Sample preparation for Tox screen in urine – how broad can it get?

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Introduction

Detection of toxic Xenobiotics is of great importance in forensic and clinical toxicology. Xenobiotics undergo metabolic transformation in the body to a more water soluble state allowing their rapid excretion from the body. In urine, xenobiotics are usually in their metabolic state (phase I and II), and sometimes in their original structure (depending on their lipophilisity and the time passed post exposure). Hence, LC-MS(/MS) is preferable to detect this type of compounds.

Objective

The nature of screening xenobiotics in urine, guided us in establishing a list of model compounds. Unlike other limited model compound lists described in the literature, 15 model compounds were chosen representing a large variety of potential xenobiotics in urine, characterized by: 1. acidic, basic or neutral properties (pKa 1-10). 2. Molecular weight in the range of 78amu to 700amu. 3. broad range of Log P (0.07-6.4). 4. Various functional groups.

Since xenobiotics and their metabolites might be present in urine as **residues**, efficient sample preparation is required to be able to detect them. However, the demand for efficient sample preparation becomes challenging due to the broad range of compounds. In the literature, most of the LC/MS tox screen procedures are based on the use of SPE with hydrophobic interaction (RP and polymeric)[1,2]. We have found that these are not effective for very small acidic molecules (such as Fluoroacetic acid (MW 78)) that are not trapped on the polymeric (RP) stationary phase. After extensively exploring various SPE technologies, followed by careful optimization of the extraction procedure, as well as solvent adaptation to LC-MS introduction, we developed a final procedure based on two routes and is a combination of clean-up and concentration. The routes are: A. Strata X - SPE cartridge for neutral and basic xenobiotics such as Aniline (MW 93), Reserpine (MW 608), and glucuronides (phase II metabolites). In this procedure the analytes are extracted and concentrated. B. Isolute SAX – SPE for acidic compounds such as Diflunisal (MW 250), and zwitterion model compounds such as Phenylglycine (MW 151). In this procedure the acidic compounds are extracted and concentrated while the zwitterion compounds are only cleaned. It is highly important that the sample is injected in aqueous medium and not in the organic solvent used for SPE extraction. Good recovery was obtained for all of the model compounds (20%-90%). We compared our preparation procedure to the well known "Dilute & Shoot" strategy, and found that the SPE procedures are superior, especially for molecules such as Aniline, Reserpine, Fluoroacetic acid, Naproxen and Phenylglycine.

Experimental

LC-MS (QTOF): Agilent 1200 coupled to QSTAR Elite – Sciex ABI

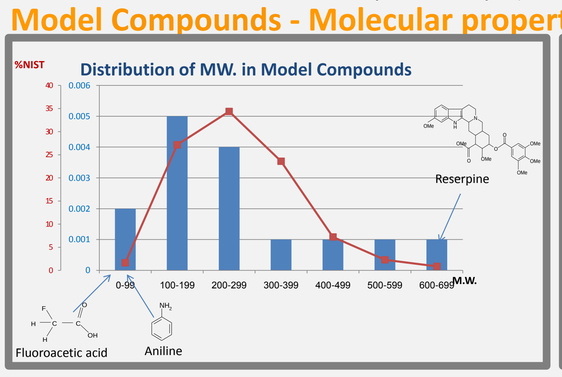
Column: Luna C18 5um, 150× 2mm, Phenomenex. Column guard: 3×4mm, Phenomenex. Used with ESI, at a flow rate of 0.3 ml/min.

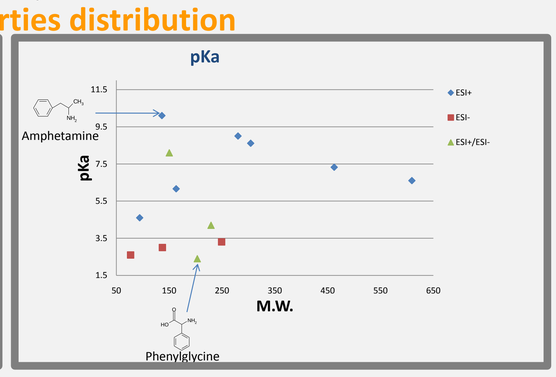
Mobile phase: A:H_2O pH 3 (0.0002M Buffer ammonium formate)
B: MeOH (0.002M ammonium formate)

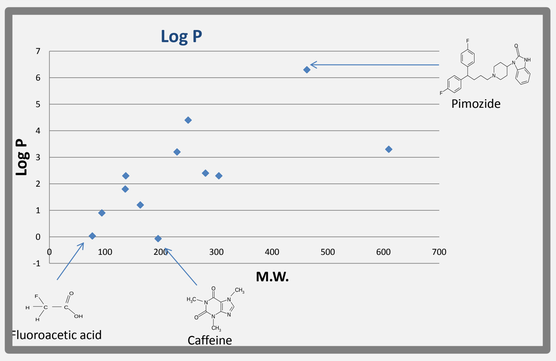
Sample preparation: **Basic and neutral:** Strata X Polymeric sorbent 6ml/500mg, Phenomenex X8B-S029-FCH. *Load: 10 ml Sample (Urine: TDW 2ml:8ml), Extract: 3ml 60:40 ACN:MeOH, Addition of 0.5ml TDW, Evaporate to 0.5ml.*

Acidic and zwitterions: Isolute PE-AX Strong anion exchange 3ml/200mg Biotage BT503- 0020B. *Load: 3 ml Sample (Urine: TDW 1:4)-collect this step. Extract: 1.5 ml MeOH +20% Formic acid, Addition of 0.5ml TDW , Evaporate to 0.5ml. Combine load and evaporate steps (50:50).*

Model Compounds - Molecular properties distribution





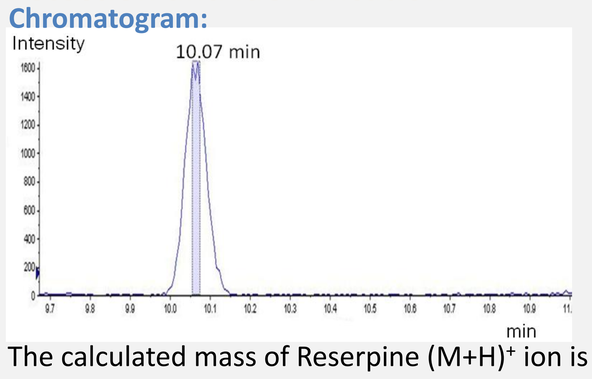


Ref:

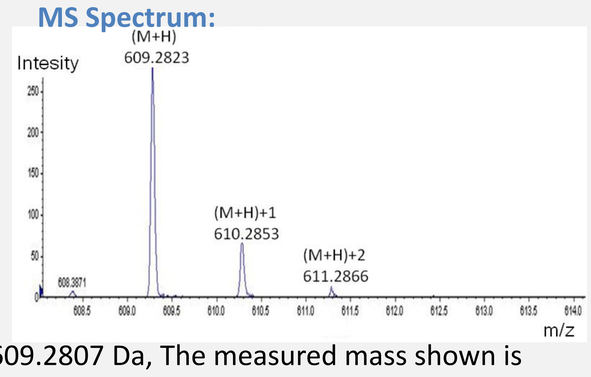
1. T.N.Decaestecker, W.E. Lambert, C.H. Van Peteghem, D. Deforce, J.F. Van Bocxaer, *J. Chromatogr. A*, 1056 : 57-65 (2004).
2. M. Rittner, F. Pragst, J. Neumann, *J. Anal. Toxicol.*, 25:115-124 (2001).

Detection and identification

Chromatogram:



MS Spectrum:



The calculated mass of Reserpine $(M+H)^+$ ion is 609.2807 Da, The measured mass shown is 609.2823 Da, deviation of 2.6 ppm.

Sample preparation

Quenching effects and urine dilution

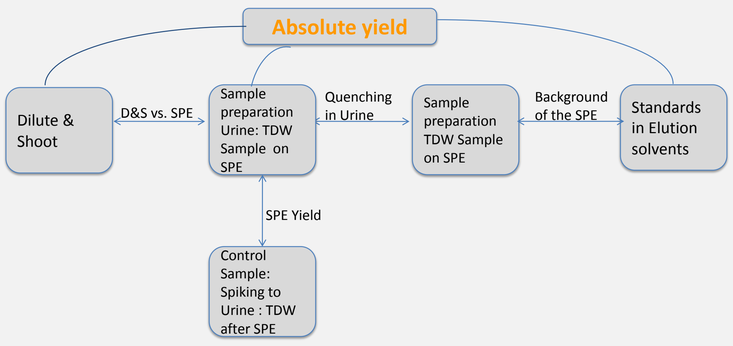
Expected peak area ratios

Compound	1:20 Peak area	1:10 Peak area	1:5 Peak area	1:2 Peak area	1:2/1:5=2.5	1:5/1:10=2	1:10/1:20=2.5
Caffeine	8.8e3	1.5e4	1.7e4	3.1e4	1.8	1.1	1.7
Cocaine	1.7e4	3.7e4	9.9e4	1.3e5	1.3	2.7	2.1
Doxepin	2.7e3	8.3e3	1.3e4	1.2e4	0.9	1.6	3.1
Amphetamine	1.8e3	5.5e3	1.5e4	1.7e4	1.2	2.7	3.0
Reserpine	1.6e2	2.6e2	6.6e2	1.5e3	2.3	2.5	1.7

Dilute1:2- the signal is much lower than expected. Hence, The recommended dilution is 1:5.

Method development strategy:

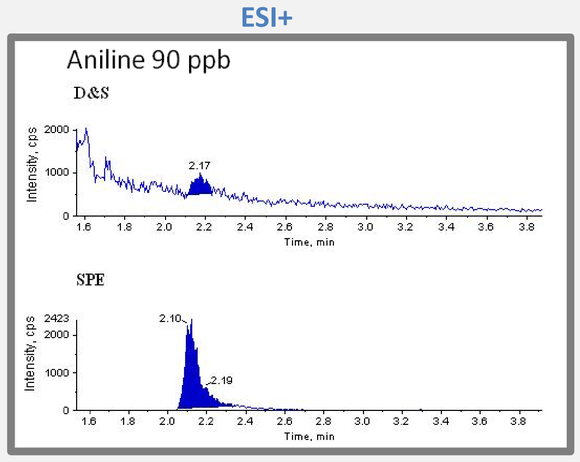
Performance comparison and recovery evaluation



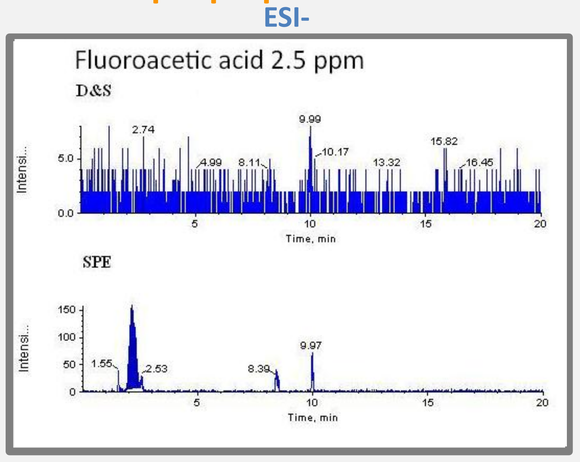
Compound	Absolute yield %
Aniline	39%
Amphetamine	53%
Cocaine	50%
Doxepin	20%
Pimozide	91%
Naproxen	88%
Diflunisal	77%
Fluoroacetic acid	42%

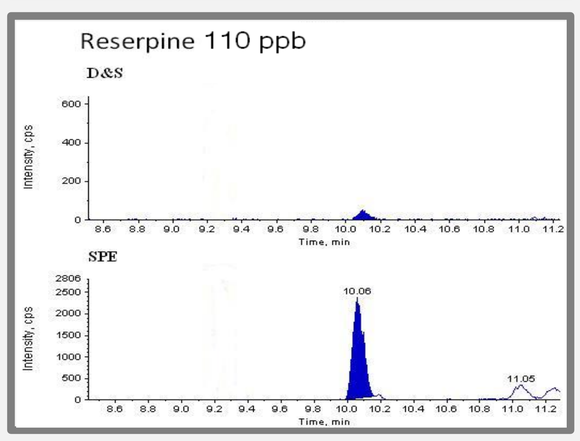
"Dilute and Shoot " vs. "Dilute and Shoot " + Sample preparation –SPE

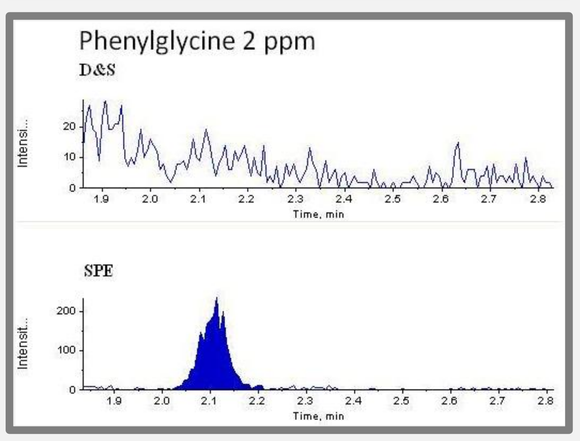
ESI+



ESI-







LC-MS analysis of Aniline, Reserpine, Fluoroacetic acid and Phenylglycine. The sample preparation protocols developed, based on SPE, are compared to "Dilute &Shoot" direct analysis. For the model compounds shown, the optimized sample preparation procedures provided good recoveries, with much better performance than "Dilute and Shoot".

Summary

- A protocol for urine preparation for general LC-MS-MS screening was developed.
- 15 acidic, neutral and basic compounds in the MW range of ~50-700 were tested, and good recoveries were obtained for all of them.
- This protocol is not applicable for very strong acids (pKa<1.8).
- Peak digging for xenobiotics in urine as well as identification schemes will be further explored.