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Professional paper

Applicability of vitreous humor as an alternative matrix versus blood for post-mortem toxicological screening

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Abstract

There is an increased interest for application of vitreous humor (VH) in forensic examinations, mainly for post-mortem interval determination, biochemical analyses as well as in forensic toxicology. When reference samples for toxicological analyses, such as blood or urine, are unavailable, the use of alternative matrices becomes essential. Due to its anatomical position and chemical properties, VH is a good candidate for suitable alternative to blood. Therefore, the aim of our study was to investigate applicability and usefulness of VH as an alternative matrix for toxicological screening in comparison to blood sample.

Paired samples of blood and VH from 51 autopsied cases were analyzed performing non-targeted, broad spectrum toxicological screening by gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography using photo diode array detector (HPLC-PDA), routinely used for blood screening.

The total number of findings in the examined samples was 207 in blood and 146 in VH. In 16 cases a 100% agreement of the number of detected compounds was noticed. Despite the lower number of findings in VH, no statistically significant difference was observed between the number of detected compounds in respective blood and VH samples.

Analyses of the blood and VH using a method for routine toxicological screening of blood revealed agreement between the numbers of findings in both samples with strong positive correlation, indicating that VH is applicable alternative matrix to blood. The full potential of the VH in forensic toxicology purposes has yet to be evaluated through further studies.

Key words: blood, vitreous humor, postmortem, toxicology, screening

Introduction

Use of vitreous humor (VH) in determination of cause of death has become increasingly common in forensic science (Baniak at al., 2015). Vitreous humor has proven to be an effective tool in diagnosing pathological conditions, hence attracts many forensic experts to discover more postmortem uses of its constituents (Kalra et al., 2016). The most important application of VH for forensic nowadays is the determination of the postmortem interval by measuring VH potassium levels (Baniak at al., 2015; Bévalot et al., 2016; Wójtowicz et al., 2020). Additionally, analyses of other electrolytes such as sodium, chloride, as well as creatinine and lactate in VH are widely used for the same purpose (Baniak at al., 2015). Glucose, ketones, urea, and insulin are other compounds of interest for biochemical analyses of VH, offering

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significant advances in diagnosis of diabetes, ketoacidosis and other ante-mortem pathological conditions (Baniak at al., 2015; Kalra et al., 2016; Wójtowicz et al., 2020). The fact that VH is relatively stable, less susceptible to rapid chemical changes, well protected from decomposition and contamination makes it more acceptable for analysis than other fluids in postmortem biochemical analyses and estimating postmortem interval (Angayarkanni, 2020; Bazmi et el., 2016; Savini et al., 2020).

Advantages of VH as a postmortem sample are due to its anatomical and physiological characteristics. Vitreous humor or vitreous body is the clear gelatinous substance that fills the posterior compartment of the eye (Bévalot et al., 2016; Chronister et al., 2008). Its position provides protection against contaminations by microorganisms and resists decomposition changes that typically occur more rapidly in other biological fluids (Chronister et al., 2008; dos Santos et al., 2016). Vitreous humor is not exposed to other biological fluids and it is also less susceptible to postmortem redistribution phenomenon, which makes it suitable matrix for postmortem toxicological examinations and it is often considered as an alternative biological matrix for the analysis, when classical matrices such as blood or urine are unavailable (dos Santos et al., 2016; Markowska et al., 2017; Wójtowicz et al., 2020). According to Bévalot et al. (2016) an ideal alternative sample should enable detection of the same xenobiotics as found in blood, at correlated concentrations, without postmortem effects and VH is the sample that probably comes closest to this ideal. Suitability of VH as an alternative sample compared to other biological samples has been examined for various groups of compounds. It is routinely used in analyzing postmortem alcohol concentration enabling determination of postmortem production of alcohol (Kalra et al., 2016; Wójtowicz et al., 2020). Widely examined is its use in determination of opiates/opioids (Al-Asmari, 2020; Chronister et al., 2008; Havig et al., 2022; Maskel et al., 2019; Thaulow et al., 2018) as well as fentanyl and its metabolites and analogues (Poklis et al., 2015; Chatterton and Scott-Ham, 2018), benzodiazepines (Bazmi et el., 2016; Øiestad et al., 2023), antidepressants (dos Santos et al., 2016; Ntoupa et al. 2020; Øiestad et al., 2018). Despite that fact, vitreous humor has rarely been used for the screening of drugs (Arora et al., 2016).

When a blood sample is unavailable or altered by postmortem changes, the use of alternative matrix becomes essential. Moreover, since substances are carried from the blood stream through the blood-retinal barrier, VH serves as a convenient alternative sample to blood (Bévalot et al., 2016; Pelander et al., 2010). To the best of our knowledge, only few articles on this topic had been published. One shares the similar conceptualization using gas chromatography mass spectrometry (GC-MS) analyses (Metushi et al., 2016), and the other one used LC time- of-flight (TOF) system for analyzing the applicability of VH as a complementary sample to urine (Pelander et al., 2010). Also, multi-targeted forensic toxicological screening in biological matrices by MRM-IDA-EPI mode had been reported (Franzin et al., 2024). However, we focused on non-targeted, broad spectrum toxicological screening, since qualitative screening of unknown substances is a key step in postmortem forensic toxicological investigation (Pelander et al., 2010). Accordingly, the aim of our study was to investigate applicability and usefulness of VH as an alternative matrix for toxicological screening in comparison to blood sample.

Material and methods

Samples of peripheral blood (femoral or subclavian) and vitreous humor (pulled from both eyes) were collected during autopsies performed at the Institute of Forensic Medicine, Criminology and Medical Deontology at the Medical Faculty in Skopje. Samples of total 51 cases were included in the study. Both samples were stored in sterile tubes containing NaF/oxalate at 2-8 ˚C if the analyses were performed within a week of sampling. All other samples were stored at -20 ˚C.

The pool of drug free whole human blood was obtained from the Institute for Transfusion Medicine, RN Macedonia. Following chemicals were used for preforming the analyses: methanol HPLC grade, Merck (Germany), acetonitrile HPLC grade, Thermo Fisher Scientific (China), formic acid 98% and ammonium hydroxide 25% VWR International (France), $NaH_2PO4 \bullet H_2O$, Merck (Germany), and in-house produced de-ionized water using Heal Force NW Ultra-pure Water System. As for the compounds included in this study, following analytical standards were used: atropine, LGC Standards (UK); ketoprofen and metoclopramide, CPAchem Ltd (Bulgaria); biperiden, cinnarizine and paracetamol, HPC Standards GmbH (Germany); 6-acetylmorphine, alprazolam, bromazepam, cocaine, diazepam, duloxetine, EDDP, ecgonine methyl ester, ketamine, methadone, metoprolol, midazolam, morphine, nordazepam, noscapine, papaverine, pentobarbital, pheniramine, propranolol, thiopental, tramadol, and venlafaxine, Lipomed (Switzerland); valproic acid and zolpidem, Cerilliant Corporation (USA); benzoylecgonine, citalopram, codeine, fluoxetine, lamotrigine, mirtazapine, olanzapine, propofol, and quetiapine, Sigma Aldrich (Germany).

One milliliter of blood and VH samples were pretreated with pH 4 phosphate buffer and 50 µL of internal standard (Procaine 1 µg/mL) was added. Then samples underwent cation exchange solid phase extraction using Oasis MCX cartridges, Waters Co. (USA), following the procedure recommended from the manufacturer. Eluates were dried under the gentle stream of nitrogen, and dry extracts were reconstituted in 0.2 mL methanol. Prepared samples were analyzed in two stages.

First, two microliters of sample were injected into a GC-MS 2010 Ultra System, Shimadzu (Japan) in split mode (1:10). Chromatographic separation of the substances

was performed using nonpolar, 30 m X 0.25 mm X 0.25 µm analytical column Agilent J&W DB-5 from Agilent Technologies (USA) with helium as carrier gas. Instrument parameters were based on previously published method with slight modifications (Metushi et al., 2016). Samples were analyzed in full scan mode (MS mass range 35.00–500.00 amu) with a method used for routine screening. Chromatograms were analyzed using GCMS solution software, Shimadzu (Japan). Peak identification was performed by retention time (RT) using the relative retention time according to the IS, and mass spectra matching with the spectra from commercial MS libraries as well as in-house created mass spectral libraries.

For the second stage of analyses, samples were filtered through a pretreated 0.2 µm syringe filter, Waters (USA) and 50 µL of the sample were injected into Waters Alliance e-2695 separation module HPLC system, using waters 2998 photo diode array detector, equipped with 5 µm, 4.6 mm x 250 mm Symmetry C8 analytical column, Waters Co. (MA, USA). Mobile phase components were pH 3.6 phosphate buffer and acetonitrile. Analyses were performed using gradient elution according to the method for toxicological screening recommended from the manufacturer. Chromatogram analyses were performed using Empower Software Version 3, Waters Co. (USA) in the scan range of 200-350 nm, with extraction at ƛ = 200.3, 230, and 254 nm and comparison of obtained RT and UV spectra with commercial UV libraries, as well as with in-house created UV spectral libraries.

Positive results were confirmed by comparison of RT, MS library match, m/z ratio, and/or UV spectra library match of blood spiked samples. The obtained data were collected, classified, and analyzed using Microsoft Excel 2003, as well as Pearson correlation and the Mann-Whitney U-test. All numerical variables were first tested for normal distribution using the Kolmogorov-Smirnov test (SPSS software 17.0). A p value of 0.05 has been considered statistically significant.

Results and discussion

Paired samples (blood and VH) of 51 autopsy cases conducted between 2023 and 2024 were included in the study. Samples were analyzed with routine screening methods combining two different chromatographic techniques. Using these methods, limit of detection (LOD) for the following compounds was established: diazepam, methadone, mirtazapine, pentobarbital, pheniramine, venlafaxine, zolpidem (0.02 mg/L); 6-acetylmorphine, alprazolam, atropine, biperiden, benzoylecgonine, cinnarizine, citalopram, cocaine, codeine, EDDP, ecgonine methyl ester, ketamine, lamotrigine, metoclopramide, midazolam, nordazepam, papaverine, propofol, thiopental, tramadol, (0.05 mg/L); bromazepam, duloxetine, fluoxetine, ketoprofen, metoprolol, morphine, olanzapine, quetiapine, valproic acid (0.1 mg/L); noscapine, paracetamol, propranolol (0.2 mg/L).

A total number of findings in examined samples were 353, more precisely, 207 in blood and 146 in vitreous humor. For sixteen cases a 100% agreement of the number of detected compounds was observed, including the seven cases with negative results obtained for both samples. The number of detected compounds in blood and VH for all examined cases is shown in Figure 1.

Evaluation of the results revealed good agreement, i.e. there was no statistically significant difference ($z = 1.93419$, $p = 0.0536$) between the number of detected compounds in respective blood and VH samples. Additionally, strong positive correlation between the number of detected compounds in both samples was observed ($r(49) = 0.898$, $p < 0.00001$), with a coefficient of determination $R^2 = 0.81$, shown in Figure 2.

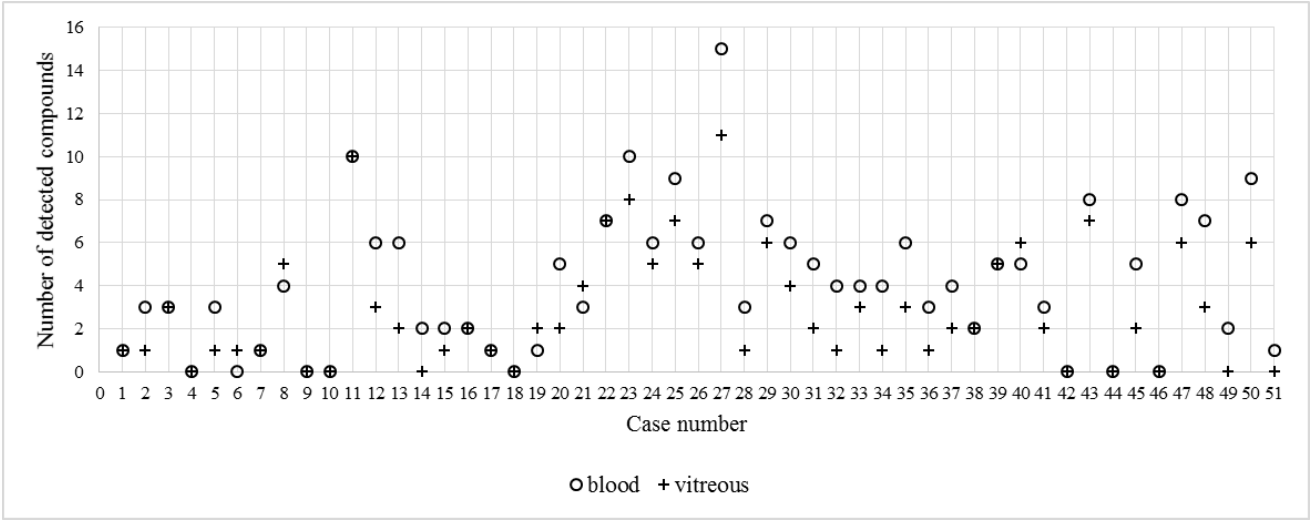

Fig. 1. Number of detected compounds in blood *vs.* vitreous humor.

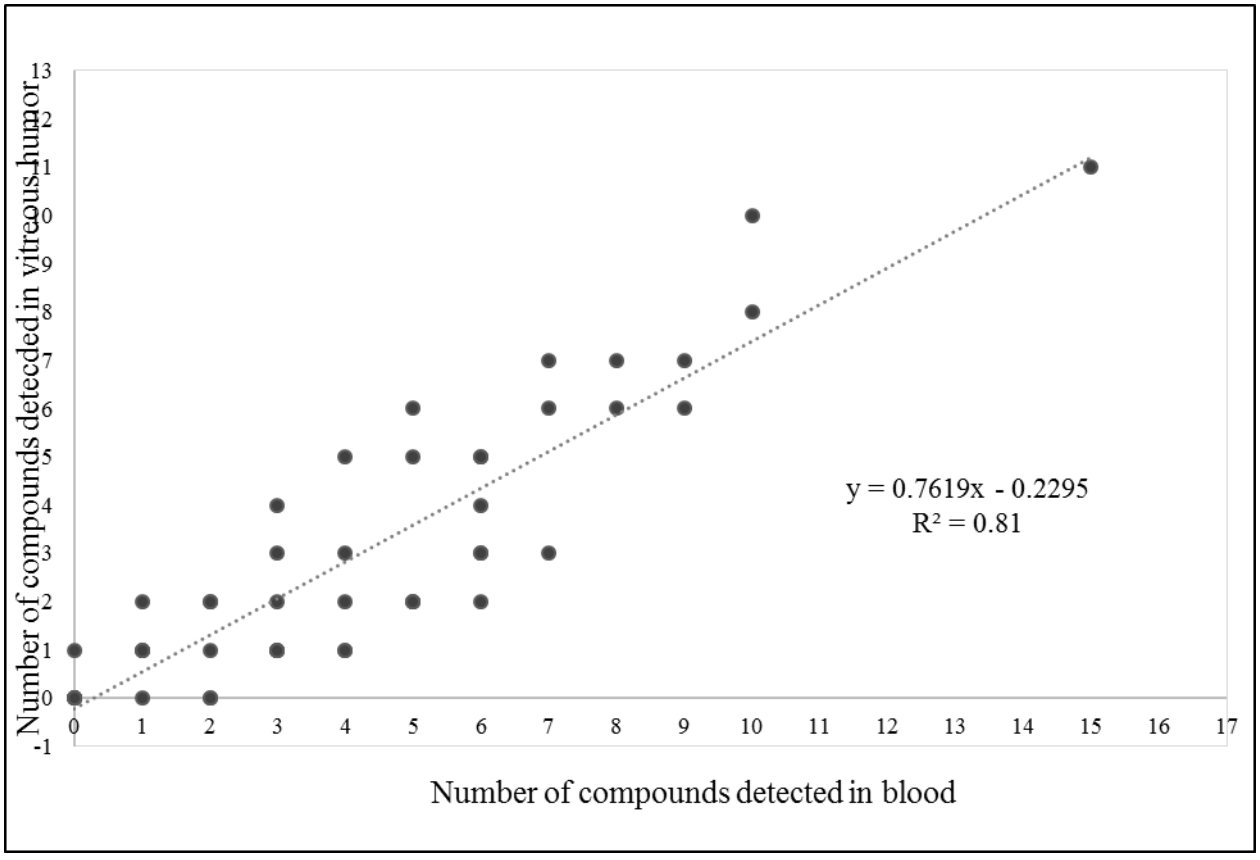

Fig. 2. Correlation between the number of detected compounds in blood vs. vitreous humor.

The study results revealed 61 different compounds of which 4 metabolites, including EDDP, metabolite of methadone, 6-acetylmorphine, metabolite of heroin, and metabolites of flurazepam and metamizole. However, identification of one or more desalkyl metabolites of flurazepam and metamizole was reported under the name of the parent compound due to their fast degradation after the intake. Frequency of detection of each compound in both examined samples is presented in Table 1. According to the criteria proposed by Methusi at al. (2016) which state that "that if a drug has been identified twice in whole blood and if it has been identified at a rate of 50% or lower in VH, then it is considered as difficult to be identified in VH", we observed that this group includes diazepam, nordazepam, alprazolam, flurazepam, metoprolol, methylprednisolone, diclofenac, ketoprofen, papaverine, and meconine. Opposite of this criteria, we propose criterion that if a compound has been detected at least twice in blood and at a rate of 100% or higher in vitreous humor, it can be considered suitable for identification in VH as a matrix comparable to blood. This group includes paracetamol, ibuprofen, cinnarizine, metronidazole, metoclopramide, ecgonine methyl ester, codeine, tramadol, methadone, lamotrigine, mirtazapine, venlafaxine, citalopram, quetiapine, and olanzapine. Compounds that do not fulfill these criteria but had shown good correlation in the findings between both samples are EDDP, atropine, propofol, and fentanyl.

Samples on which this study was conducted, blood and VH, were collected during autopsy and stored in tubes with NaF as preservative. While some authors consider the storage of VH in clean tubes without a preservative as a suitable, supported by the fact that VH is protected of bacterial contamination and postmortem degradation, significant number of studies recommend the use of a preservative. This approach has been proven to enhance the stability of xenobiotics, especially of benzodiazepines, 6-acetylmoprphine, and cocaine (Bévalot et al., 2016; Markowska et al., 2017; Wójtowicz et al., 2020). Samples that were not analyzed within a few days after sampling were immediately frozen in accordance to the findings suggesting that lower temperature also improve the stability of the compounds of interest (Wójtowicz et al., 2020). During collection, the total amount of VH was combined into the single tube, based on data confirming that there are no significant between eyes differences (Baniak at al., 2015).

In spite of the fact that GC-MS is an instrument of choice in forensic toxicology screening process, in our study screening on both samples was conducted in parallel with two methods using GC-MS and HPLC-PDA, which offers advantages over using only one technique. Possibility of detection of nonvolatile compounds when HPLC is included is worth to mention, although sensitivity of this technique is lower than GC-MS. Furthermore,

conducting a non-targeted screening using various commercial libraries provides an opportunity for detection of compounds that are not regularly included in the method. This approach emphasizes the importance of screening of unknown compounds, guiding a further toxicology analysis in a more specific direction. In that manner, a total of 207 and 146 compounds were detected in blood and VH respectively, which is comparable with results reported in the previous studies on this topic, i.e. 209 and 169 in blood and VH, respectively, in the study of Metushi et al. (2016), and 376 and 245 compounds in urine and VH, respectively, reported by Paulaner, et al. (2010). Results of our study are in line with literature data, indicating that VH is suitable matrix for screening when blood is not available. The strong positive correlation of the number of compounds detected in blood versus VH supports this finding. Nevertheless, lower number of compounds detected in VH is notable. The reasons for this occurrence are primarily related to the physicochemical properties of the compounds, which largely determine their ability to cross the blood-brain barrier. Blood concentration, protein binding, and volume of distribution are additional factors that have important influence on transmission of the compounds from blood to the VH (Bévalot et al., 2016; Wójtowicz et al., 2020).

Table 1. Frequency of detected compounds in blood and vitreous humor of 51 cases

Compound	Number of findings in blood	Number of findings in vitreous	Compound	Number of findings in blood	Number of findings in vitreous
6-Acetylmorphine	0	1	Lidocaine	1	1
Alphamethrin	0	1	Meconine	2	0
Alprazolam	3	0	Memantine	1	1
Amiodarone	1	0	Metamizole**	13	14
Atropine	10	8	Methadone	12	12
Biperiden	1	0	Methylprednisolone	2	1
Bromazepam	1	0	Metoclopramide	4	4
Benzoylecgonine	2	1	Metoprolol	3	1
Cinnarizine	3	3	Metronidazole	5	6
Citalopram	3	3	Midazolam	9	6
Clomipramine	1	1	Mirtazapine	2	2
Cocaethylene	0	1	Morphine	1	2
Cocaine	0	1	Naproxen	3	2
Codeine	3	3	Nimesulide	1	0
Diazepam	24	8	Nordazepam	23	7
Diclofenac	5	1	Noscapine	1	0
Donepezil	1	0	Olanzapine	3	4
Duloxetine	1	0	Papaverine	2	0
EDDP*	8	6	Paracetamol	9	9
Ecgonine methyl ester	2	2	Pentobarbital	1	1
Fentanyl	5	4	Pheniramine	1	2
Fluconazole	1	1	Pregabalin	1	1
Fluoxetine	1	1	Propofol	3	2
Flurazepam**	4	1	Propranolol	1	1
Furosemide	1	1	Quetiapine	2	2
Ibuprofen	2	2	Thiopental	1	1
Ketamine	1	1	Tramadol	3	3
Ketoprofen	5	1	Valproic acid	1	1
Lamotrigine	2	2	Venlafaxine	2	3
Laudanosine	1	1	Zolpidem	1	1
Levamisole	1	1			

*Metabolite of methadone
**Detected as desalkyl metabolites of a compound

We have observed that benzodiazepines, generally, were detected in lower rate in VH than in blood, and this finding was confirmed for all examined medications from this group. Similar findings have been observed from other authors, suggesting that due to neutral or weak acidic chemical structure and high protein binding, benzodiazepines have low diffusion rate in VH (Bazmi et el., 2016; Øiestad et al., 2023). Comparable results were obtained for opium alkaloids, papaverine, noscapine, meconine, even so they were detected in only 1-3 blood samples and none of them in VH. Notwithstanding, good correlation for morphine and codeine was observed. Heroin main metabolite, 6-acetylmorphine, was detected only once in VH without positive findings in blood. Although the applied method is not highly sensitive for morphine and additionally the small sample size with positive findings for opiates that avert us of making a conclusion, according to the literature, VH is considered as a preferred postmortem sample for heroin use determination. Heroine and 6-acetylmorphine are unstable compounds but due to their high lipophilicity can easily penetrate trough membranes, and achieve higher concentration in this medium since esterase activity in VH is much lower than in blood (Al-Asmari, 2020; Maskel et al., 2019; Thaulow et al., 2018). Based on the results of our study straightforward conclusion can be made for medications that belong to the opioid analgesic group, like methadone and tramadol, for which the number of findings in VH and blood is equal. Moreover, there is a sufficient number of cases with positive result for methadone. Literature data support our findings, emphasizing the caution in interpretation of quantitative results for these compounds in VH (Havig et al., 2022). Good correlation in positive findings between blood and VH was observed for anesthetics also, with fentanyl being the most often detected representative of this pharmacological group. In the Chatterton and Scott-Ham (2018) study, VH has been highlighted as a useful matrix for qualitative and quantitative analyses of fentanyl and its metabolites. In relation to antidepressants, there are few studies pointing out that VH is a relevant matrix for identification, though not equally reliable for quantification as blood, especially for tricyclic antidepressants, probably as a result of their high plasma protein binding (dos Santos et al., 2016; Ntoupa et al., 2020; Øiestad et al., 2018). Evaluation of our results revealed good agreement between the findings of antidepressants in blood and VH, as well as for quetiapine and olanzapine as the only representatives of the antipsychotic group of medication in presented study. Paracetamol and metamizole were the most abundant anti-inflammatory medications determined in examined samples, both with almost equal findings in blood and VH. Ibuprofen has been targeted as a difficult compound to be identified in VH according to previous studies (Hubbard et al., 2021; Metushi et al., 2016), however, we detected ibuprofen in the both samples of two cases. Ketoprofen and diclofenac were obviously found in a lesser extent in VH. This finding can be a result of an inappropriate sample preparation procedure for acid substances, or due to their physicochemical properties causing inability for penetration trough blood-brain barrier (Hubbard et al., 2021).

Altogether, VH demonstrated satisfactory results for the aim of the study. Still, more extensive investigations including higher number of samples, along with broader spectrum of compounds are needed. Cut-off values and extraction efficiency were not determined for all mentioned compounds, which can be considered as the limitation of our study, because analyses were performed using not fully validated method, routinely used for screening in blood, without adaptation to VH sample. Recognition of the properties of the applied method, especially its limitations, is crucial for interpretation of the results. Regarding toxicological results obtained from VH analyses, compound`s characteristics presumably have greater importance than the method itself. Therapeutic index, blood concentration, plasma protein binding, physicochemical properties, and metabolic pathways of compounds, postmortem redistribution and postmortem period, are factors that can also affect presence and concentration of compounds in the VH. Hence, many factors ought to be considered, especially in interpretation of the quantitative analyses. Scientific literature data about the importance of VH in forensic toxicology are scarce, therefore including VH in routine toxicological analyses is encouraged. The applicability of VH as an alternative matrix for screening compared to blood is persuasive, furthermore, its use as a routine sample in forensic toxicology provides more detailed insights about toxicological profile of examined cases.

Conclusion

A method used for routine toxicological screening of blood was successfully applied on vitreous humor samples. Results shown agreement between the number of findings in both samples with strong positive correlation. Presence of compounds in vitreous humor depends on many factors which should be considered during results interpretation. Our study provided comparative results for several pharmacological groups indicating that benzodiazepines are difficult to be detected in vitreous humor, in contrast to methadone, tramadol and fentanyl. More detailed studies are needed to investigate the full potential of the vitreous humor in forensic toxicology purposes.

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Резиме

Применливост на очната вода како алтернативен примерок во споредба со крв за пост-мортем токсиколошки скрининг

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Клучни зборови: крв, очна вода, пост-мортем, токсикологија, скрининг

Употребата на очната вода во форензичките испитувања наидува на голем интерес, најчесто за определување на пост-морталниот интервал, биохемиски анализи, како и во форензичката токсикологија. Во случаи каде крв и урина, како референтни биолошки примероци, не се достапни, неопходна е употребата на алтернативни примероци. Како резултат на анатомската позиција и хемиските особини, очната вода е добар кандидат за алтернативен примерок. Целта на ова истражување беше да се испита соодветноста и применливоста на очната вода како алтернативен примерок за токсиколошки скрининг споредено со примероци крв.

Примероци крв и очна вода од вкупно 51 обдукција беа анализирани со не-таргетиран токсиколошки скрининг со широк опсег, со примена на методи што се користат за рутински скрининг во крв користејќи гасна хроматографија со масена спектрометрија (gas chromatography mass spectrometry, GC-MS) и високоефикасна течна хроматографија комбинирана со детектор со низа диоди (high-performance liquid chromatography - photo diode array detector, HPLC-PDA).

Во испитуваните примероци од крв беа идентификувани вкупно 207 соединенија и и 146 соединенија во примероците од очна вода. Во 16 од испитуваните случаи беше забележано 100% совпаѓање на бројот на идентификувани соединенија во двата примероци. И покрај помалиот број идентификувани соединенија во примероците очна вода, не беше забележана статистички значајна разлика помеѓу бројот на соединенија идентификувани во комплементарните примероци крв и очна вода.

Анализата на крв и очна вода со примена на метод за рутински скрининг во крв покажа совпаѓање помеѓу бројот на идентификувани соединенија во двата примероци со силна позитивна корелација, укажувајќи на тоа дека очната вода е соодветен алтернативен примерок на примероците од крв. Потенцијалот на очната вода како примерок за широка примена во форензичната токискологија треба дополнително да се евалуира во понатамошни студии.