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Approaches to eliminate elution solvent modifiers in the mixed-mode solid-phase extraction (SPE) of drug residues for on-line GCMS and LCMS analysis

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Introduction

Mixed mode SPE has been used successfully for the extraction of a variety of drugs from biofluids and specifically from urine. The primary elution and washing solvents and even the exhausted urine have been used in multi-eluate strategies. The need to use modifiers such as ammonia in the organic elution solvent limits the use of mixed-mode sorbents for on-line and rapid throughput analysis because the modifier must be removed or neutralised prior to chromatography. We describe a modification of the mixed-mode extraction of urine on C8/SCX type sorbents that allows the recovery of the basic fraction with modifier free solvents (Fig. 1).

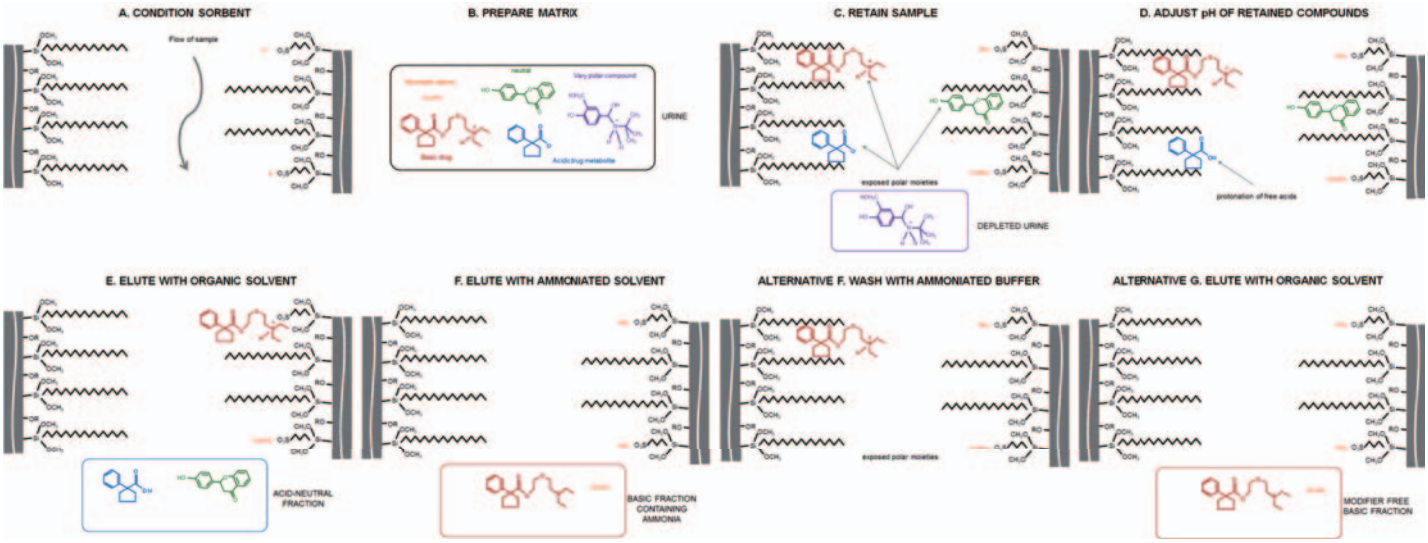

Fig. 1 Steps A – F show a stepwise approach to conventional mixed mode SPE using a C8/SCX type sorbent. Alternative steps F-G show an alternative strategy for base elution from C8/SCX and other mixed mode sorbents involves flipping bases from ion-exchange sites back to reversed-phase retention for subsequent elution in non-ammoniated solvents.

Method

Methanol was HPLC grade and water purified using a Millipore synergy system. Drug free urine samples were maintained at 4°C until required for analysis. Portions of urine (3 mL) were spiked with the drugs listed in table 1, diluted with 0.5 M ammonium acetate buffer (pH 5.5, 4.5 mL) and the pH adjusted to 5.5-6. The samples were subject to protease and beta-glucuronidase treatment, centrifuged to remove sediment and extracted (Fig. 2A) on mixed-mode C8-SCX columns (Bond Elut-Certify, 130 mg, 3 mL, Agilent, CA, USA) previously conditioned with methanol (2 mL) and water (2 mL). Each sample was passed through a column. The column was washed with water (4 mL) then 1 M acetic acid (2 mL) for pH adjustment and dried with nitrogen at 200 mL/sec for 6 minutes. The sorbent was washed with methanol (2 mL) and again dried with nitrogen for 6 minutes. The base fraction was eluted with ethyl acetate /dichloromethane /2-propanol (5:4:1v/v) containing 2% concentrated aqueous ammonia (2 mL).

Alternatively (Fig. 2B), after washing with methanol, the sorbent was washed with 2 mL of a 0.5 M aqueous ammonium acetate solution that had been adjusted to pH 8, 9 or 10 with ammonia. The column was optionally washed with water, dried with air and the retained bases eluted with methanol (2 mL). The basic fractions were evaporated to dryness under a stream of nitrogen at 20°C and a flow rate of 1 mL/min and the residues were reconstituted in 200 µL of a 95:5 mixture of 0.1% v/v aqueous formic acid and methanol containing 0.1% formic acid prior to analysis by LCMS.

Heated electrospray Ionisation (HESI) - LCMS experiments were performed on a Prominence LC- 20A (Shimadzu Corp., Kyoto, Japan) coupled to an Exactive High Resolution Mass Spectrometer (Thermo Fisher, Braeman, Germany). The column was an Eclipse Plus C18 (2.1 mmID × 100 mm × 3.5 µm, Agilent, CA, USA) maintained at 35 °C and the mobile phase was a mixture of 0.1% v/v

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aqueous formic acid (solvent A) and 0.1% formic acid in methanol (solvent B) at a flowrate of 400 µL/min. A solvent gradient of A:B of 95:5 (0 min), initially held for 30 seconds then ramped to 2:98 (0.5 - 10 min), held at to 2:98 (2 min). Re-equilibration at 95:5 was continued for 3 min. The injection volume was 10 µL. The heated electrospray ion (HESI) source operated with a vapourizer temperature of 350°C, capillary temperature of 300°C, spray voltage of 3 kV, sheath gas at 50 units and auxiliary gas at 20 units. The mass spectrometer was run in positive ion mode at 50,000 resolution, scanning from 100 - 7 00 Da and a scan speed of 1000 µ/sec.

The recovery of each substance and percentage matrix effect (% ME) was measured using both methods at pH 8 (Table 1) and recovery was measured for two separate batches of urine using a wash of pH 8-10 (Table 2). Recovery was calculated as the area ratio of the peak calculated for urine samples spiked before extraction versus the area obtained when the same quantity of standard was spiked into a drug free urine extract. The %ME was calculated according to the formula:

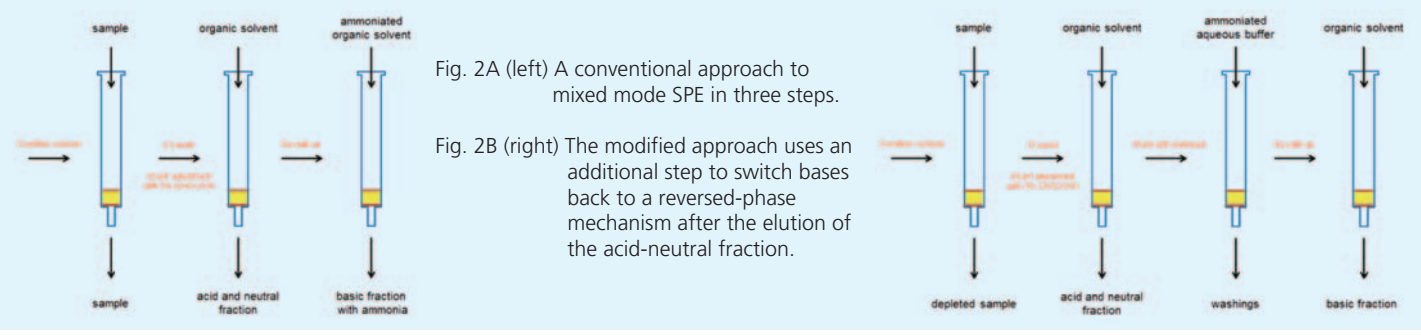

Fig. 2A (left) A conventional approach to mixed mode SPE in three steps.

Fig. 2B (right) The modified approach uses an additional step to switch bases back to a reversed-phase mechanism after the elution of the acid-neutral fraction.

% R = 100 × (area urine spiked before extraction/area urine spiked after extraction)

% ME = 100 × ((area for urine spiked after extraction/area of standard) - 1)

Discussion

In these preliminary experiments, our modification to the conventional SPE method does not significantly affect the utility of the method (Table 1 and 2). The modified method used methanol as the base elution solvent while the conventional method used a less polar solvent blend and we anticipated an increase in the matrix influences but wished to avoid incomplete elution in the case the sorbent was not completely dried. Both methods showed useful recovery of basic drugs although there was individual variation between the methods for individual analytes. We believe that lower recovery using the conventional method is related to the efficiency of sorbent drying and the power of the elution solvent. Conversely, poorer recovery from the modified method (e.g. acebutolol, benzylpiperazine and norfentanyl) may be attributed to analyte hydrophilicity contributing to poor transfer back to the reversed-phase retention sites.

As anticipated, methanol elution contributes to more significant ion-supression by the matrix. However, we have no explanation other than poor solubility in solvents other than methanol to account for the improved recovery of the sartans and fexofenidine by the modified method. Our ongoing study of this method is examining the %ME of different urine specimens and the influence of elution solvent polarity in controlling %ME.

Continuing studies are examining the influence of the sorbent, elution solvent polarity and ion-source design to reduce the matrix effects shown by both methods.

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	modified method		conventional method	
	recovery	% ME	recovery	%ME
16-hydroxystanozolol	72	-84	37	-51
acebutolol	54	-38	70	-36
acepromazine	62	-77	78	-36
atenolol	68	-39	64	-24
benzylpiperazine *	20	-67	59	-28
betaxolol	71	-69	75	-36
clenbuterol	69	-68	66	-23
clonidine	99	-42	78	-14
cyclobenzaprine	81	-86	80	-38
doxapram	93	-81	76	-32
drofenine	86	-73	91	-22
eprosartan	59	-69	0	
fexofenadine	81	-63	0	
flumazenil	48	-66	61	-23
hydromorphone*	30	-62	34	-37
isoxsuprine	88	-71	65	-35
ketamine	99	-67	97	-22
loperamide	97	-87	57	-43
losartan	78	-56	14	-65
nafronyl	65	-65	41	-25
nalbuphine	93	-60	82	-47
norbuprenorphine	63	-75	58	-53
norfentanyl	26	-64	72	-29
nylidrin	92	-72	62	-32
oxymorphone*	56	-67	30	-42

Table 1 The %ME and recovery for a series of basic drugs at 25 ng/mL in hydrolysed equine urine using the modified and conventional approaches to mixed mode SPE.

	pH 8 buffer		pH 9 buffer		pH 10 buffer		pH 10 buffer*	
	batch 1	batch2	batch 1	batch2	batch 1	batch2	batch 1	batch2
16-hydroxystanozolol	61	74	62	68	81	76	50	51
Acebutolol	56	44	54	46	68	53	69	59
Acepromazine	43	76	66	82	4	102	42	105
Atenolol	58	58	57	58	64	68	68	64
Benzylpiperazine *	18	20	15	16	27	21	16	15
Betaxolol	57	71	71	60	94	83	60	61
clenbuterol	60	60	70	55	78	52	62	54
clonidine	89	99	95	94	93	81	82	91
cyclobenzaprine	68	75	87	52	222	63	63	79
doxapram	91	93	104	95	116	124	78	99
drofenine	94	93	84	77	277	165	63	89
eprosartan	87	83	84	90	81	100	85	104

Table 2 Recovery shown for a limited panel of basic drugs extracted using the modified SPE method and a basic wash (Step F) of pH 8-10. The asterisk (*) indicates that the pH 10 wash was followed by a wash with distilled water. Elution in each case was with methanol.

Conclusion

This study shows that there is no significant loss of recovery for bases in mixed mode strategies when the analytes are shunted from ion-exchange retention back to reversed-phase retention. This characteristic of the mixed mode method opens the way for the recovery of fractions that are free of modifiers such as ammonia and therefore suitable for on-line analysis by GCMS or LCMS without risk of column damage (for GC) or uncontrolled sample pH (for LC). The hydrolysed horse urine gives rise to significant matrix effects for both methods.

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