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Screening of 30 acidic and neutral pharmaceuticals in whole blood by fully automated SPE and UPLC-TOF-MS^E

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Introduction

A number of compounds encountered in the framework of forensic toxicology belong to a disperse group of drug classes and may be relevant in very diverse cases. Acidic and neutral compounds have relatively high therapeutic levels (over 1 mg/kg) compared to basic drugs, with only a few reaching 0.1 mg/kg, and their therapeutic intervals and therapeutic indexes vary.

Over-the-counter drugs, such as mild pain relievers and anti-inflammatory drugs (diclofenac, etodolac, ibuprofen, naproxen, paracetamol (acetaminophen), or salicylic acid and salicylamide), are available for self-medication and frequently reported in cases of intended or unintended overdose. Prescribed drugs, such as anti-convulsants and anti-epileptics, skeletal muscle relaxants, and sedatives (amobarbital, barbital (diemal), carbamazepine, carisoprodol, chlorzoxazone, enphenemal, levetiracetam, meprobamate, oxcarbazepine, pentobarbital, phenobarbital, phenytoin, primidone, thiopental, topiramate, or zonisamide) are relevant with respect to impaired performance (e.g. in traffic cases, drug rape cases, violence cases) and toxicological investigation. Other commonly reported drug classes are bronchodilators (theophylline), anticoagulants (warfarin), loop-diuretics (furosemide), hypoglycaemic agents (tolbutamide), and the stimulant caffeine.

The compounds vary in their chemical structure. However, acidic and neutral compounds can be defined as compounds that possess acidic functional groups with low pK_a values and/or have relatively hydrophobic properties, which are commonly exploited by liquid-liquid extractions.[1,2] Sample preparation represents the first task in retrieving the various compounds from a complex matrix, such as whole-blood samples, and removing interfering agents. Multi-targeted methods benefit from the development of solid-phase extraction (SPE) sorbents[3,4] by achieving increased retention for a number of drugs,[5] and precipitation combined with filtering preserves the compounds but removes interferences,[6] such as phospholipids. The second task is to secure sufficient sensitivity, minimizing co-elution with other compounds or matrix constituents. The third task is to acquire sensitive and specific signals. Forensic and clinical laboratories previously performed general unknown screening for acidic and neutral compounds using high performance liquid chromatography with diode-array detection (HPLC-DAD) spectra comparison[1] or gas chromatography-mass spectrometry (GC-MS).[7] Acidic and neutral drugs can be identified and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)[2,5,6] in a targeted set-up.

We investigated how a well-described gradient developed by Humbert[8] and the Waters Corporation works for target screening of acidic and neutral compounds in a fraction from a mixed mode SPE application in which the acidic and neutral compounds are eluted before the basic compounds. We also investigated how time-of-flight data acquired in MS^E works with the software ChromaLynx XS for targeted compound identification. The target drugs were selected from reporting frequencies and case relevance, including three metabolites (10,11-epoxid-carbamazepine, 10-hydroxy-carbazepine, demoxepam). The method was validated for extraction recoveries, matrix effect, process efficiency, and RT variation.

Materials and methods

Chemicals and reagents

Phenobarbital and zonisamid were purchased from Sigma-Aldrich (St Louis, MO, USA), and salicylamide and salicylic acid from Alfa Aesar (Lawrence, KS, USA). The remaining drugs were supplied by different pharmaceutical companies. All reference standards were ≥98% purity (except thiopental 85%). Stock solutions of 1 or 10 mg/ml in methanol were prepared and stored at -25°C. Paracetamol-D4 (100 µg/ml) purchased from Cerilliant (Round Rock, TX, USA) and hexemal (cyclobarbital) (10 mg/ml) provided by a local Pharmacy (Copenhagen, Denmark) were used as internal standards.

LC-MS grade methanol and acetonitrile were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Aqueous ammonia (25%) solution was obtained from Merck (Darmstadt, Germany). Purified water was produced by a Millipore Synergy UV water purification system (Millipore A/S, Hellerup, Denmark). The LC mobile phase was prepared weekly. Mobile phases were 5 mM ammonium formate adjusted to pH 3 using formic acid (solvent A) and acetonitrile containing 0.1% (*v/v*) formic acid (solvent B).

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Samples

Blank human whole blood was purchased from the blood bank at Copenhagen University Hospital (Copenhagen, Denmark) and stabilized by the addition of 1% sodium fluoride. The absence of licit and illicit drugs was confirmed using in-house screening procedures comprising GC-MS, HPLC-DAD, and LC-MS methods.

Preparation of standard solutions and quality control (QC) samples

Quality control cut-off ($QC_{cut\text{-}off}$) samples containing all target compounds at concentrations of 1 mg/kg were prepared from the working solution (25 mg/L) in pooled blank whole blood and stored in 350 μl aliquots at −80°C.

A single internal standard solution (IS) containing paracetamol-D4 at a concentration of 6 mg/L and hexemal at a concentration of 200 mg/L was prepared, divided into aliquots of 1000 μl, and kept at -80°C. A total of 20 μl of IS was added to all $QC_{cut\text{-}off}$ samples.

Instrumentation for fully automated solid-phase extraction

The fully automated extraction process was performed on a Tecan Freedom EVO 200 robotic platform (Tecan, Männedorf, Switzerland) fitted with the following modules: a Tecan liquid handling arm with eight independent pipetting channels using disposable filter tips, a Tecan robot manipulator arm for plate movements, a Tecan Te-Vacs vacuum station for solid phase extraction, a Tecan Te-Shake orbital plateshaker, a Mettler Toledo XP203S balance (Mettler Toledo, Schwerzenbach, Switzerland) for weighing samples, an Agilent Microcentrifuge (Agilent Technologies, Wilmington, Germany), a Porvair Ultravap evaporator (Porvair Sciences, Leatherhead, United Kingdom), and a FluidX Xat-384 automated thermal sealer (FluidX, Cheshire, United Kingdom). All modules were programmed and controlled by Tecan Evoware 2.3 SP3 (Tecan, Männedorf, Switzerland). Polymeric mixed-mode cation-exchange resin Strata-X-C SPE cartridges (30 mg, 2 ml) were from Phenomenex (Torrance, CA, USA).

Solid-phase extraction

During the first step, approximately 200 μl of each whole-blood sample was transferred to the first 96-well deep-well plate on a balance. The plate was re-weighed after each aliquot of whole blood was transferred to accurately record the amount of sample dispensed.

In the next step, 20 μl of IS was added to each sample. Following dilution of the samples with 800 μl of 1% formic acid in water, the plate was heat-sealed, shaken for 1 min on the orbital shaker, and centrifuged at 1000 g for 10 min. After centrifugation, the supernatant was loaded onto pre-conditioned SPE columns (pre-conditioned and equilibrated with methanol followed by water).

The columns were washed consecutively with 900 μl water, followed by 900 μl 2% formic acid in 5% methanol. Elution was performed with 250 μl of methanol. This eluate was further diluted with 250 μl mobile phase solvent A before analysis.

Liquid chromatographic conditions

Chromatography was performed with an ACQUITY UPLC system (Waters Corporation, Milford, USA) using the chromatographic method described by Humbert *et al.*,[8] Rosano *et al.*,[9] and in the application notes from Waters (Waters Corporation, Milford, USA). The column was a 150 mm × 2.1 mm, 1.8 μm ACQUITY UPLC HSS C_{18} maintained at a column temperature of 50°C and eluted at a constant flow rate of 0.4 ml/min. The gradient was 13% solvent B (0–0.5 min), 13–50% solvent B (0.5–10 min), and 50–95% solvent B (10–10.75 min); the column was then flushed with 95% solvent B (10.75–12.25 min). The total run time was 15 min. The injection volume was 15 μl.

Mass spectrometry

Mass spectrometry was performed using a SYNAPT G2 (Waters MS Technologies, Manchester, UK) orthogonal acceleration quadrupole time-of-flight mass spectrometer. The mass spectrometer was operated in positive or negative ion mode with electrospray ionisation (Z-spray). The nebulisation gas was set to 800 L/h at a temperature of 550°C. The cone gas was set to 10 L/h and the source temperature to 120°C. The capillary voltage was set to 300 V and 2600 V in positive and negative mode, respectively. The cone voltage was set to 20 V and 16 V for positive and negative mode, respectively. Argon was used as the collision gas. For the MS^E experiment, two acquisition functions with different collision energies were used in the trap collision cell. The low energy function (LE) was set to 4 eV, and the high energy (HE) function was set to a collision energy ramp from 10 eV to 40 eV. The SYNAPT G2 was operated in resolution mode (V optics mode) with a resolution of 18,000 for *m/z* 556 (FWHM). The data acquisition rate was 0.15 sec/scan in the LE and HE function with 0.024 sec interscan delay; data was collected from 1 min to 13 min. The mass spectrometer was calibrated to <2 mDa mass error prior to each batch. All analyses were acquired using LockSpray to ensure accuracy and reproducibility; leucine-enkephalin was used as the lock mass (m/z 556.2771 and 554.2615 for positive and negative mode, respectively) at a concentration of 400 ng/ml and a flow rate of 50 μl/min. Data were collected in centroid mode from *m/z* 50–950.

MassLynx version 4.1, SCN 803 software (Waters Corporation, Milford, USA) with automated data processing (ChromaLynx XS) was used for data processing. The analytes were identified by the exact mass of the precursor and fragment ion, as well as the retention time. Tolerance was set to 3 mDa for the precursor ion and ± 0.2 min for the retention time. The retention times, precursor, and fragment ions are shown in Table 1.

Method validation

Matrix effects and extraction recoveries were evaluated on the basis of absolute peak areas. Eight authentic case samples of whole blood (four post-mortem samples and four ante-mortem samples) and two blank blood samples from the blood bank were used. Two sets of the 10 whole-blood samples were extracted according to the SPE procedure. The first set was spiked with 32 compounds (the 30 target compounds and the two internal standards, hexemal and paracetamol-D4) after SPE (B), and the second set was spiked before extraction (C) to obtain corresponding concentrations in whole blood (5 mg/kg). The B and C experiments were performed in duplicate for all 10 samples. A reference solution (A) was prepared in quadruplicate by "extracting" purified water using the SPE set-up, and then spiking the extract in the same manner as the B samples in whole blood. Matrix effect (ME), extraction recovery (RE), and process efficiency (PE) were calculated for all 32 compounds as described by Matuszewski *et al.*[10]

Table 1. Ionization mode (IM), retention time (RT), molecular formula (MF), precursor ion (PI), fragment ion (FI), recovery (RE), matrix effect (ME), process efficiency (PE), RT variation within-run and between-run

Analyte	IM	RT	MF	PI*	FI*	RE	ME	PE	RT variation within-run	RT variation between-run
		min		*m/z*	*m/z*	% Mean (range)	% Mean (range)	% Mean (range)	CV (%)	CV (%)
Caffeine	ES +	2.00	C8H10N4O2	195.0882	138.0669	30 (16-53)	108 (95-137)	34 (15-62)	0.21	0.35
Carbamazepine	ES +	7.10	C15H12N2O	237.1028	194.0970	72 (67-76)	97 (94-105)	70 (64-73)	0.06	0.12
Carbamazepine, 10,11-epoxid-	ES +	5.45	C15H12N2O2	253.0977	180.0813	36 (29-47)	95 (86-104)	34 (28-45)	0.09	0.14
Carbazepine, 10-hydroxy-	ES +	4.64	C15H14N2O2	255.1134	194.0970	67 (58-73)	98 (93-117)	65 (60-70)	0.09	0.07
Carisoprodol	ES +	8.25	C12H24N2O4	261.1814	176.1287	71 (60-82)	95 (80-123)	67 (50-81)	0.08	0.08
Chlorzoxazone	ES -	6.12	C7H4ClNO2	167.9852	132.0079	67 (63-73)	94 (86-104)	63 (57-71)	0.10	0.16
Demoxepam	ES +	6.79	C15H11ClN2O2	287.0587	269.0482	25 (20-30)	97 (92-110)	24 (19-31)	0.07	0.11
Diclofenac	ES -	11.27	C14H11Cl2NO2	294.0089	250.0187	19 (10-26)	78 (45-119)	14 (9-22)	0.00	0.05
Diemal (Barbital)	ES -	2.82	C8H12N2O3	183.0770	183.0770	37 (30-44)	100 (86-113)	37 (26-45)	0.00	0.29
Enphenemal	ES -	7.32	C13H14N2O3	245.0926	245.0926	63 (49-75)	97 (76-110)	61 (51-71)	0.08	0.14
Etodolac	ES -	11.16	C17H21NO3	286.1443	242.1544	37 (32-41)	81 (62-97)	30 (22-37)	0.00	0.04
Furosemide	ES -	6.58	C12H11ClN2O5S	328.9999	285.0098	43 (34-50)	83 (53-104)	35 (22-46)	0.00	0.12
Ibuprofen	ES -	11.39	C13H18O2	205.1229	161.1344	35 (14-53)	75 (39-101)	30 (13-54)	0.00	0.05
Levetiracetam	ES +	1.62	C8H14N2O2	171.1134	126.0919	17 (11-22)	98 (92-113)	17 (10-22)	0.25	0.27
Meprobamate	ES +	4.81	C9H18N2O4	219.1345	158.1181	70 (62-81)	97 (92-113)	68 (60-74)	0.06	0.12
Naproxen	ES -	9.30	C14H14O3	229.0865	170.0726	59 (39-93)	132 (95-201)	70 (48-94)	0.05	0.1
Oxcarbazepine	ES +	5.85	C15H12N2O2	253.0977	208.0762	64 (53-74)	83 (67-97)	53 (43-62)	0.07	0.07
Paracetamol	ES +	1.45	C8H9NO2	152.0712	110.0606	51 (44-58)	96 (91-112)	49 (43-57)	0.20	0.26
Pentobarbital	ES -	7.25	C11H18N2O3	225.1239	225.1239	66 (55-90)	91 (81-102)	60 (55-66)	0.04	0.13
Phenobarbital	ES -	5.08	C12H12N2O3	231.0770	231.0770	71 (55-90)	99 (84-116)	70 (54-88)	0.06	0.18
Phenytoin	ES -	7.10	C15H12N2O2	251.0820	208.0791	56 (29-71)	84 (75-96)	47 (28-63)	0.07	0.14
Primidone	ES +	3.42	C12H14N2O2	219.1134	162.0919	69 (60-75)	98 (93-120)	67 (59-74)	0.19	0.05
Salicylamide	ES +	3.16	C7H7NO2	138.0555	121.0290	68 (58-76)	100 (90-128)	68 (61-77)	0.09	0.20
Salicylic acid	ES -	4.45	C7H6O3	137.0239	93.0337	70 (66-76)	96 (88-106)	67 (59-76)	0.09	0.3
Theophylline	ES +	1.39	C7H8N4O2	181.0726	124.0511	23 (16-39)	103 (96-139)	24 (16-43)	0.36	0.34
Thiopental	ES -	8.89	C11H18N2O2S	241.1011	241.1011	67 (61-75)	130 (113-141)	87 (76-94)	0.06	0.12
Tolbutamide	ES +	8.52	C12H18N2O3S	271.1116	91.0540	57 (51-68)	60 (49-70)	35 (27-42)	0.07	0.12
Topiramate	ES +	6.04	C12H21NO8S	340.1066	264.0542	66 (53-74)	102 (85-132)	67 (56-76)	0.07	0.14
Warfarin	ES +	10.31	C19H16O4	309.1127	251.0708	31 (25-38)	81 (74-88)	25 (20-33)	0.10	0.06
Zonisamide	ES +	3.67	C8H8N2O3S	213.0334	132.0449	68 (54-82)	102 (94-125)	69 (56-82)	0.11	0.19
Hexemal (Internal Standard)	ES -	5.91	C12H16N2O3	235.1083		68 (57-81)	93 (83-100)	63 (56-73)		
Paracetamol D4 (Internal Standard)	ES +	1.47	C8H5D4NO2	156.0961		51 (44-58)	96 (91-112)	49 (43-57)		

* There is a difference of around 0.5 mDa between the theoretical mass and the calculated mass, as the software adds the mass of a hydrogen atom instead of the mass of a proton.

The retention time variation within and between runs was tested twice in each run over six days.

The limit of detection was not established directly, but a QC cut-off level was monitored in each series by running a quality QC cut-off sample spiked at one level (1 mg/kg) at the start and end in each series.

Results and discussion

Sample preparation

The fully automated sample preparation used in this study was part of a composite robotic set-up in which the mixed mode SPE fractions were divided into acid/neutral drugs and basic drugs. This set-up has a big advantage of only 200 μl of whole blood being used for multiple LC-MS instruments. Other procedures require as much as 1 ml[1] of whole blood, or 200 μl[2] and 100 μl[6] for acidic and neutral screening only.

The fully automated sample preparation worked well for both ante-mortem and post-mortem whole-blood samples, as the weight of the samples were checked on a balance. If the blood amount was less than 150 mg, the pipetting was repeated automatically up to three times. This step prevented clotted or viscous blood samples to fail. A study showed that less than 2% of the blood samples failed three times in the same pipetting system.[11]

Using the robotic set-up, a run or batch with 96 whole-blood samples can be fully processed to injection-ready samples in roughly 3 h. This result presents a significant reduction in both overall preparation time and manual steps. Furthermore, the 96-well plate format offers a cost-saving and time-efficient alternative to using individual vials and eliminates the risk of sample mix-up during the transfer of vials to the UPLC autosampler system.

Screening by UPLC-TOF-MS^E and ChromaLynx XS

The chromatographic separation method used in this study was developed by Humbert *et al*. and has been successfully used by a number of groups,[8,9] as well as our in-house screening of basic drugs. In our in-house library, we have the retention times for more than 850 compounds and this acidic and neutral drug screening can easily be expanded to more drugs as long as they elute in the acidic and neutral fraction from the automated SPE set-up. The retention time of each acidic and neutral compound is checked in every batch, and has proven to be stable within and between days (Table 1).

When setting up a screening method on a TOF instrument, universal values for capillary, cone, and collision energy are applied. We found that the optimal capillary voltage in positive mode should be set at a very low voltage (0.3 kV), whereas the optimal capillary voltage in negative mode was set at a high voltage (2.6 kV). The cone voltage was optimized to low voltages for positive mode (20 V) and negative mode (16 V). The collision energy (MS^E) was set to ramp from 10 eV to 40 eV in both positive and negative mode, and the most abundant fragment ion for each compound was determined (Table 1) and used as additional identification in ChromaLynx XS.

We tested peak saturation by injecting the concentrated standard mixture (working solution). This did not cause any saturation problems.

The SYNAPT G2 does not have the ability to do positive/negative switching; therefore, each sample was injected twice, one time in each mode. The total runtime of 30 min is considered adequate for this analysis, when less than 50 samples are screened every week.

The data processing software ChromaLynx XS is easy to use for data acquired in MS^E mode. The compound names, molecular formulas, expected retention times, and exact mass of the fragment ions were typed in a text file (Library or Database). ChromaLynx XS was then set to display targets that fulfilled the criteria: ± 0.2 min retention time, ± 3 mDa, and area count above 10 arbitrary units. The displayed targets were considered positive findings if a fragment were identified; or in the case of no fragment, the retention time, mass accuracy, and isotopic pattern was studied closer. When we use this screening methodology on general cases, normally none to two targets are identified in each case, so each data set is easy to go through.

The data processing is similar to procedures based on LC-MS/MS with TargetLynx. TargetLynx is great for quantitation but less suitable for screening, as every peak has to be evaluated.

Validation

The average and range of matrix effects, extraction recoveries, and process efficiency for the 30 tested target compounds and two internal standards from the measurement of 10 samples (both ante-mortem and post-mortem) are provided in Table 1. Even though compounds such as levetiracetam had a process efficiency as low as 10% because of low recovery, the method is still sufficient as a screening method, as the lower therapeutic level of levetiracetam is ten times higher than the QC cut-off.

The RT variation within and between runs was less than 0.37 (%CV) for all compounds, which can be considered to be very robust (Table 1).

The target compounds were always found in the QC cut-off samples in each series. Therefore, we can conclude that the LOD is at least 1 mg/kg. This result is similar to other procedures[2] and is sufficient in regards to acidic and neutral drugs.

Conclusion

We present a validated UPLC-TOF-MS^E method for screening of 30 acidic and neutral drugs in whole blood with a run time of 2 x 15 min and fully automated sample preparation using 200 μl of whole blood. The procedure can easily be upgraded to include more drugs.

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