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Journal

Journal of Analytical Toxicology, 40(4)

ISSN

0146-4760

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Publication Date

2016-05-01

DOI

10.1093/jat/bkw009

Peer reviewed

Journal of Analytical Toxicology Advance Access published March 5, 2016

Journal of Analytical Toxicology, 2016, 1–5
doi: 10.1093/jat/bkw009
Article

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Assessment and Comparison of Vitreous Humor as an Alternative Matrix for Forensic Toxicology Screening by GC–MS

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Abstract

Alternative specimens have been occasionally considered as substitutes for whole blood for postmortem toxicology testing. We studied the applicability of vitreous humor, and evaluated whether it would be suitable to replace (or augment) whole blood for routine drug screening. Results showed that from 51 autopsy cases, we were able to identify an aggregate of 209 findings in whole blood compared with 169 in vitreous. The total number of compounds identified was 71 for whole blood and 60 for vitreous humor. Quantitative analysis showed that whole-blood concentrations of trazodone were several fold higher than vitreous humor concentrations (1.42 ± 0.57 vs. 0.15 ± 0.05 mg/L, respectively) and similar results were also obtained for diazepam (0.37 ± 0.06 vs. 0.13 ± 0.01, respectively). For other drugs such as oxycodone, hydrocodone and doxylamine, a trend suggesting higher concentrations in vitreous humor vs. whole blood was observed; however, this was not significant. Our results are consistent with the limited work of other investigators, and suggest that vitreous humor could be an appropriate matrix for drug screening in postmortem toxicology.

Introduction

Mass spectrometry (MS) has gained a leading role as the technique of choice used in forensic toxicology. The choice of specimen for MS analysis includes blood, tissue, urine, saliva, hair and vitreous humor. For quantitative results, blood has been the primary matrix utilized in postmortem toxicology (1). However, one substantial issue with interpreting concentrations of drugs in blood specimens is that postmortem redistribution can change concentrations after death (2). Vitreous humor has been proposed as an alternative matrix to blood for postmortem toxicology testing (3, 4). Vitreous fluid offers several advantages over other specimens such as longer sample stability and easier pretreatment (1). However, major limitations that have hampered the use of vitreous fluid as an alternative matrix for postmortem toxicological analyses include limited specimen volume, and the blood–retinal barrier that limits the passage of drugs in and out of the vitreous fluid (5). Some reports have used targeted analysis to compare drug concentrations between whole blood and vitreous fluid, such as for oxycodone (6) designer amphetamines (7), benzodiazepines (1) cocaine (8, 9), 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (10) and opiate (9), but there are no reports comparing broad-spectrum drug screening results between whole blood and vitreous fluid.

Electron impact gas chromatography (GC)–MS has been traditionally used for postmortem forensic toxicological studies. When utilizing GC–MS, full scan spectra from the analyte are matched against a pre-existing library and a score which describes how well the two spectra match is generated. The efficiency of GC when combined with simple extraction protocols and a large spectral library has established GC–MS as a commonly used technique for postmortem drug identification.

In this study, GC–MS was employed to investigate whether vitreous fluid could serve as an alternative matrix for postmortem toxicology studies for broad-spectrum postmortem drug screening. Results obtained in vitreous were compared with those of whole blood.

Experimental methods

Sample collection

Whole-blood and vitreous fluid samples from 51 autopsy cases were collected during forensic autopsies. Peripheral blood (~20 mL) was drawn from the left common iliac vein (blood returning from the leg and visually identified in the pelvis at autopsy) and stored in standard glass tubes containing sodium fluoride (100 mg) and potassium oxalate (20 mg). Vitreous humor samples (~5 mL) were pulled from both eyes with a syringe and stored in a glass tube without preservative. All specimens were stored at 4°C.

Detection of drugs in whole blood was performed through a validated method used routinely in autopsy cases. Drug-free porcine blood or human drug-free pooled vitreous humor was used as the alternative matrix for quality control (QC) and calibrator preparation. An initial investigation was performed to assess the validity of replacing vitreous fluid with water or phosphate-buffered saline (pH 7.4) for calibrators and QC material. However, due to unsatisfactory recovery studies (data not shown), none of these matrixes were appropriate to substitute vitreous fluid. Consequently, a pool of human drug-free vitreous fluid was collected to prepare calibrators and QC.

Materials

Dichloromethane, methanol, ethyl acetate and isopropanol were EMD Chemicals OmniSolv grade, purchased through VWR International (Radnor, PA, USA). Ammonium hydroxide (ACS), cyclizine hydrochloride and glacial acetic acid (ACS) were obtained from VWR International. Zinc sulfate heptahydrate (certified ACS) was obtained from Fisher Scientific (Pittsburgh, PA, USA).

Sample preparation

The procedure for drug screening was as previously described (11, 12). Calibrators, patient samples and QC were fortified at the appropriate concentration and the final volume was made up to 2 mL in the appropriate matrix. To this was added 200 µL of internal standard (1.0 µg: cyclizine) and 200 µL of 2% ascorbic acid. Samples were mixed, precipitated with 5 mL of zinc sulfate (5% methanolic solution), centrifuged at 2,400 *g* for 10 min and the supernatant was treated with 4 mL of sodium acetate (pH 6.0) buffer.

The supernatant was passed through an SPE column (SPEWare, Baldwin Park, CA, USA) which was initially preconditioned with 3 mL of methanol, 3 mL of distilled water and 2 mL of sodium acetate (pH 6.0). Following sample extraction, the column was washed with 3 mL of distilled water, 2 mL of 100 mmol/L of acetic acid and 3 mL of methanol. Cartridges were dried and the analytes were eluted with a mixture of dichloromethane : isopropanol : ammonium hydroxide (78 : 20 : 2). Samples extracts were then dried under nitrogen and reconstituted with 150 µL of ethyl acetate for GC–MS analysis.

GC–MS analysis

One microliter of sample was injected into 7890A GC–MS system (Agilent Technologies, Santa Clara, CA, USA) consisting of a 15-m, 0.25-mm diameter and 0.25-µm film thickness analytical column (Zebron, ZB-5MS; Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas with a flow rate of 1.1 mL/min. The inlet temperature of the GC was 250°C with an initial oven temperature of 85°C which increased to 170°C at the rate of 40°C/min (held 4 min), then 40°C/min to 190°C (held 5 min) and finally 10°C/min up to 300°C (held 7 min). The MS Aux was 280°C. The mass selective detector (5975C, Agilent Technologies) was set in scan mode with a solvent delay of 2.64 min. The MS mass range was 43.00–550.00 amu, the threshold was 150 counts and the sampling rate was 2 (2^N).

Data analysis

By using peak area ratios (including IS), the procedures employed enabled quantification of several commonly detected compounds by the utilization of appropriate calibration curves. The compounds that could be quantified (or semi-quantified) were split into three panels (Supplementary Table I). Panels A and B consisted of a five-point calibration curve (0.1–1.0 mg/L), whereas Panel C consisted of a single-point calibrator (generally 0.50 mg/mL) which only provided a semi-quantitative estimate of drug concentrations. Relevant drugs from Panel C were routinely quantified with a second specific analytical technique. For drug Panels A and B, samples with low concentrations that were unlikely to be related to cause of death (toxicologically insignificant) were routinely reported directly from the results obtained with this screening procedure.

Data were analyzed using the analyst software (Agilent Technologies). Peak identification was determined by relative retention time (relative to the internal standard ±0.08), and then mass spectral matching from a commercial MS library and/or SWGDRUG mass spectral library (http://www.swgdrug.org; at least 70% match).

Validation

During each run, two different QC samples fortified in vitreous fluid at 0.5 mg/L were run (QC A and QC B; Supplementary Table II) in duplicates. The calibration curves were linear and the concentration of QC samples was expected to be within 20% of the 0.50 mg/L target.

Statistical analysis

Data were analyzed by the GraphPad prism software (La Jolla, CA, USA) using nonparametric two-tailed *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

To the best of our knowledge, only one other laboratory has performed a broad-spectrum drug screening study, and compared drug concentrations between urine and vitreous humor using an LC-time-of-flight (TOF) system (3). This earlier investigation reported that the number of drugs in vitreous fluid and the concentration of such drugs were lower than urine (3). We compared the number of drugs that were identified in whole blood with that determined in vitreous fluid from the same decedents. Furthermore, for those drugs that were able to be quantified, we also compared their relative concentrations. The aggregate number of findings in whole blood and vitreous fluid was 209 and 169, respectively. The total number of different compounds identified was 71 for whole blood and 60 for vitreous. Results are given in Table I.

Some drugs proved to be more difficult to identify in vitreous humor compared with whole blood. We applied criteria that if a drug was identified twice in whole blood and if it was identified at a rate of 50% or lower in vitreous, then we considered it as difficult to be identified in vitreous humor. Using these criteria, drugs that were difficult to identify included: 7-aminoclonazepam, benztropine, cyclobenzaprine, morphine, norvenlafaxine, phenytoin, promethazine, zolpidem and zopiclone. Possible explanations for difficulty in detecting these drugs include: low concentrations of drugs in vitreous humor compared with whole blood, inability of the drug to cross the blood–retina barrier, drug redistribution to other tissues than vitreous humor

Table I Frequency of Drug/Metabolite Occurrence in 51 Autopsy Cases

Compound	Number of findings in blood	Number of findings in vitreous	Compound	Number of findings in blood	Number of findings in vitreous
7-Aminoclonazepam	2	0	Lidocaine	4	3
Acetaminophen	2	2	Meprobamate	1	1
Alprazolam	6	5	Methadone	3	3
Amitriptyline	3	3	Metronidazole	1	1
Benzoylecgonine	2	2	Mirtazapine	5	5
Benztropine	2	0	Mitragynine	1	1
Brompheniramine	1	1	Morphine	10	5
Bupropion	1	1	Naproxen	1	0
Bupropion metabolites	1	1	*n*-Desmethyltramadol	1	1
Carisoprodol	1	1	Nordazepam	9	6
Chlordiazepoxide	3	3	Norfluoxetine	1	1
Chlorpromazine	1	1	Norpromethazine	1	1
Chlorpheniramine	2	2	Nortriptyline	5	4
Citalopram	6	6	Norvenlafaxine	2	1
Chlorpheniramine	1	1	*O*-desmethyltramadol	2	2
Cocaethylene	2	2	Olanzapine	2	2
Codeine	3	3	Oxycodone	6	5
Cyclobenzaprine	2	1	Oxymorphone	3	3
Desmethylcitalopram	1	0	Paroxetine	1	0
Desmethylvenlafaxine	1	0	Phenytoin	2	0
Dextromethorphan	4	4	Promethazine	2	1
Diazepam	9	6	Propranolol	1	1
Dihydrocodeine	4	3	Quetiapine	3	3
Diphenhydramine	11	11	Quetiapine metabolites	6	5
Diphenhydramine metabolite	2	2	Quinine	1	0
Doxylamine	5	5	Sertraline	3	2
Fentanyl	2	2	Tapentadol	1	1
Fluconazole	2	2	Topiramate	2	2
Fluoxetine	4	4	Tramadol	2	2
Gabapentin	7	7	Trazodone	12	11
Haloperidol	1	0	Trihexyphenidyl	1	0
Hydrocodone	6	5	Trimethoprim	1	1
Hydromorphone	1	1	Venlafaxine	3	3
Ibuprofen	2	0	Zolpidem	2	1
Levamisole	2	2	Zopiclone	2	1

and matrix effects. Since the number of specimens included in this study was small, a larger study would be necessary to further determine if such drugs are routinely difficult to identify in vitreous humor.

A comparison of the concentrations for those compounds that were quantified ($n \geq 3$) was then performed. A statistically significant increase in the concentration of trazodone and diazepam was observed in whole blood when compared with vitreous fluid (Figure 1), with a trend being observed for fluoxetine (however, this was not significant). A trend suggesting higher drug concentrations in vitreous fluid compared with whole blood was observed for oxycodone, hydrocodone and doxylamine; however, these results were not significant. The small non-statistical increase in oxycodone concentrations is consistent with other investigations which have found a ratio of 1.16 in vitreous humor/blood (6). Pelander *et al.* (3) investigated broad drug screening in vitreous by using LC-TOF–MS and compared it with urine. In that study, the authors were able to identify 45 compounds and 24 metabolites in vitreous humor compared with 55 compounds and 39 metabolites in urine. The total number of findings in vitreous was 245 compared with 376 in urine. These findings were similar to the findings of the present study where there was a reduction in the number of drugs identified when analyzing vitreous humor compared with whole blood.

Only some of the drugs that were detected could be quantified. In order for a drug to be quantified, it had to meet the detection criteria outlined in the methods; in addition, the concentration of the drug had to be within the concentration range of the calibrators. Our results showed that the concentrations of trazodone and diazepam were several fold lower in vitreous fluid compared with whole blood. Trazodone has high plasma protein binding (13), which could account for the higher concentrations observed here. Diazepam and other benzodiazepines have also been found to have lower concentrations in vitreous compared with whole blood (1).

There are a number of limitations to our study. First, our vitreous assay was based on an analytical method developed for measuring drugs in whole blood and we did not perform an extensive validation of using the technique for vitreous fluid. For validation of the vitreous fluid assay, we established that calibration curves were linear and that the QC samples demonstrated a similar response (area) as blood QC with appropriate recovery of target concentrations. However, the extraction efficiency of all compounds was not specifically determined. In addition, detection and quantification limits were not determined and remain the subject of additional investigations. Second, this 'screening method' has not been optimized for the detection of opiates, which may help explain why morphine was a difficult drug to identify.

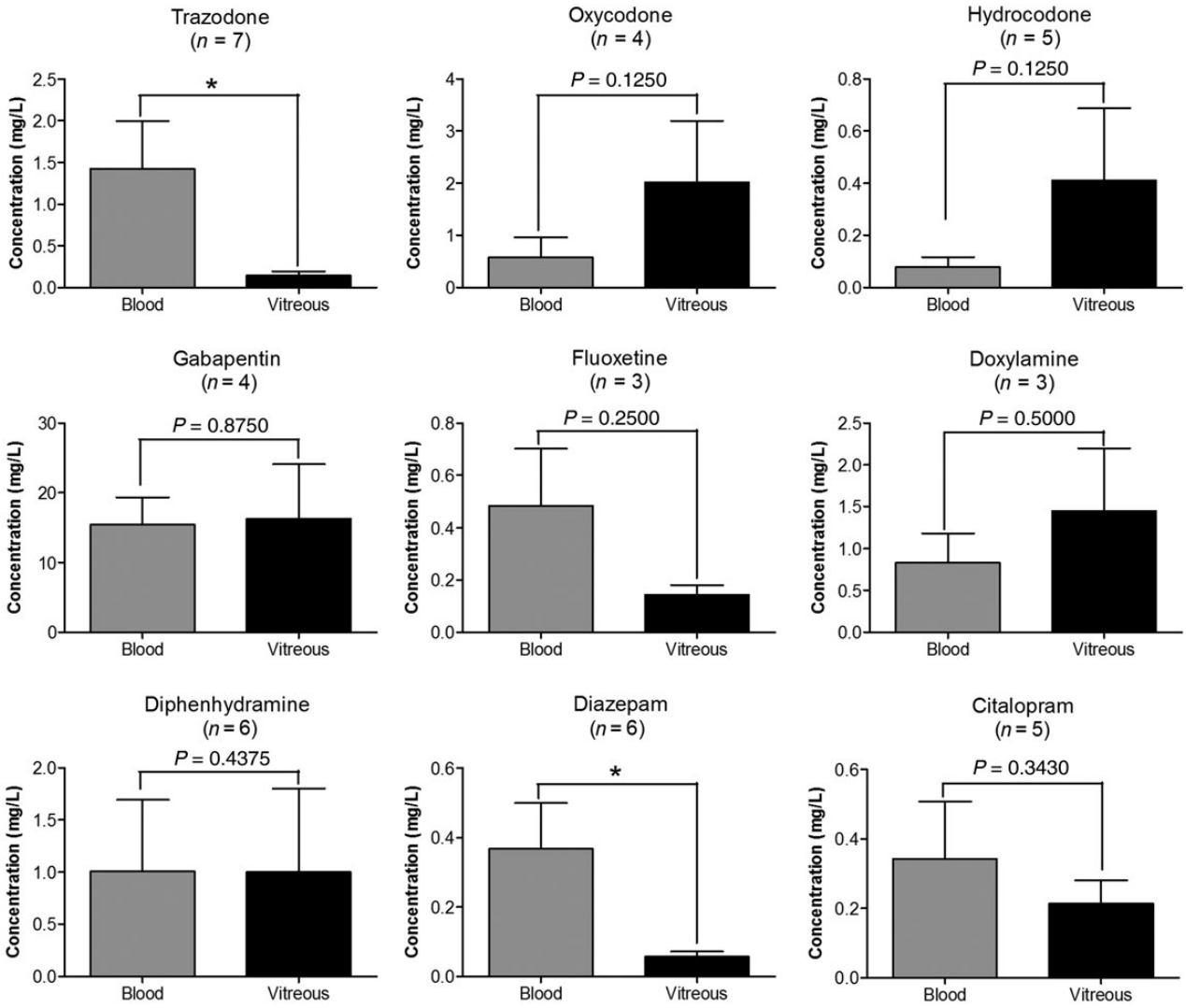

Figure 1. Concentration differences between whole blood and vitreous fluid for nine drugs. A statistically significant decrease in vitreous fluid concentrations was observed for trazodone and diazepam when compared with whole-blood concentrations. *$P < 0.05$.

Third, the gabapentin QC failed to be within the 20% CV of the target (Supplementary Table II), indicating that quantitation for this drug was not optimal and in need of additional investigation.

Our results are consistent with those of other investigators (3), suggesting that vitreous humor can be an alternative matrix for qualitative postmortem drug screening. Although some of the drugs were difficult to identify, the vast majority of drugs identified in blood were also found in vitreous fluid.

Supplementary data

Supplementary data are available at *Journal of Analytical Toxicology* online.

Acknowledgments

The authors thank the San Diego County Chief Medical Examiner, Dr Glenn Wagner, for making available case details described in this report.

Funding

I.G.M.'s fellowship in Clinical Chemistry is funded by Roche Diagnostics.

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