TECHNICAL NOTE

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The Use of Vitreous Humor as an Alternative to Whole Blood for the Analysis of Benzodiazepines

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ABSTRACT: In postmortem drug analysis, the most commonly used sample matrix is whole blood. However, postmortem changes can denature this matrix, resulting in a loss or degradation of drugs, thus biasing analytical findings. Vitreous humor is thought to be less affected by these changes and should, therefore, have the potential to provide a more reliable estimation of antemortem drug concentrations.

To assess the usefulness of vitreous humor for the analysis of benzodiazepine drugs, vitreous humor and whole blood were obtained postmortem in 27 cases. Three benzodiazepine drugs were investigated—temazepam, diazepam, and desmethyldiazepam. For temazepam and diazepam, some correlation was found between the matrices (R^2 = 0.789 and 0.724, respectively). However, for desmethyldiazepam, no correlation was observed (R^2 = 0.068). Regression analysis on plots of vitreous humor versus blood concentrations produced gradients of less than 1.0 showing that, in general, levels in blood are higher than the corresponding levels in vitreous humor.

KEYWORDS: forensic science, forensic toxicology, vitreous humor, whole blood, benzodiazepines, SFE, HPLC

The use of vitreous humor as an alternative sample to postmortem blood is of particular importance when the body has undergone considerable bleeding, decomposition, or burning (1). In comparison to postmortem blood, which is often putrefied, vitreous humor is a relatively simple matrix (Table 1) (2). It is much less susceptible to postmortem changes than blood. The transport of drugs across the blood vitreous humor barrier is limited by the lipid solubility of the drug at physiological pH (3).

Most studies on the analysis of vitreous humor have been concerned with time-since-death determinations (4). It is only recently that interest has focused on the use of vitreous humor for the determination of drug levels to assist with the investigation of the cause of death. Some of the drugs that have been investigated in vitreous humor include morphine (5), flurazepam (6), methadone (5), and cocaine (1,7). The methodologies reported usually involve solid phase extraction followed by an appropriate analysis technique.

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In the West of Scotland the abuse of benzodiazepines either alone or in conjunction with heroin, has greatly increased over the last decade. The most commonly encountered benzodiazepines over the time period of this study were temazepam and diazepam. Consequently, the investigation focused on the analysis of these two drugs and desmethyldiazepam, the major metabolite of diazepam. The 27 cases detailed in this study were found to be positive for benzodiazepines by immunoassay. Of these cases, only 15 were suspected to have used benzodiazepines prior to death from the previous medical or drug abuse histories.

Experimental

Chemicals

Temazepam, diazepam, desmethyldiazepam, and prazepam (internal standard) were supplied by Wyeth Laboratories (Hants, U.K.). Methanol, ethyl acetate, dichloromethane and distilled water were high-performance liquid chromatography grade supplied by Lab Scan Analytical Sciences, (Dublin, Ireland). Na_2HPO_4 was GPR grade (Merck, Poole, U.K.). Extrelut® (Merck) was prewashed using dichloromethane. CO_2 was obtained from Air Products (Walton-On-Thames, U.K.) in 25 kg cylinders fitted with a dip-tube. Collection vials for SFE were 6 mL Hypovial sealed with butyl rubber septa (Pierce and Warriner, Chester, U.K.).

Samples

Blood and vitreous humor samples obtained at postmortem were placed in 25 mL glass vials and refrigerated until used. Spiked blood samples were prepared in drug free whole blood. Since no blank vitreous humor was available, spiked standards for analysis were prepared in distilled water. The extraction method was linear for all drugs from 0.01 to 10 μg/mL in both matrices ($r^2 > 0.99$) and the limit of quantitation set for all drugs was 0.01 μg/mL, for a 100 μL sample.

Apparatus

A Supercritical Fluid Extraction (SFE) system was used for the extraction of the drugs from both blood and vitreous humor. This consisted of two Gilson (Middleton, WI) 306 pumps and a PYE Unicam (Cambridge, U.K) series 104 GC oven fitted with a Rheodyne 7037 back pressure regulator. All components were adapted to suit the supercritical fluid phase. The CO_2 pumphead was cooled

using a Gilson model SFC3 refrigeration unit. Extracts were collect by expansion into methanol at the flow outlet of the system. The supercritical fluid used was CO_2:EtAc (90:10) at a flowrate of 2.0 mL/min. The temperature and pressure were 65°C and 3000 psi respectively.

TABLE 1—*Comparison of putrefied blood and vitreous humor.*

Putrefied Blood (pH 4–9)	Vitreous Humor (pH 7–7.8)
Water (20–70%)	Water (98–99.7%)
Lysate	Glucose
Clots—denatured & bacterial	Hyaluronic acid
Fat droplets	Simple anions & cations
Steroids	Collagen
Putrefactive bases	
Protein microagglutinates	
Enzymes	

TABLE 2—*Results of blood and vitreous humor benzodiazepine analysis.*

	Blood (mg/L)			Vitreous Humor (mg/L)		
Case	Tem.*	DMD†	Dzp.‡	Tem.	DMD	Dzp.
KS/02	1.22	Nd§	0.18	1.19	Nd	Nd
KS/07	0.72	0.06	1.98	0.71	0.42	0.63
KS/08	Nd	0.14	0.44	Nd	0.17	0.39
KS/11	0.48	0.47	1.01	Nd	0.39	0.74
KS/13	Nd	0.55	0.47	Nd	Nd	0.47
KS/14	Nd	0.29	0.15	Nd	Nd	0.15
KS/15	Nd	0.20	0.29	Nd	0.37	Nd
KS/21	0.01	0.39	0.55	Nd	0.35	Nd
KS/22	1.30	0.68	Nd	0.76	0.27	0.62
KS/23	Nd	0.18	0.45	Nd	0.03	0.07
KS/24	0.25	0.29	0.03	0.08	0.10	0.01
KS/25	Nd	0.08	0.27	Nd	0.07	0.10
KS/26	0.06	0.23	Nd	Nd	0.10	Nd
KS/27	0.78	0.22	0.08	0.35	0.12	0.10
KS/28	0.11	0.11	1.51	0.11	Nd	1.25
KS/29	0.36	0.52	Nd	0.17	0.52	Nd
KS/30	0.23	0.20	2.62	0.19	0.28	2.48

* Tem: Temazepam; † DMD: Desmethyldiazepam; ‡ Dzp: Diazepam; § Nd: Not detected.

Final analysis was carried using HPLC. The system consisted of a Gilson 305 pump with a Gilson 115 UV detector. The column (25 cm × 4.6 mm) and guard column (2 cm × 4.6 mm) were prepacked with Hyposil ODS (5 μm) (Capital HPLC Specialists, Bathgate U.K.). The mobile phase Na_2HPO_4—methanol (30:70 v/v) was pumped at a flowrate of 1.0 mL/min and the analytes detected at a wavelength of 254 nm.

Sample Preparation

Approximately 0.2 g of Extrelut® was placed in a plastic weighing boat. To this 100 μL of blood or vitreous humor and 100 μL of internal standard were added and mixed. The mixture was allowed to dry until a friable consistency was achieved. The dried mixture was then transferred to a stainless steel extraction column (3 cm by 4.6 mm) which was placed inside the equilibrated SFE system.

Results and Discussion

Of the 27 cases analyzed, 17 were found to be positive for one or a combination of the benzodiazepines investigated by HPLC. The remaining 13 immunoassay positive cases may have been positive for other benzodiazepines, not investigated in this study. The drugs and levels detected are shown in Table 2. In summary, the levels detected were as follows: temazepam 0.01 to 1.30 mg/L (mean 0.50 mg/L) in blood and 0.08 to 1.19 mg/L (mean 0.45 mg/L) in vitreous humor; diazepam 0.03 to 2.62 mg/L (mean 0.72 mg/L) in blood and 0.01 to 2.48 mg/L (mean 0.58 mg/L) in vitreous humor; desmethyldiazepam 0.06 to 0.68 (mean 0.29 mg/L) in blood and 0.03 to 0.52 (mean 0.25 mg/L) in vitreous humor. In all cases, the highest and mean concentrations in vitreous humor were lower than in blood.

Despite benzodiazepines being detected in both matrices in all cases, there were seven cases where one or more of the benzodiazepines were not detected in vitreous humor. In the case of vitreous humor, only one analysis could be carried out due to limited volume and in each case only 100 μL was analyzed. In the case of blood, where levels were approaching the limit of detection, the analysis was repeated using a higher volume. In only one case was a drug detected in vitreous humor that was absent in blood (case KS/22). For each drug, the results of vitreous humor analysis were plotted against the blood results in order to visualize the correlation between the two matrices. These graphs are shown in Figs. 1–3 with the corresponding coefficients of correlation and equations of lines.

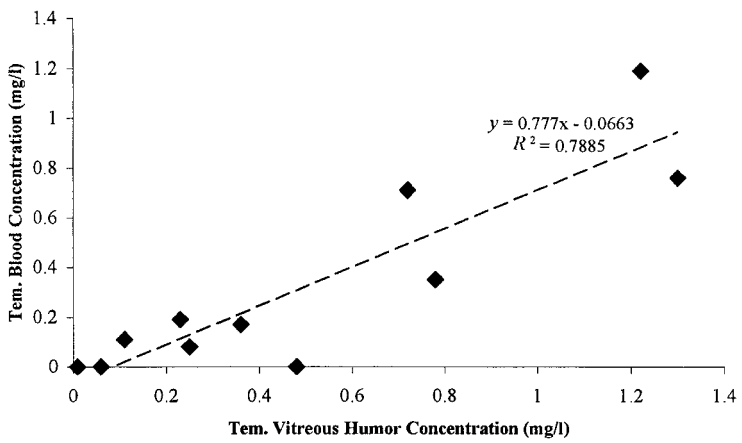

FIG. 1—*Blood versus vitreous humor-temazepam.*

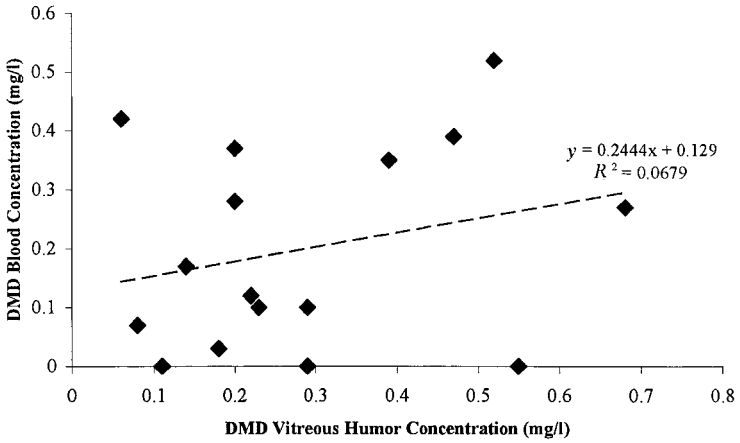

FIG. 2—*Blood versus vitreous humor-desmethyl diazepam.*

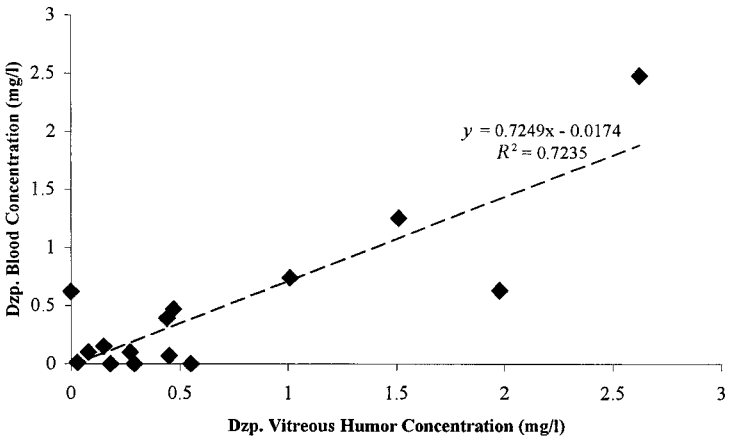

FIG. 3—*Blood versus vitreous humor-diazepam.*

From the graphs it is apparent that although the correlation coefficients are reasonably high for temazepam and diazepam, a large amount of variation still exists. It was not possible to determine the exact time from ingestion of the benzodiazepines until death or the exact time between death and sampling. However, from the postmortem case reports it was found that eight of the deceased had taken either prescribed or illicit benzodiazepines on the day of death. For example, case KS/07 was prescribed temazepam and case KS/08 diazepam and in both cases the prescription was consumed at the pharmacy. In these two cases, the prescription was said to have been taken several hours before death, which may be indicated in the similar levels of these drugs in blood and vitreous humor (KS/07 0.72 and 0.71 mg/L temazepam; KS/08 0.44 and 0.39 mg/L diazepam).

Following this hypothesis, it is thought that for those cases who were prescribed benzodiazepines and had high blood and low vitreous humor results, e.g., case KS/23 (0.45 and 0.07 mg/L diazepam), that the benzodiazepines had been taken soon before death. However, as the postmortem redistribution from blood to vitreous humor is little understood, it is not known what the effects of a long delay time from death until the sample collection would have on these comparative values.

Conclusions

We have shown that it is possible to detect and quantitate levels of benzodiazepines in vitreous humor and that this sample is suitable as an alternative when blood or urine are not available postmortem. Reasonable correlation was found between blood and vitreous humor for temazepam and diazepam and in all cases benzodiazepines could be detected in both matrices. In order to interpret the results of vitreous humor testing, it will be helpful to evaluate drug concentrations in this media under controlled conditions. As can be seen from the results of this paper, many factors can affect the drug concentrations in postmortem vitreous humor, including time from ingestion to death, time from death to collection of sample, and multiple dosing. However, if, as in this case the purpose of analysis is to determine which, if any, drugs contributed to death, then vitreous humor is a suitable alternative to conventional samples.

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