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Acidic Drug Concentrations in Postmortem Vitreous Humor and Peripheral Blood

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Abstract

Vitreous humor is a potential alternative matrix for postmortem toxicology drug screens when peripheral blood is unavailable. It is easily and reliably collected and may not suffer from the same postmortem redistribution as seen in blood. Here, we compared the concentrations of 7 acidic drugs (acetaminophen, ibuprofen, naproxen, salicylic acid, carbamazepine, phenobarbital and phenytoin) in peripheral blood and vitreous fluid collected in 89 autopsy cases. Analysis was done by high-performance liquid chromatography with diode-array detection. Overall, we found that vitreous drug concentrations were significantly lower than peripheral blood with median vitreous to peripheral blood (V/PB) ratios ranging from 0.0 to 0.6 (mean, 0.1–0.6). The correlations between the concentrations of over-the-counter analgesics in peripheral blood versus vitreous fluid were poor, with acetaminophen exhibiting the best linearity ($R^2 = 0.72$). The antiepileptic drugs (carbamazepine, phenytoin and phenobarbital) exhibited good correlations between peripheral blood and vitreous humor, with all exhibiting an $R^2 \geq 0.95$. Overall, we have demonstrated the potential of vitreous fluid as an alternative matrix for the detection of select acidic drugs.

Introduction

The most commonly used matrix in postmortem toxicology is peripheral blood. However, this matrix may suffer from changes in drug concentrations due to postmortem redistribution (PMR) (1–5) making it difficult to interpret. This redistribution of chemicals from tissue (high concentration) to blood (low concentration) was first described several decades ago (6, 7). Since then, several groups have tried to estimate antemortem concentrations based on postmortem values. Although none have yet to find a high degree of correlation (8–10), a recent investigation has proposed a technique to estimate potential for PMR (11). Cases of suspected overdose, homicidal poisonings, suicides and motor vehicle deaths are all highly dependent on toxicology screens in peripheral blood. This matrix may not be available in cases with massive blood loss. Furthermore, delayed collection of blood after death may result in PMR, which may complicate interpretation of quantitative drug concentrations (1).

Vitreous humor is a clear gelatinous substance that is routinely collected from the eye during autopsy. It has been proposed as an alternative matrix when peripheral blood or urine is not available (12, 13). It is thought to be less susceptible to redistribution due to its isolated nature (14); however, this has been debated (2). Vitreous fluid is also protected from putrefaction, rarely suffers from trauma and generally lacks microorganisms that may alter certain drug concentration such as ethanol (2). Limitations of vitreous fluid include small specimen volume and the variability of drug penetration of the blood–retinal barrier (15). Several methods are being developed to measure a variety of compounds in vitreous humor (13, 16–20), but the relationship to peripheral blood is not well understood.

Previously, we compared drug concentrations between human vitreous fluid and peripheral blood using a gas chromatography-mass spectrometry (GC–MS) broad spectrum screen for 71 identified drugs (21). In this study, we examined concentrations of a subset of acidic drugs in these two matrices using high-performance liquid chro-

matography with diode-array detection (HPLC-DAD). These include the over-the-counter (OTC) analgesics, acetaminophen, ibuprofen, naproxen and salicylic acid, as well as the antiepileptic drugs (AEDs), carbamazepine, phenobarbital and phenytoin. We employed HPLC-DAD to investigate the potential of vitreous humor as an alternative matrix for postmortem analysis. Specifically, we compared concentrations of these seven acidic drugs in peripheral blood to vitreous fluid and determined the correlations between the two matrices.

Experimental methods

Sample collection

Peripheral blood and vitreous humor samples from 89 autopsy cases were collected during forensic autopsies. Peripheral blood was collected from the common iliac vein after being visually identified in the pelvis. Blood (up to 20 mL) was stored in glass tubes containing sodium fluoride (100 mg) and potassium oxalate (20 mg). Vitreous fluid was collected from both eyes using a syringe and was stored in glass tubes without preservatives. All samples were stored at 4°C until analysis.

Materials and reagent preparation

OmniSolv grade acetonitrile was purchased from EMD Millipore (Temecula, CA). Dibasic anhydrous potassium phosphate and concentrated phosphoric acid were purchased from Fisher Scientific (Pittsburg, PA). Drug standards were purchased from Alltech (Hesperia, CA) and used to prepare calibrators at three different concentrations by transferring stock standard solution into a volumetric flask consisting of the appropriate matrix. The calibration curve for peripheral blood was created in 50:50 pork blood: deionized water, and the curve for vitreous fluid was made in blank human vitreous humor. Both an external (UTAK TDM control 1) and an internal (matrix spiked with drugs) quality control (QC) samples were prepared and run with each batch of peripheral blood samples. An internal QC was run with each vitreous humor batch. Drug standards used to prepare the internal QC were purchased from Cerilliant. The blank control consisted of drug-free pork blood or drug-free vitreous fluid. The negative control was drug-free matrix with 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) added as internal standard.

Sample preparation and analysis

Samples were analyzed via HPLC-DAD. Specimen samples were added at a volume of 250 µL to Eppendorf microtubes. Then, 500 µL of extraction solvent containing 16.7 mg/L MPPH as internal standard in acetonitrile was added to each tube. Samples were vortexed and allowed to sit at room temperature for 10 minutes. All tubes were centrifuged (10,000 × g maximum) for 10 minutes, and the supernatant was transferred to autosampler vials and placed into the autosampler. Calibrators, QCs, a negative control and a blank control were prepared in the same manner.

HPLC-DAD analysis was based on a previously published method (22). Briefly, an aliquot of 12 µL of sample was injected onto a Zorbax Eclipse XDB-C18 column (2.1 × 150 mm, 5 µm particle size with a guard column) on an 1100 series HPLC-DAD (Agilent Technologies, Santa Clara, CA). A gradient elution was performed using mobile phase A (15 mM phosphate buffer pH 2.9) and mobile phase B (acetonitrile) at a flow rate of 0.45 mL per minute over a 30 minute run. Relative retention times to MPPH were calculated. Compounds were identified and confirmed by their relative retention time to the corresponding standard (±0.05) as well as a ultraviolet (UV) spectra library match of ≥95%. A calibration curve (linear regression) was generated using the relative peak areas of the calibrators compared to that of the internal standard. A minimum of 3 non-zero calibrators with a coefficient of determination (R^2) ≥ 0.98 were used. Calculations of unknown concentrations were performed using this calibration curve. All calibrators and controls back-calculated to within 20% of the target concentration. The lower limits of quantification of the 7 drugs included in this study were as follows: acetaminophen, 2.0 mg/L; carbamazepine, 2.5 mg/L; ibuprofen, 2.0 mg/L; naproxen, 5 mg/L; phenobarbital, 5 mg/L; phenytoin, 5 mg/L; and salicylic acid, 20 mg/L.

Statistical analysis

Data were analyzed using GraphPad Prism version 7.03 (La Jolla, CA, USA). Drug concentrations between vitreous fluid and peripheral blood were analyzed using a paired two-tailed nonparametric *t*-test. Significant difference was defined with a $P < 0.05$ and denoted with ** if $P < 0.01$ and with **** if $P < 0.0001$. Linear regressions were used to generate the coefficients of determination for the X-Y scatter plots.

Results

Prevalence of drug presence in peripheral blood and vitreous solution

The concentrations of 7 different drugs were analyzed in peripheral blood and vitreous fluid from 89 decedents over 2 years (Table I). Cases originally tested for drugs routinely determined in peripheral blood by this analytical method, and which had sufficient vitreous fluid, were included in this investigation. Of those, acetaminophen exhibited the highest frequency, being present in 50 of the 89 autopsy cases (56%). In all of these cases, acetaminophen was also detected in vitreous fluid. The next most abundant drug finding was naproxen, found in 19% of cases, which was also detected in both peripheral blood and vitreous fluid in all cases. Although ibuprofen and carbamazepine exhibited similar frequency in peripheral blood (10 and

Table I. Frequency of drug occurrence in peripheral blood and vitreous humor in 89 autopsy cases

Drug	Matrix	Number of findings	Frequency (%)
Acetaminophen	Blood	50	56.2
	Vitreous	50	56.2
Carbamazepine	Blood	8	9.0
	Vitreous	8	9.0
Ibuprofen	Blood	9	10.1
	Vitreous	4	4.5
Naproxen	Blood	17	19.1
	Vitreous	17	19.1
Phenobarbital	Blood	4	4.5
	Vitreous	4	4.5
Phenytoin	Blood	3	3.4
	Vitreous	2	2.2
Salicylic acid	Blood	4	4.5
	Vitreous	4	4.5

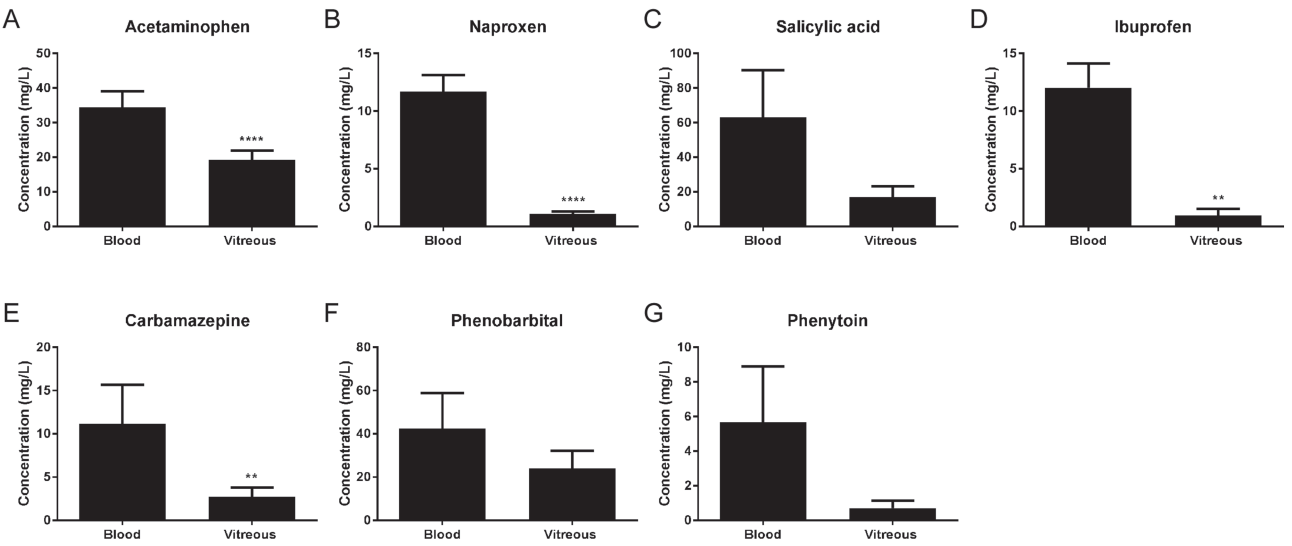

Figure 1. Mean concentrations of acidic drugs in peripheral blood and vitreous humor. Mean concentrations of (A) acetaminophen, (B) naproxen, (C) salicylic acid, (D), ibuprofen, (E) carbamazepine, (F) phenobarbital and (G) phenytoin in peripheral blood and vitreous humor. Concentrations are in mg/L, and error bars represent the standard error of the mean. Statistical significance was defined with a $P < 0.05$ and denoted with ** if $P < 0.01$ and with **** if $P < 0.0001$.

9%, respectively), carbamazepine was detected in vitreous fluid in all 8 cases whereas ibuprofen was only detected in 4/9 (44%) cases. Salicylic acid, phenobarbital and phenytoin were all detected in <5% of autopsy cases. Only three cases had detectable levels of phenytoin in peripheral blood and 2/3 had detectable levels in vitreous fluid.

The average concentrations of the seven acidic drugs were higher in peripheral blood than in the vitreous fluid (Figure 1). Despite the large intra-individual variation, this was statistically significant (non-parametric *t*-test) for acetaminophen, carbamazepine, naproxen and ibuprofen. Statistical significance was not reached for salicylic acid, phenobarbital or phenytoin. This is likely due to the small sample size (frequency of ≤4) and wide range of observed concentrations for each of these drugs.

The average and range of concentrations of drugs in all cases with detectable levels in peripheral blood are presented in Table II. For all seven acidic compounds examined, the median V/PB ratio was less than one, confirming that each drug exhibited higher concentrations in peripheral blood than in the vitreous fluid. Concentrations of acetaminophen in peripheral blood ranged from 5 to 170 mg/L with a median concentration of 19.5 mg/L. The corresponding values in vitreous fluid ranged from 1.1 to 96.0 mg/L. In the case with an acetaminophen concentration of 170 mg/L, the corresponding concentration in the vitreous fluid was 96 mg/L. The cause of death for this particular case was a multi-drug intoxication. Acetaminophen vitreous concentrations were 1–5-fold lower than concentrations in peripheral blood in the majority of cases. In 3 of the 50 autopsy cases with measurable levels of acetaminophen, the concentrations were higher in vitreous fluid than in peripheral blood (peripheral blood concentrations of 61, 12 and 16 mg/L and corresponding vitreous fluid concentrations of 65, 15 and 25 mg/L, respectively). No obvious explanation for this can be gleaned from the causes of death (accidental drug overdose, natural and suicide/jump, respectively) or presence of other substances.

Ibuprofen exhibited the poorest ability to enter the vitreous fluid, with less than half of the cases with measurable ibuprofen in peripheral blood exhibiting detectable amounts in the vitreous. Those with detectable concentrations exhibited a 4–14-fold reduction in concentration in vitreous fluid compared to peripheral blood. Phenytoin, ranging from 1.4 to 12 mg/L in peripheral blood, exhibited a similar inability to enter into the vitreous fluid. Of the 3 cases where phenytoin was detected in peripheral blood, phenytoin was undetectable in vitreous fluid of 1 case and exhibited a 6–8-fold reduction compared to peripheral blood in the remaining 2 cases. Salicylic acid, phenobarbital, carbamazepine and naproxen all demonstrated at least a 2-fold higher concentration in peripheral blood than vitreous fluid in all cases except 1. Naproxen measured at 8.1 mg/L in peripheral blood and 25 mg/L in vitreous fluid in 1

Table II. Average and range of drug concentrations in peripheral blood and vitreous humor

Drug	Matrix	Mean	Median	Minimum	Maximum
Acetaminophen	Blood	34.4	19.5	5.0	170.0
	Vitreous	19.2	14.5	1.1	96.0
	V/PB ratio	0.6	0.6	0.1	1.5
Carbamazepine	Blood	11.1	7.3	2.6	42.0
	Vitreous	2.7	1.8	0.5	10.0
	V/PB ratio	0.2	0.2	0.2	0.3
Ibuprofen	Blood	12.0	11.0	3.8	23.0
	Vitreous	0.9	0.0	0.0	5.4
	V/B ratio	0.1	0.0	0.0	0.2
Naproxen	Blood	11.7	9.3	5.1	22.0
	Vitreous	1.1	0.8	0.3	3.5
	V/PB ratio	0.1	0.1	0.0	0.3
Phenobarbital	Blood	42.5	45.5	8.0	71.0
	Vitreous	24.0	23.7	6.4	42.0
	V/PB ratio	0.6	0.6	0.5	0.8
Phenytoin	Blood	5.7	3.6	1.4	12.0
	Vitreous	0.7	0.6	0.0	1.5
	V/PB ratio	0.1	0.1	0.1	0.2
Salicylic acid	Blood	63.0	55.0	12.0	130.0
	Vitreous	16.8	16.0	2.3	33.0
	V/PB ratio	0.3	0.3	0.2	0.5

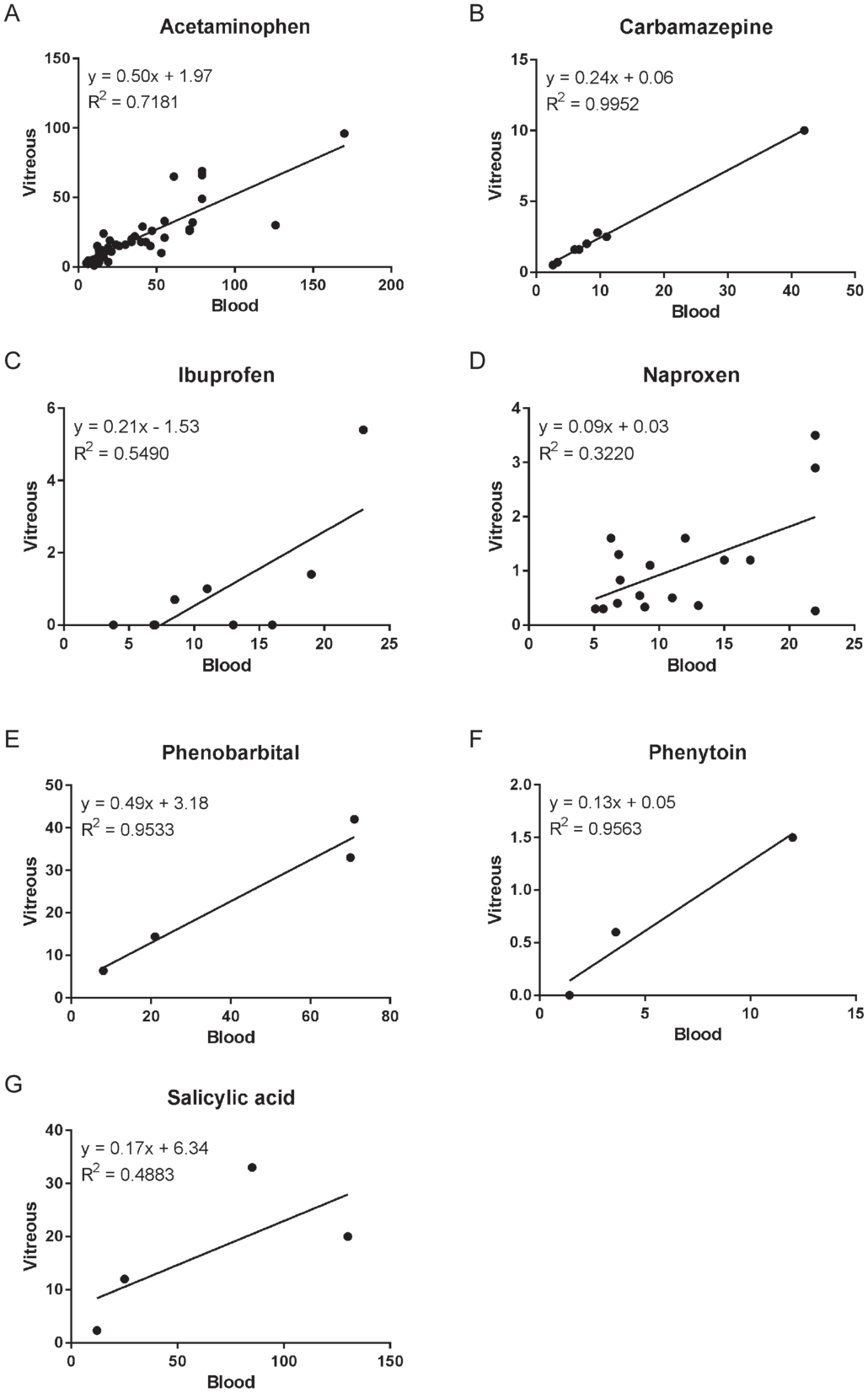

Figure 2. Correlations of drug concentrations between peripheral blood and vitreous humor. Scatter plots of peripheral blood (*x*-axis) and vitreous humor (*y*-axis) concentrations (mg/L) of (A) acetaminophen, (B) naproxen, (C) salicylic acid, (D), ibuprofen, (E) carbamazepine, (F) phenobarbital and (G) phenytoin. Linear regression equations and the coefficient of determination (R^2) for the line of best fit are written within each graph.

autopsy case. All other cases had a 5–85-fold higher concentration of naproxen in peripheral blood as compared with vitreous fluid. The median value for naproxen in vitreous fluid was 0.8 mg/L. The vitreous fluid specimen with a measured concentration of 25 mg/L was greater than 3 standard deviations from the mean and a Dixon's Q test with >99% confidence interval determined this to be an outlier. Therefore, this specimen was removed from all analyses and is not presented in any of the figures.

Correlations between matrices

The linear agreement between peripheral blood and vitreous fluid is shown in Figure 2. The best correlation observed was for carbamazepine, with a coefficient of determination (R^2) of 0.995. The next best correlations were seen with phenobarbital and phenytoin, both exhibiting an R^2 value >0.95. This suggests that vitreous fluid can be used to estimate peripheral blood concentrations when necessary for these acidic AEDs. However, caution should be taken when interpreting these results due to the low sample size ($n \leq 4$) of both phenobarbital and phenytoin. Of the OTC analgesics, acetaminophen exhibited the best correlation with an R^2 of 0.718, whereas naproxen exhibited the worst correlation with an R^2 of 0.322. Therefore, acidic OTC analgesic concentrations do not correlate well between peripheral blood and vitreous fluid.

Discussion

Overall, we observed decreased concentrations of acidic drugs in vitreous fluid compared to peripheral blood resulting in low V/PB ratios. Ibuprofen exhibited the lowest detection rate and concentration in the vitreous humor. Acetaminophen was detected in more than half the autopsy cases presented. This is logical because acetaminophen is widely used as an analgesic and antipyretic, is occasionally used to commit suicide and is coformulated with opioids, which have high addiction and overdose potential (23). Vitreous humor showed potential as an alternative matrix for detection of all drugs included in this study with the exception of ibuprofen and phenytoin. Ibuprofen and phenytoin exhibited low concentrations in the vitreous humor and was detected in 4/9 and 2/3 cases where it was measurable in peripheral blood, respectively. Finally, a good linear correlation between vitreous humor and peripheral blood for the AEDs suggest a potential mechanism to predict peripheral blood concentrations when this matrix is unavailable.

Size, rate of clearance, volume of distribution, half-life, acidity (pKa) and lipophilicity (partition coefficient, logP) all may affect the ability of a drug to cross the blood–retinal barrier (Table III) (24, 25). In general, the OTC analgesics tend to be absorbed rapidly and reach peak concentrations shortly thereafter; the peak concentration of acetaminophen, ibuprofen and salicylic acid are all within 1 hour, whereas naproxen peaks within 2–4 hours (25). AEDs, on the other hand, tend to exhibit slow absorption, have limited aqueous solubility, distribute rapidly and display more variable pharmacokinetics (25). Carbamazepine is absorbed erratically and is rapidly distributed into all tissues. The concentrations of carbamazepine in cerebrospinal fluid correlate well with free drug levels in plasma, indicating it is capable of crossing these biological barriers. Phenytoin does not exhibit linear kinetics, and the rate of elimination depends on the concentration. These delayed pharmacokinetic properties may contribute to their ability to better equilibrate between the blood and vitreous humor (26).

In a previous study, we demonstrated that many drugs were similar in concentration or trended toward higher concentrations in vitreous fluid compared to peripheral blood (21). These included opioids such as oxycodone and hydrocodone. These alkaloid drugs are largely neutral and are highly lipid soluble and known to penetrate the blood–brain barrier (27), which may contribute to their ability to cross the blood–retinal barrier. Antidepressants such as trazodone, sertraline and venlafaxine, as well as diazepam, a benzodiazepine, exhibit lower concentrations in vitreous humor compared to peripheral blood (21, 28). Relative to opioids, these drugs exhibit a slightly higher polarity, which may contribute to their inability to penetrate the blood–retinal barrier. Similarly, the acidic drugs in this study all exhibited low V/PB ratios, indicating chemical properties that reduce its ability to enter the vitreous humor. Acetaminophen exhibited a relatively high detection rate in the vitreous humor compared to ibuprofen and is minimally protein bound (<20%) (25). Ibuprofen exhibited a poor detection rate in vitreous humor and is almost entirely protein bound (>99%) (25). The V/PB ratio is likely influenced by both lipophilicity and protein binding (29). Finally, PMR is greatest with lipid-soluble drugs, whereas neutral and acidic drugs are less affected (2). Therefore, lower concentrations of acidic drugs may be due to—or a reflection of—reduced redistribution.

When blood is not available for postmortem analysis, a toxicologist may have to rely on vitreous humor for drug screens. However, interpretation is difficult without established reference ranges or correlative data to blood concentrations. Bevalot and

Table III. Drug pharmacokinetic characteristics

Drug	Molecular weight (g/mol)	Bound in plasma (%)	Clearance (mL/min/kg)	Volume of distribution (L/kg)	Half-life (hours)	pKa	LogP
Acetaminophen	151	<20	5± 1.4	0.95± 0.12	2± 1.4	9.759	0.55
Carbamazepine	236	74± 3	1.3± 0.5	1.4± 0.4	15± 5	N/A	2.93
Ibuprofen	206	>99	0.75± 0.20	0.15± 0.02	2± 0.5	4.366	3.75
Naproxen	230	99.7± 0.1	0.13± 0.02	0.16± 0.02	14± 1	4.234	2.97
Phenobarbital	232	51± 3	0.062± 0.013	0.54± 0.03	99± 18	N/A	1.52
Phenytoin	252	89± 23	Nonlinear, V_{max} = 5.9± 1.2 mg/kg/day; K_m = 5.7± 2.9 mg/L	0.64± 0.04	6–24	N/A	2.14
Salicylic acid	131	80–90	N/A	0.17	2–30	13.038, 2.983	1.2

Pharmacokinetic properties were obtained from Hardman and Limbird (2001) and physical constants from ChemDraw (1985) (24, 25).

colleagues (2011) showed that meprobamate, a carbamate derivative, exhibits a linear relationship between peripheral blood and vitreous humor concentrations using a logarithmic model with an R^2 of 0.94 (12). Here, we found that carbamazepine, a much more commonly prescribed drug, also exhibits a linear relationship between vitreous humor and peripheral blood and can serve as a means to estimate blood concentrations when necessary. However, a direct correlation between OTC analgesic vitreous humor and peripheral blood concentrations was not observed. Therefore, vitreous humor concentrations of these drugs may be used as an alternative matrix for detection of acetaminophen, naproxen and salicylic acid, but not to necessarily appropriate to estimate blood concentrations. The low detection rate of ibuprofen in vitreous humor limits its potential as both an alternative matrix and a means to correlate to blood concentrations.

AEDs often have a narrow therapeutic index and are frequently measured to optimize dosing. Phenytoin has a therapeutic index of 10–20 mg/L (30) and is considered toxic when concentrations exceed 30 mg/L. None of the cases presented here exceeded 12 mg/L in peripheral blood. Carbamazepine has a therapeutic range of 4–12 mg/L and exhibits adverse central nervous system (CNS) effects at concentrations >12 mg/L (30). Here, the median observed blood concentration of carbamazepine was 7.3 mg/L, and only 1 case had carbamazepine peripheral blood concentrations above the therapeutic range. The therapeutic range of phenobarbital is 15–40 mg/L and has toxic effects at concentrations >40 mg/L (30). Of the 4 cases with detectable levels of phenobarbital included in this study, 2 decedents had concentrations above 40 mg/L in peripheral blood (70 and 71 mg/L), whereas the other 2 fell within or below the therapeutic range (8 and 21 mg/L). In all cases, concentration of AEDs in vitreous humor was lower in peripheral blood but correlated well ($R^2 > 0.95$). This highlights the importance of monitoring AEDs and emphasizes the significance of vitreous humor as an alternative matrix when necessary.

Limitations

First, the vitreous assay was based on analytical method development for measuring drugs in blood, and an extensive validation was not performed. Calibrators and QCs were spiked into blank vitreous humor. A linear calibration curve was established, and QCs with acceptable concentrations (within 20% of the target) and similar responses (area counts) as the blood QCs were demonstrated. The extraction efficiency and appropriate detection limits were not investigated. Second, some compounds, particularly phenytoin, suffered from a low number of case specimens due to its low prevalence in autopsy cases. Third, antemortem values were not available to establish the extent of PMR in either matrix.

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