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# Development of a Systematic Toxicological Screening Method using an automated online-SPE-LC-QqTOF System (XLC-QqTOF)

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## Abstract

Aim: The aim was to develop a highly automated online-SPE-LC-QqTOF (XLC-QqTOF) screening method for small neutral and basic molecules and to test an in-house mass spectral reference library for compound identification.

Methods: All analyses were carried out on a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer coupled to an automated online-SPE-HPLC system. A checkmix was designed for system control, to optimize mobile phase and to test different SPE types. The MS/MS spectra acquired in positive ESI mode were automatically searched against the high resolution mass spectral library GKB Berlin 1.0 (400 toxicologically relevant compounds) via the data management software Smile MS.

Results and Discussion: Best analytical performance in terms of peak areas and asymmetries was found using a weak cation exchanger sorbent (OASIS WCX, Waters) at pH 6. In the STA of the first incoming 20 intoxication samples 21 analytes were additionally found compared to routine STA methods (IA, GC-NPD/FID, LC-DAD, TOX.I.S.).

Conclusion: An automated XLC-QqTOF method was capable of identifying xenobiotics in human plasma within 30 min from sample receipt to the final, comprehensive report.

## 1. Introduction

The liquid chromatographic-hybrid quadrupole time-of-flight mass spectrometric (LC-QqTOF) instrumentation is on the rise in routine systematic toxicological analysis (STA) which requires a fast and reliable detection of toxicologically relevant compounds [1]. Several LC-MS/MS methods have been developed in the last few years [2, 3]. Due to safer sampling handling, higher throughput and time-savings an increasing attention in the toxicological field is devoted to online-SPE [4]. The new XLC-QqTOF system combines automated sample preparation by online-solid phase extraction (SPE), liquid chromatography (LC) and detection of the accurate masses of parent and product ions.

Our purpose was the development of an analytical method for the detection of neutral and basic compounds in human plasma. An automated mass calibration procedure and the analysis of a checkmix-solution were introduced to test and maintain the performance of the whole analytical system. The extraction performance of several weak cation exchange (WCX)- and reversed phase (RP)-sorbents were tested. The final analytical procedure was applied to the first incoming authentic samples from intoxications.

## 2. Material and Methods

### 2.1. Chemicals

Formic acid (>98%, p.a.) were from Fluka (Buchs, Switzerland) and methanol were from ULC-MS grade (Biosolve, Valkenswaard, The Netherlands). Acetic acid (96%, p.a.) and ammonium acetate (p.a.) were from Merck (Darmstadt, Germany). The deionized water was produced by an in-house water purification system Aqua RO 5-20 (membraPure, Bodenheim, Germany). Stock solutions of the standards were used at the highest available purity.

### 2.2. Standard solutions

For the mass calibration an ethanolic mass calibration solution of purine at 0.01 g/L, hexakis(1H,1H,3H-tetrafluoropropoxy)-phosphazine at 0.01 g/L and tris(heptafluoropropyl)-1,3,4-triazine at at 0.1 g/L was used. For the system performance control and the optimization of the analytical method a methanolic checkmix-solution containing following analytes (concentration in mg/L) with a broad spectrum of physicochemical properties was applied: Histamine (12.5), MDMA (5.0), rocuronium (5.0), haloperidol (1.25), carbamazepine (1.25), diazepam (1.25), amiodarone (1.25).

### 2.3. System performance control

The chromatographic system as well as the QqTOF mass spectrometer was tested by injecting 1 µL of checkmix-solution. Retention times and m/z masses of the analytes were documented.

### 2.4. Extraction procedure

Following five weak cation exchange (WCX) and four reversed phase (RP) sorbents were compared by analyzing an aqueous solution of the checkmix-compounds: OASIS WCX/HLB, 10 mm x 1 mm (Waters, Milford, US); STRATA XWC, 10 mm x 2 mm, (Phenomenex, Torrance, US); HySphere MMWC, 10 mm x 1 mm, 25 µm / 10 mm x 2 mm, 10/25 µm and HySphere C8 EC-SE/Resin GP/Resin SH, 10 mm x 2 mm (Spark Holland, Emmen, Netherlands). In the first stage of SPE optimization the SPE-cartridge with the highest peak areas of the checkmix-anayltes were selected from the group of WCX- and RP-sorbents. After optimization, both SPE-procedures were compared in terms of peak areas and peak asymmetries (=[Rt (min)-peak start (min)]/[peakend (min)-Rt (min)]). The online-SPE was carried out with 10 mM ammonium acetate solution at pH 6 for WCX sorbents and with 10 mM ammonium hydrogencarbonate solution at pH 9 for RP sorbents using XLC Symbiosis Pico system (Spark Holland, Emmen, Netherlands). For elution the SPE cartridge was switched into the flow of the LC gradient. In order to reduce matrix effects a protein precipitation was applied to plasma samples prior online-SPE:

### 2.5. Liquid Chromatography

The chromatographic separation including equilibration time for the LC-run was achieved within 17 minutes run time on a Luna Pentafluorophenyl PFP column (150 x 2.0 mm, 5 µm) from Phenomenex (Aschaffenburg, Germany) at 50°C using a gradient consisting of a mixture of 0.2% formic acid (A) and methanol (B) at a flow rate of 0.3 mL/min.

2.6. QqTOF mass spectrometry

The accurate mass measurement was performed on a QStar Elite (AB Sciex, Foster City, US) in positive electrospray ionization mode (+ESI). Prior to each analytical run the mass calibration solution (1 µL) was directly injected into the mass spectrometer for automated calibration. The IDA method generated a survey scan in single MS mode (TOF MS) at *m/z* 101-950 followed by product ion spectrum acquisition (TOF MS/MS) at *m/z* 50-950 of two most intense ions. The intensity threshold was set to 100 counts and exclusion time to 6 sec. The cycle time was 3 sec at maximum.

2.7. Data management

The acquired data were exported, processed and evaluated using SmileMS software (version 1.1) from GeneBio (Geneva, Switzerland). The experimental QqTOF MS/MS spectra were compared to the in-house mass spectral library containing high resolution mass spectra of 400 compounds at both automatic collision energy (ACE) and collision energy spread (CES: 20/35/50 eV) conditions. A mass tolerance of 0.01 Da, a retention time tolerance of 0.5 min and a score above 0.2 were empirically determined for positive matching. The library search results (score, Δm/z, Δ Rt) were displayed in a comprehensive report.

## 3. Results and Discussion

The checkmix-analytes were appropriate to cover a broad range of retention times (1.8-11.5 min) and m/z masses (112.086923- 646.03094 Da). In combination with the automated external mass calibration the system performance control over a period of six months the resolution were 15,000 at maximum, the mean mass accuracy below 5 ppm and the CV of the Rts below 5% for all checkmix-analytes [5].

Highest peak areas were observed using OASIS WCX at pH 6 and OASIS HLB at pH 9. Peak areas and asymmetries of the checkmix-analytes after SPE-optimization are monitored in table 1.

Tab. 1. Average peak areas (x E+03 counts) and peak asymmetries of the checkmix-analytes.

| **Sorbent** | | **OASIS HLB** | | **OASIS WCX** | |
|---|---|---|---|---|---|
| Dimension | | 10 mm x 1 mm | | 10 mm x 1 mm | |
| SPE conditions | | RP at pH 9 | | WCX at pH 6 | |
| **Tested parameters (average, n=3)** | | **Peak area ($xE^3$ counts)** | **Peak asymmetry** | **Peak area ($xE^3$ counts)** | **Peak asymmetry** |
| **Checkmix-analytes** | Paracetamol | 107,3 | 3,04 | 150,0 | 11,01 |
| | MDMA | 121,0 | 2,66 | 224,7 | 5,67 |
| | Rocuronium | 83,7 | 2,42 | 21,9 | 15,27 |
| | Haloperidol | 31,0 | 3,38 | 36,2 | 7,50 |
| | Carbamazepine | 66,6 | 2,81 | 145,7 | 2,31 |
| | Diazepam | 45,8 | 3,30 | 57,7 | 4,56 |
| | Amiodarone | 150,7 | 6,56 | 259,0 | 12,78 |

The OASIS WCX cartridge showed the highest peak areas for 6 of 7 analytes. It is believed that the high extraction performance but also the high peak asymmetry of the WCX-cartridge was due to the ionic interaction between the positively charged analytes and the negative carboxylate-anchor groups of the sorbent. Nevertheless the uncompleted elution of the quaternary ammonium compound rocuronium as well as the extreme peaktailing of the analytes could be improved by increasing the formic acid concentration in the mobile phase from 0.2% up to 1.0%.

The intoxication samples were analyzed by routine STA (IA, GC-NPD/FID, LC-DAD, TOX.I.S.) and by the new XLC-QqTOF method described above. All neutral and basic compounds found in routine STA. In addition to all neutral and basic compounds found in routine STA, 21 substances of toxicological interest (e.g. 7-aminoflunirazepam, amiodarone, bisoprolol, fentanyl, ketamine, metformine, methadone, venlafaxine) were detected. An example for the detection of a very broad compound spectrum was case nr. 1. Even the polar metformine (c=9.46 mg/L), bisoprolol (c=0.38 mg/L) and the lipophilic amiodarone (c=0.18 mg/L) were found (see fig.1).

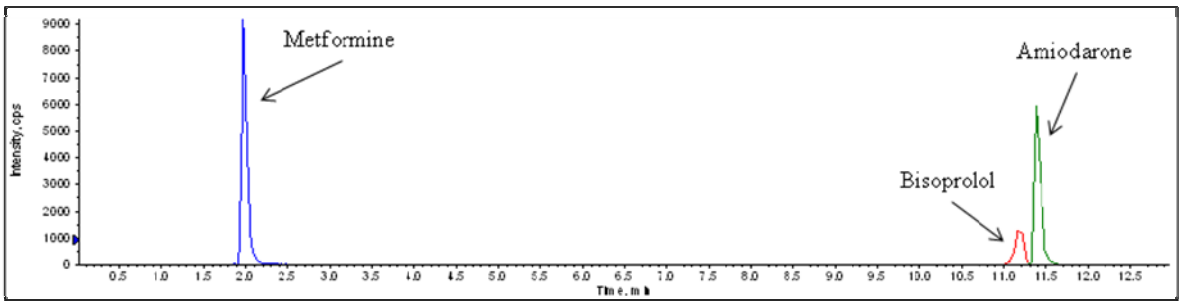


Fig.1. Extracted chromatograms of the analytes found in an intoxication sample (case nr. 1) by XLC-QqTOF.

Also the detection of the acidic Ibuprofen at 41.6 mg/L (case nr. 2) showed that the universal STA-procedure was suitable for the identification of basic, neutral and acidic xenobiotics of toxicological interest. In this case beside the parent ion (m/z=207.137956 Da) the in-source-fragment (m/z=161.132477 Da) were detected.

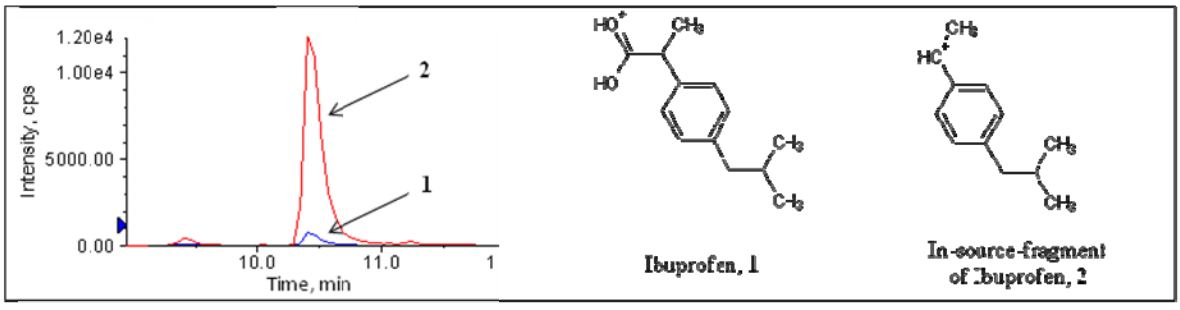


Fig. 2. Extracted chromatograms and chemical structures of ibuprofen and its in-source-fragment found in an intoxication sample (case nr. 2) by XLC-QqTOF.

## 4. Conclusion

A highly automated screening method for the detection of xenobiotics in human plasma samples using XLC-QqTOF was developed. The whole analytical procedure including sample preparation and final report was completed within 30 min. Compared to routine STA using multiple methods the analysis of authentic intoxication samples showed good correlation. In some cases true positives were additionally identified. The in-house high resolution MS/MS-library applied in this new screening method contained 800 spectra of 400 toxicological relevant compounds and will be expanded continuously.

## 5. References

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