of loading conditons on analyte recovery (n=5)

Table 4 shows analyte recoveries and relative RSDs obtained using the 20 mM NH_4OAc pH 5 buffer method. This method was used going forward to investigate the amount of ion suppression using the various wash protocols and subsequently the effect on polar analyte recovery from these protocols.

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

Figure 3. shows the ion suppression obtained from the various wash protocols. When using a 3 mL wash increasing the MeOH content above 5% (v/v) did not show any improvements in overall ion suppression. When using successive 1 mL washes increasing the MeOH content in the second wash above 10% (v/v) did not show improvements in ion suppression.

Figure 3. FIA-MS/MS TIC obtained for the final extracts using various interference wash steps compared to pure spiked mobile phase (caffeine spiked at 1 µg/mL).

No significant effect on ion suppression/enhancement was observed for any of the other analytes in the suite, apart from the most polar analytes amiloride and acetazolamide. The effect of these wash protocols on these two analytes can be seen in **Figure 4**.

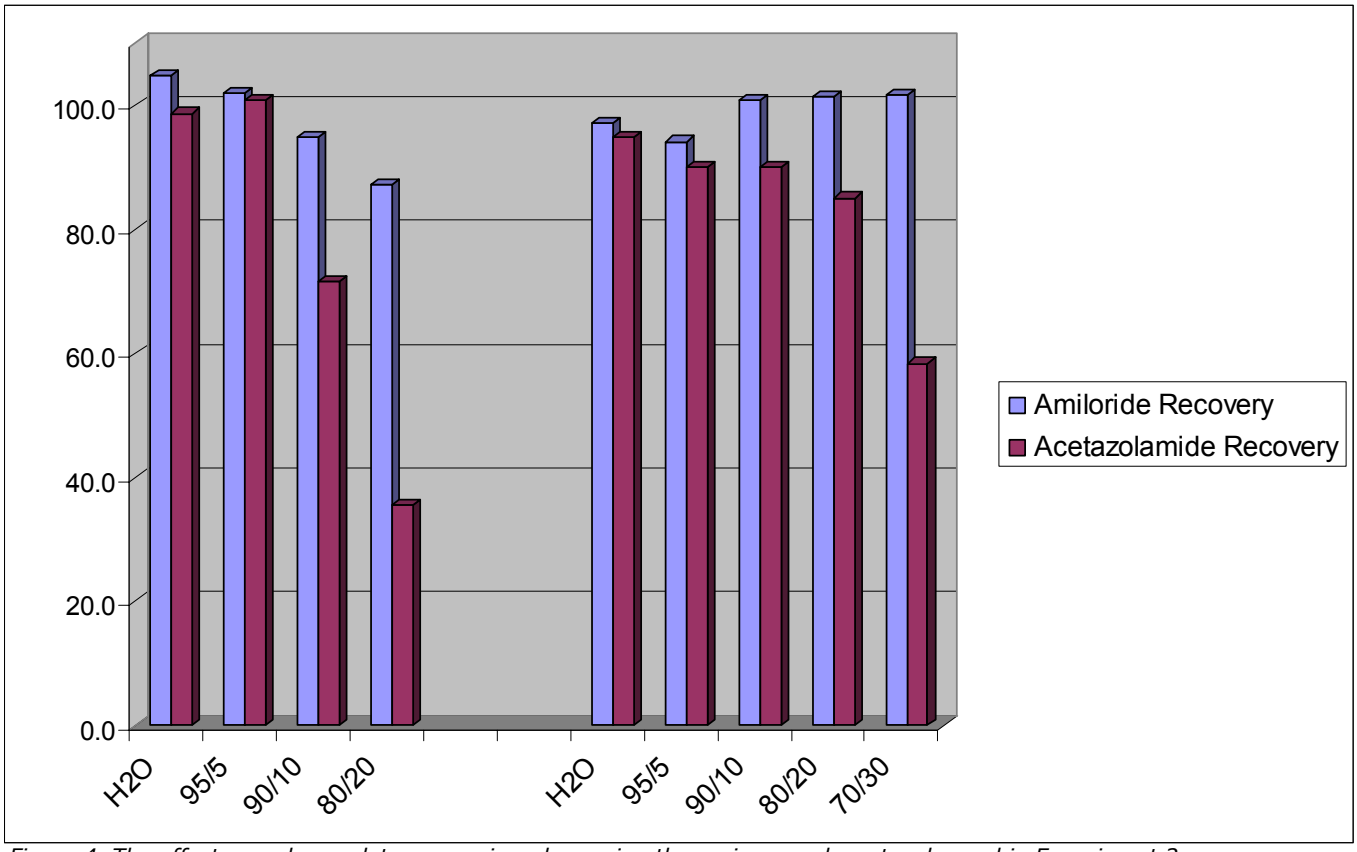


Figure 4. The effect on polar analyte recoveries when using the various wash protocols used in Experiment 2.

Conclusions

1. Good recoveries and RSD's were obtained for all analytes using the four different methods outlined.
2. The highly acidic loading conditions show more interfering matrix components in the early part of the chromatogram.
3. Increasing the MeOH content to 5% in the 3 mL interference wash step gave approximately 10% lower ion suppression; however, increasing beyond this did not show any improvements.
4. For the case of the successive 1 mL wash steps, increasing the second wash to 10% (v/v) MeOH again showed an approximate decrease of 10% suppression, however, no improvements were observed with greater than 10% (v/v) MeOH.
5. Decreases in recovery for the two polar analytes were observed when increasing the MeOH beyond 5% in the 3 mL interference wash step.
6. When using 2 successive wash steps up to 20% MeOH can be used in the second wash before polar analyte recovery is compromised.

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