Postmortem Vitreous Humor Analysis for Xenobiotics and their Metabolites

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Abstract: This article discusses the current state of knowledge on quantifying xenobiotics and their metabolites in vitreous humor as part of postmortem toxicological analysis. The evaluated compounds included: opiates and their metabolites, cocaine and its metabolites, amphetamine and its metabolites, cannabinoids, phencyclidine, benzodiazepines, and ethyl alcohol and biomarkers of its abuse.

Keywords: vitreous humor, detection, quantification, xenobiotics

Introduction

Vitreous humor (VH) is a very useful, alternative biological specimen for postmortem toxicology analysis and, as such, has many advantages. Due to its location in the body, VH is relatively well protected from postmortem degradation and contamination. It remains mostly unaffected by postmortem biochemical changes occurring in the body. In the cases where other biological fluids, such as blood and urine, have been altered by postmortem chemical processes, VH is often the only available specimen for toxicology analysis (Kovatsi, Rentifis, Giannakis, Njau, & Samanidou, 2011). VH contains no active enzymes which could destabilize the evaluated compounds. VH is the only fluid in the body where some compounds (e.g. 6-acetylmorphine) resist decomposition; thus, only VH analysis can verify antemortem drug (heroin) use (Pragst, Spiegel, Leuschner, & Hager, 1999). The vitreous body has no direct blood supply, which is why VH is free from microbiological contamination and the effects of hemolysis. Moreover, its breakdown occurs considerably slower than that of other biological fluids and tissues. This has a particularly practical application, considering the fact that a postmortem examination is typically conducted between 24 and 48 hours after death (Lin, Chen, Shaw, Havier, & Lin, 1997). VH analysis may be also useful when blood samples are unavailable or their quantity is inadequate or limited (Knittel, Clay, Bailey, Gebhardt, & Kraner, 2009). Laboratory processing of VH samples is easier than that of blood or urine samples (Carvalho et al., 2013). VH concentrations of many analyzed compounds are similar to those in blood (Letter et
al., 2002; Peres, Pelição, Caleffi, Martinis, & Spinosa, 2014). VH contains less protein than urine. Since the degree of protein binding in VH is minimal, it only very slightly affects quantitative analysis results (Letter et al., 2000).

**Structure vitreous body**

The vitreous body is a gelatinous mass which consists of 98% water. The volume of the vitreous chamber is approximately 4 mL (Bévalot, Cartiser, Bottinelli, Fanton, & Guitton, 2016). The inside of the vitreous body is composed of the stroma and humor. The vitreous stroma is a meshwork of fine connective-tissue fibers, with the space between them filled with VH, whose composition is similar to that of aqueous humor. The vitreous body is devoid of blood vessels and contains few cells; it plays an important role in the period of growth and enlargement of the globe of the eye (Michajilik & Ramotowski, 2009).

Like the blood-brain barrier, the blood-retina barrier (BRB) is a selective barrier (Mannermaa, Vellonen, & Urtti, 2006). The BRB consists of two structures: avascular and vascularized retina. The avascular retina, which separates the retina from the choroid, comprises the outer component of the BRB (Mannermaa, Vellonen, & Urtti, 2006). The avascular retina is composed of retinal pigmented epithelium (RPE) and sensory epithelium (Klaassen et al., 2014). Sensory epithelium is formed by outer and inner photoreceptor segments (rods and cones) (Klaassen et al., 2014). While RPE comprises a single layer of polarized cells, the inner layer of BRB can be divided into nine layers (Mannermaa, Vellonen, & Urtti, 2006).

The avascular retina receives its blood supply via a layer of capillaries that pass through the adjacent choroid. These fenestrated capillaries derive from the ciliary arteries and form a dense vascular network (the choriocapillaris). These capillaries are characterized by loose junctions and a large number of pores (fenestrations) in their endothelial-cell wall. These features allow capillary walls to be permeable to large-molecule proteins (Klaassen et al., 2014).

The vascularized retinal layer is the inner component of the BRB (Mannermaa, Vellonen, & Urtti, 2006). This layer is composed mainly of 5–10-μm arterioles that have no tunica intima or autonomic innervation. These arterioles can maintain sustained blood flow even under conditions of high intraocular pressure. The endothelial cells making up capillary walls are tightly connected, and it is these tight endothelial junctions that constitute the inner component of the BRB (Raczyńska, 2011).

Retinal capillary permeability is similar to that of cerebral vessels, with only minimal amounts of fluorescein and small ions (e.g. sodium) getting through. Retinal capillary vessels are highly permeable to gases (oxygen, CO₂) and water but they constitute a barrier to large molecules, such as proteins, and smaller molecules, such as amino acids and glucose. Transport across the BRB is achieved via facilitated diffusion, involving the use of carrier proteins (Raczyńska, 2011).

The cornea, which is an avascular structure, serves as a protective layer for the inner structures of the eye. Xenobiotics absorption occurs via the vascularized conjunctiva (Klaassen et al., 2014).

The BRB allows the necessary nutrients to enter the retina but protects the retina against harmful agents (e.g. pathogens, enzymes, anaphylatoxins) (Mannermaa, Vellonen, & Urtti, 2006). The BRB is absent at the level of the optic disc. As a result, hydrophilic molecules can diffuse from the extravascular space into the optic disc. This may cause selective damage to the optic disc (Klaassen et al., 2014).

BRB function may be weakened by various pathogens and conditions. The most common causes of BRB dysfunction are diabetic retinopathy and age-related macular degeneration (Mannermaa, Vellonen, & Urtti, 2006).
The mechanisms responsible for transfer of molecules into VH are: diffusion, hydrostatic and osmotic pressure gradients, convection and active transport. High-molecular-weight molecules and colloidal molecules are transported via convection. Low-molecular-weight molecules move into and out of VH mainly via diffusion; however, there is evidence that larger molecules may also be transferred this way. Due to the small size of most drug molecules, they can move in and out of VH via diffusion. Only unbound drug molecules can leave the blood and be transferred into VH. Thus, drugs characterized by a low degree of protein binding can be detected in VH in high concentrations. Conversely, drugs characterized by a high degree of protein binding appear to accumulate in VH to a much lesser extent (Hosoya & Tachikawa, 2009).

Drug transfer into the retina depends on a number of factors: drug concentration gradient, barrier area, physicochemical and pharmacokinetic properties of the drug, volume of distribution, plasma-protein binding capacity of the drug, and relative permeability of the BRB (Mannermaa, Vellonen, & Urtti, 2006).

After a drug enters the body, either orally, transdermally, parenterally, or via inhalation, it is absorbed and then distributed by circulating blood. The drug reaches all structures of the eye. The RPE and retinal photoreceptors are target structures for systemic drugs, despite the fact that RPE cells are connected with tight junctions (zonulae occludentes), which preclude transfer of large molecules into the retina. Choroid capillary endothelial cells are not considered to be target sites because they prevent transfer of molecules of over 50–70 Da (Klaassen et al., 2014).

Ocular drugs can be administered via anterior or posterior delivery. Anterior ocular drug delivery involves crossing the corneal barrier. The anterior route plays an important role in treating anterior ocular segment conditions, such as inflammation, infection, glaucoma. In such cases it is the cornea that constitutes the main barrier to drug uptake. Posterior ocular drug delivery involves crossing the BRB. In the posterior ocular segment it is the BRB that limits drug uptake (Mannermaa, Vellonen, & Urtti, 2006).

Drugs can be transferred via either simple or facilitated diffusion. Larger and/or more hydrophilic (or polar) drug molecules are more likely to require active transport to cross the BRB. A study by Holmgren et al. on a number of compounds commonly evaluated for their practical use in forensic pathology demonstrated an evident correlation between the blood and VH concentration of a drug and its protein-binding capacity. The BRB contains a number of transporter proteins (pumps), which play a significant role in drug bioavailability to the posterior chamber of the eye. There are two main types of transporters: export pumps, which are members of the ATP-binding cassette (ABC) transporters, and uptake pumps, which belong to the superfamily of solute carriers (SLCs) (Mannermaa, Vellonen, & Urtti, 2006).

### Evaluating VH for xenobiotics and their metabolites

This paper presents the current state of knowledge on evaluating VH for xenobiotics and their metabolites as part of postmortem toxicology analysis. The purpose of this study was to find information on the usefulness of VH as a biological specimen in toxicology analysis. Earlier studies on the topic typically focused on individual compounds or other research aspects (Hosoya & Tachikawa, 2009; Jabłoński & Sybirská, 2000).

### VH analysis for opiates

VH is frequently used in postmortem toxicology analysis. All compounds detected in the blood can be also detected in VH. There is a wide variety of opioids that can be analyzed in a VH sample, which makes VH a very practical and, in some cases, the only available forensic toxicology specimen. VH is relatively well protected against contaminants and relatively resistant to postmortem decomposition, as it is only minimally affected by postmortem biochemical changes. VH analysis can provide crucial information in those cases where other
biological samples have undergone postmortem chemical processes (Kovatsi, Rentifis, Giannakis, Njau, & Samanidou, 2011).

There have been many studies to identify the optimal biological specimen for postmortem toxicology analysis for opiates. There have been problematic cases where morphine was detected in the body but its source could not be definitively identified as codeine, heroin, or morphine itself. In such cases choosing the appropriate biological sample is of crucial importance. Toxicological analysis findings can be confounded by the presence of codeine, which is metabolized to morphine. Codeine as well as acetylcodine can be found in narcotics, as they are common additive materials that are supposed to substitute for heroin. If there is no 6-acetylmorphine, it is difficult to determine whether or not the toxicity profile including codeine and morphine is a result of codeine-based treatment or illegal heroin use. A number of studies have aimed to improve toxicology analysis by evaluating the usefulness of testing for 6-acetylmorphine in such specimens as cerebrospinal fluid, VH, and urine. Recent studies indicated that heroin exposure was detected via VH analysis in a higher (by 54%) proportion of cases than those where the only analyzed specimen was blood (Wyman & Bultman, 2004).

Opiates and their metabolites are relatively easy to detect in VH. Testing VH for opiates and their metabolites as part of toxicology analysis may provide additional data on the cause of death and help estimate the time from drug exposure to death. The concentration of opiates and their metabolites in VH should not serve as an indicator of the concentration of those drugs in blood or other specimens (urine, cerebrospinal fluid). VH may be an alternative biological specimen and an additional source of information in toxicology analysis. Nonetheless, assessing opiate or opiate metabolite levels in VH alone is currently considered insufficient.

**VH analysis for cocaine**

Due to the low metabolic activity of VH, its samples seem to be more suitable for qualitative and quantitative analysis of cocaine and its metabolites. VH analysis findings should be interpreted only while considering factors that could affect the obtained values (such as the effect of ethanol on benzoylecgonine levels) (Carvalho et al., 2013). VH concentrations should not be used to estimate blood concentrations of the relevant compounds, as the direction and extent of metabolic changes in the blood is unpredictable. Postmortem VH analysis cannot serve as a basis for estimating antemortem blood concentrations of cocaine or its metabolites. If blood samples are unavailable, VH analysis may provide qualitative data on the presence of cocaine and its metabolites in the body (Mackey-Bojack, Kloss, & Apple, 2000). Further studies are necessary to confirm the usefulness of VH as an alternative biological specimen and verify the reliability of toxicology analysis findings.

**VH analysis for methamphetamine**

The usefulness of VH as an alternative biological test specimen for methamphetamine and its metabolites has been confirmed in a number of studies on animal models. VH seems to be a suitable specimen for both qualitative and quantitative MDMA analysis. Postmortem MDMA concentrations in VH help estimate antemortem blood MDMA levels. Moreover, VH seems to be a specimen more suitable than blood for systemic MDMA quantification. However, all these promising conclusions were drawn based on animal studies (a rabbit model) (E. A. De Letter et al., 2000; Els A. De Letter et al., 2002). Analogous studies conducted in humans yielded results that were not always consistent with animal study findings (Mackey-Bojack, Kloss, & Apple, 2000). VH seems to be a promising specimen for qualitative and quantitative postmortem analysis of methamphetamine and its metabolites. However, further human studies are needed.

**VH analysis for cannabinoids**

So far, VH cannabinoid levels have been either undetectable or detectable at very low, threshold levels, despite their high concentrations in blood. Due to its high plasma protein-binding capacity, tetrahydrocannabinol (THC)
does not cross the BRB into VH. Thus, VH is not a suitable specimen for either qualitative or quantitative cannabinoid analysis (Jenkins & Oblock, 2008; Lin & Lin, 2005).

**VH analysis for phencyclidine**

Phencyclidine is stable in VH and its qualitative analysis yields reliable results (Mokhlesi, Garimella, Joffe, & Velho, 2004). However, phencyclidine concentrations in VH should not be used to assess concentrations of this compound in blood, as findings obtained from these specimens tend to be inconsistent (Cox et al., 2007).

**VH benzodiazepine analysis**

There have been relatively few studies on benzodiazepine analysis in VH. Human studies on qualitative and quantitative analysis yielded reliable results for five benzodiazepines (bromazepam, alprazolam, lorazepam, lormetazepam, diazepam). Tetrazepam cannot be easily detected in VH (Cabarcos et al., 2010). More detailed studies for diazepam and desmethyldiazepam have been conducted on a rabbit model. The obtained VH concentrations should not be used to estimate blood levels of the drug at the time of death. VH drug concentration values may help estimate the blood concentration; however, it is impossible to determine the time between drug exposure and death or time between death and specimen analysis. Data on VH analysis for benzodiazepines are based on a rabbit model and should not be relied on in forensic toxicology. Further studies on human-derived specimens are needed (H M Teixeira et al., 2004).

**Ethyl alcohol and its biomarkers**

The topic of ethanol kinetics and detection in VH has been studied in detail, with VH successfully used for postmortem ethanol detection. There have been many studies involving hundreds of human-derived VH and blood specimens. They showed a strong correlation between ethanol concentrations in VH and those in blood. Based on several studies, a ratio range of mean VH/blood ethanol concentrations of 1.15–1.20 was established (Ioan, Jitaru, Damian, & Damian, 2015; Jones & Holmgren, 2001). Despite numerous studies on this topic, VH ethanol concentrations are not a recommended basis for estimating blood ethanol levels, due to considerable variations in the obtained results and the fact that these results depend on a number of factors (Jones & Holmgren, 2001).

Ethyl sulfate (EtS) and ethyl glucuronide (EtG) are stable VH markers of ethanol consumption and are used in toxicology analyses. EtG analyses provide mainly the data on whether or not the ethanol present in the body came from antemortem alcohol consumption or postmortem alcohol synthesis (in the process of putrefaction). EtG analysis seems to yield more reliable results on antemortem ethanol consumption than an analysis of ethanol itself (Keten, Turner, & Balance-0dabasi, 2009; Theriauf et al., 2011; Vezzoli, Bernini, & De Ferrari, 2015). EtG and EtS analysis provides supplementary information on time from ethanol exposure to death. No consistent relationships between EtG, EtS, and ethanol concentrations have been established.

Carbohydrate-deficient transferrin (CDT) is stable in VH. VH CDT analysis may be a valuable tool in preliminary forensic diagnosis of antemortem alcohol abuse and in verifying potential alcohol addiction. Unfortunately, there have been very few studies on CDT analysis in VH specimens. Moreover, the mechanism of CDT synthesis in the body and the factors affecting CDT levels are unknown. Although CDT seems to be a promising marker of alcohol abuse, it should not be used in toxicology analyses due to the scarcity of data (Berkowicz, Wallerstedt, Wall, & Denison, 2003).

**Gamma-hydroxybutiric acid (GHB)**

GHB is a naturally occurring neurotransmitter in the mammalian brain. It is an endogenous precursor and metabolite of the main inhibitory neurotransmitter γ-aminobutyric acid (GABA). For many years it has been used as an anesthetic, and is currently used to treat alcoholism and as an aid to detoxify the body. This depressant is
a recreational club drug known and used as a pill for rape (Bévalot, Cartiser, Bottinelli, Fanton, & Guitton, 2016; Busardò et al., 2017). Significant GHB postmortem production is overstating the results and difficult correct interpretation. VH is one of the alternative biological materials proposed to confirm elevated GHB levels in the blood (Kintz, Villain, Cirimele, & Ludes, 2004). Busardo et al. showed significantly greater stability of GHB in the VH, as compared to its stability in the peripheral blood (Bévalot, Cartiser, Bottinelli, Fanton, & Guitton, 2016; Busardò et al., 2017).

**Conclusions**

In summary, it is sometimes necessary to conduct postmortem tests for xenobiotics using alternative biological specimens (e.g. in case of inadequate blood sample volume). VH is a good, alternative specimen and can provide additional information in toxicology analysis. However, it must be emphasized that the obtained VH xenobiotic concentrations cannot always be a basis for estimating xenobiotic concentrations in other biological specimens (blood, urine, cerebrospinal fluid). Many of the studies on this topic were conducted in animal models, which also contributed to the variability of findings. Therefore, verifying the usefulness of VH in forensic toxicology analyses requires further studies in humans.

**References**


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