

Automated ion trap LC-MS screening for xenobiotics in vitreous humour

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Aims: The aim of this project was to evaluate the feasibility of a previously developed automated LC-MSⁿ screening approach for the detection of xenobiotics in vitreous humour to extend its application to post-mortem analysis. **Methods:** After addition of deuterated standards, 1 ml of vitreous humour was extracted using a previously published solid-phase extraction protocol [1]. Analysis was performed on a Dionex Ultimate RSLC coupled to a Bruker amaZon speed ion trap. Identification of analytes and reporting was carried out fully automated using the Toxtyper workflow. Drug-free bovine vitreous humour fortified with 57 compounds of forensic interest at three different concentrations (levels were adjusted to requirements in post-mortem cases) were analysed for method evaluation. In addition, the presented workflow was applied to vitreous humour from autopsy cases. **Results:** Limit of automated identification was set to the lowest concentration level that could be identified correctly in duplicate determination. Approximately 90 % of the compounds could be detected and identified correctly at or below typical 'therapeutic' concentrations reported in the literature (5 to 50 ng/ml). In vitreous humour of 24 autopsy cases 74 % of the active substances consumed by the deceased could be identified in accordance to routine post-mortem analysis. Half of the analytes not detected in vitreous humour could only be detected at very low serum concentrations or exclusively in the corresponding urine sample. **Conclusion:** The applied screening approach is a suitable tool for the detection and identification of xenobiotics in vitreous humour. For further evaluation, a larger set of cases where blood, urine and vitreous humour are available will be analysed to compare the different findings and assess the effectiveness of this new post-mortem screening approach.

1. Introduction

Comprehensive screening for xenobiotics is a crucial part of post-mortem toxicological analysis. Urine and/or blood are the matrices of choice for systematic toxicological analysis but the sample volume preserved during autopsy is sometimes very limited. In such cases, analysing tissue samples like liver can be a suitable but laborious option. Vitreous humour is an easy-to-handle body fluid only little affected by putrefaction and therefore a smart alternative to urine or blood. Due to the blood-retinal barrier, comparability of quantitative results in vitreous humour with serum concentrations is complicated, but still it can be regarded as a suitable matrix for qualitative screening analysis.

When analysing body fluids, liquid chromatography - mass spectrometry has become the method of choice for a wide range of analytical questions. In this project, a previously developed automated LC-MSⁿ approach was evaluated for the detection of drugs and drugs of abuse in vitreous humour to extend its application to post-mortem analysis.

2. Material and Methods

For evaluation of the limits of detection, bovine vitreous humour was fortified with different mixtures of analytes.

In total, 57 substances distributed to six mixtures were analysed at three different concentrations (low, medium and high). Medium concentration levels were adjusted to vitreous humour concentrations reported in the literature [2-4] and cut-offs of other established screening methods [1]. In addition, vitreous humour of 24 autopsy cases was analysed with the presented method. For evaluation, the screening results were compared with the findings of the routine post-mortem analysis.

Human and fortified bovine vitreous humour (1 ml each) was extracted using an established two-step solid phase extraction procedure (SPE) [1]. A mixture of three deuterated standards (D5-Diazepam, D3-Doxepin, D4-Halperidol) and 2 ml of phosphate buffer (pH 6) were added to the vitreous humour sample. SPE-cartridges (Isolute[®] HXC-5, 100 mg, 3 ml, Biotage Uppsala, Sweden) were conditioned using 2 ml methanol, 2 ml water and finally 3 ml phosphate buffer (pH 6). After loading the sample, the cartridge was washed with 1 ml phosphate buffer (pH 6) and 1 ml acetic acid (1 M). After each of the two washing steps the cartridge was dried for 5 min. The first elution step was performed by 3 ml ethyl acetate/hexane (25:25, v/v). The cartridge was dried for 2 min, washed with 1 ml methanol and dried again for 2 min. The second elution step was performed by 3 ml ethyl acetate/ammonia (98:2, v/v), 25 % ammonia solution in water. Both eluates were combined, evaporated to dryness at 40 °C under a gentle stream of nitrogen and reconstituted with 25 µl LC-eluent A/B (50:50, v/v). 2 ml of the extract were injected into the LC-MS system.

The screening analysis was performed using the Toxyper[®] approach [5]. The LC was a Dionex UltiMate 3000 LC-system (Thermo Fisher Scientific, Idstein, Germany), including a degasser, an autosampler, a binary pump and a column oven. All analyses were performed using solvent A (0.1% formic acid with 2 mmol/L ammonium formate and 1 % acetonitrile (v/v)) and solvent B (acetonitrile: 0.1% formic acid with 2 mmol/L ammonium formate and 1 % H₂O dest.). Separation was performed using an Acclaim[®] RSLC 120 C18 2,2 µm 120A 2.1x100 mm column (Thermo Fisher Scientific, Idstein, Germany) using the following gradient: 0-1 min: 1% B; 1-8 min: 1-95% B linear; 8-9 min 95% B; 9-9.06 min 95-1% B linear; 9.06-11 min: 95% B. The total flow rate was set to 0.5 ml per minute.

The MS was an amaZon speedTM ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) fitted with a standard ESI-source and was operated in autoMSⁿ mode using UltraScan with a mass range from 70 to 800 amu at 32,500 amu/s. AutoMSⁿ spectra were generated up to n = 3 if possible.

3. Results and Discussion

3.1. Detection limits in bovine vitreous humour

Approximately 94 % of the compounds could be detected and identified correctly at each concentration level investigated. Mirtazepine could only be detected at medium (10 ng/ml) and high concentration levels (20 ng/ml) while tramadol and olanzapine could only be identified correctly at high concentration levels (25 and 150 ng/ml, respectively). LSD was the only compound that could not be detected at all, probably due to its sensitivity to light. Limit of detection was set to the lowest concentration level that could be identified correctly in duplicate determination.

3.2. Analysis of vitreous humour from autopsy cases

The results from real cases were in good agreement with the findings from routine post-mortem analysis. In vitreous humour of 24 autopsy cases (c1 - c24) 76 % of the active agent consumed by the deceased could be identified in accordance to routine post-mortem analysis.

Tab. 1. Limits of detection evaluated in bovine vitreous humour.

Limit of detection	Analyte
5.0 ng/ml	Buprenorphine, carbamazepine, cocaine, diazepam, melperone, methadone, metoprolol, phenazone, propranolol, venlafaxine, zolpidem
10 ng/ml	Acebutolol, amitriptyline, bisoprolol, codeine, fentanyl, flunitrazepam, methamphetamine, mianserine, mirtazepine, moclobemide, phencyclidine, trimipramine, sulpiride, warfarin
12.5 ng/ml	6-MAM, atenolol, clozapine, diltiazem, MDE, MDMA, midazolam, morphine, nortriptyline, oxycodone
25 ng/ml	Amphetamine, fluoxetine, fluvoxamine, risperidone, sotalol, tramadol
50 ng/ml	Carvedilol, chloroquine, citalopram, doxepin, paroxetine, verapamil, zopiclone
150 ng/ml	Benzoyllecgonine, dixyazine, olanzapine, phenytoin, sildenafil

The almost ubiquitous compounds caffeine, nicotine and their metabolites as well as alcohol and its metabolite ethyl glucuronide were excluded from this evaluation. In six cases (25 %) screening results from vitreous humour perfectly matched the results of routine post mortem analysis.

In cases c2, c6 and c24, paracetamol and/or ibuprofen were the only compounds not detected in vitreous humour. It is known, that compounds with high polarity (e.g. paracetamol or oxazepam) and/or a high plasma protein binding rate (e.g. ibuprofen) only poorly pass the blood-retinal barrier and therefore are not necessarily detectable in vitreous humour by a general screening approach.

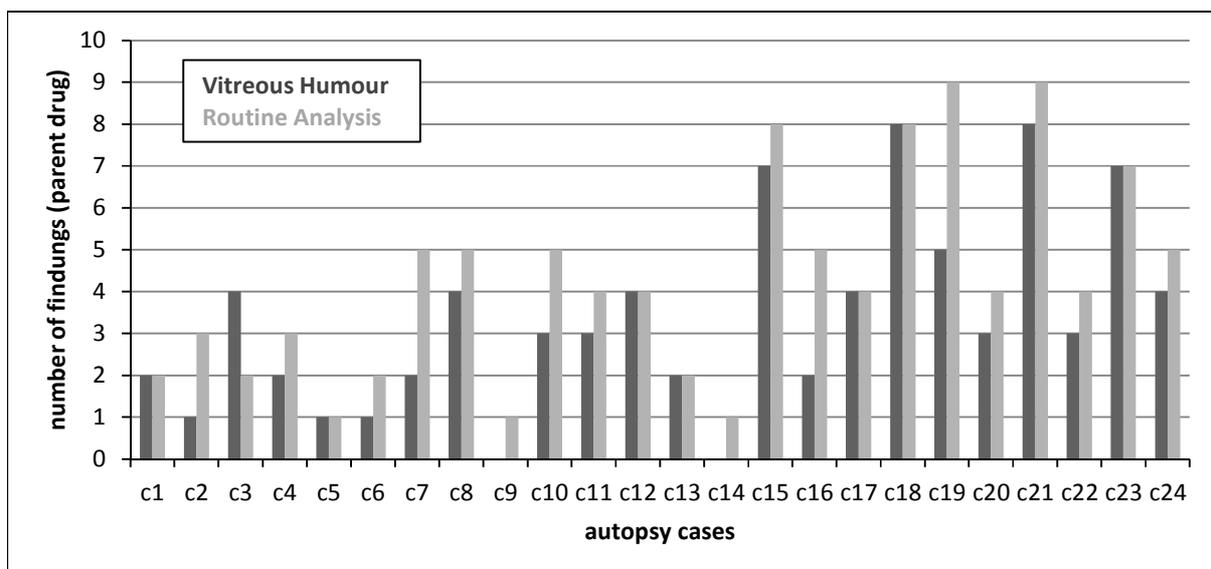


Fig. 1. Number of findings (parent drugs only) in routine post-mortem analysis and vitreous humour of 24 autopsy cases.

Olanzapine was the only compound not detectable in vitreous humour in cases c9 and c22. Serum concentrations of olanzapine were 94 and 190 ng/ml. Nevertheless, sample c15 shows

that olanzapine is detectable in vitreous humour although only identified in urine and stomach content of the deceased. Sample c15 illustrates, that the ingestion of multiple drugs can be detected easily in vitreous humour. Promethazine could not be identified in vitreous humour but serum levels at the time of death turned out to be below 10 ng/ml.

Although norbuprenorphine and THC-COOH could be detected in some of the cases investigated, a targeted LC-MS approach seems mandatory for the detection of low dosed drugs like buprenorphine ($C_{SERUM} < 0.8$ ng/ml in c10) and other selected analytes like THC and GHB.

Tab. 2. Results of selected post-mortem cases.

Case	Findings in vitreous humour	Results of routine post-mortem analysis
c3	Midazolam Lidocain Sildenafil Flecainide	Midazolam and metabolite Lidocain
c10	Diazepam Nordazepam Sertraline Lorazepam	Diazepam Nordazepam Sertraline Lorazepam Temazepam Oxazepam Buprenorphine and metabolite THC and metabolites
c11	Citalopram Methadon and EDDP	Citalopram Methadon and EDDP THC metabolites GHB (endogenous)
c15	Trimipramine Zopiclone Quetiapine 7-Aminoflunitrazepam Oxazepam Olanzapine Desmethylvenlafaxine	Trimipramine and metabolite Zopiclone Quetiapine and metabolite Flunitrazepam and metabolites Oxazepam Olanzapine Venlafaxine and Metabolite Promethazine

In some cases the intake of a substance could neither be confirmed in the corresponding body fluids nor by the case history: In case c3 a male person (age 21) died in hospital one day after a cardiac seizure. Routine post-mortem analysis revealed the intake of midazolam and lidocaine, probably administered during hospitalization. In addition, the antiarrhythmic flecainide and sildenafil could be identified in vitreous humour. Nevertheless, there were no additional findings or information from the case history that would have confirmed the intake of these two compounds. In case c12, zopiclone was detected in vitreous humour besides various others drugs but not identified during routine analysis. According to the police record, the deceased was under treatment and self medication with unspecified narcotics and analgesics.

For final method evaluation a confirmatory analysis to determine vitreous humour concentrations of at least the most common substances found in post-mortem analysis is mandatory.

4. Conclusions

The applied screening approach is a suitable tool for the detection of xenobiotics in vitreous humour. Besides the known physiological limitations of the matrix itself, the obtained limits of detection seem to be adequate for forensic casework. The easy handling of vitreous humour when compared to sample preparation of tissue samples combined with the LC-MSⁿ analysis and automated data evaluation of the Toxtyper present a time- and cost-effective alternative if no urine and/or only a limited volume of blood is available.

Due to the lack of human samples acquirable under controlled conditions, analysis of fortified bovine vitreous humour to evaluate LODs of additional compounds of interest and further comparison of urine, blood and vitreous humour findings in post-mortem cases are necessary to assess the effectiveness of this new post-mortem screening approach.

5. References

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