A Preliminary Investigation into Retrospective Calculation of In-Vivo Drug Concentrations in Dried Crime Scene Blood

Dwain C. Fuller*, Patricia Pisana, Veterans Affairs North Texas Health Care System, Dallas, TX, U.S.A.

Abstract:
Dried blood left at a crime scene is at times of forensic toxicological interest. Such as in determining if an injured motorist who has left the scene of an accident, and not apprehended until many hours or days later, may have been under the influence of drugs at the time of the accident. Or in determining if a wounded individual who has escaped the scene may have been under the influence of drugs during a violent dispute.
Dried blood spots have been used for many years in clinical laboratory testing. These tests rely on premeasured volumes of blood or standardized blotter material for calculating in-vivo concentration. However, dried blood discovered at a crime scene can rely on no such convention to determine original specimen volume. Furthermore, dried crime scene blood is an unfamiliar matrix, which by its nature is unpreserved and unrefrigerated. These factors raise the questions of drug recovery and stability in this matrix. The objective of this study is to provide forensic toxicologists with a conversion factor to accurately estimate blood volume from the weight of a dried blood specimen as well as to provide data on recovery and stability of drugs in this matrix.

The authors investigate the potential for back calculating in-vivo drug concentration using the weight of dried blood to predict the original volume. The authors also investigate the recovery and stability of several drugs of abuse in dried blood. The present study evaluates the recovery and stability of amphetamine, methamphetamine, MDA, MDMA, codeine, morphine, hydrocodone, hydromorphone, cocaine, ecgonine methyl ester, and benzoylecgonine over a period of weeks and months.

While this is a limited study and further investigations are warranted, it was found that the volume from which a specimen of dried blood originated could be calculated within 23% with a confidence level of 95%. Furthermore, with the expected exception of cocaine, ecgonine methyl ester and perhaps benzoylecgonine, the drugs investigated were quite stable in a dried blood matrix over a period of months. Recovery for most drugs investigated was well above 90%. These findings along with future investigations may allow for the calculation of original drug concentration from the analysis of dried crime scene blood and provide an estimate of the uncertainty of this process.

Introduction:
Blood from a suspect or victim that is left at the scene of a crime or accident may at times be crucial to the prosecution or defense of a crime. This blood by its very nature will be unpreserved and unrefrigerated and in usually in a short period of time will be dried. The purpose of this investigation is to determine if it is feasible to detect and quantitate drugs in unpreserved dried blood and to convert the mass/mass concentration of dried blood to the commonly understood mass/volume concentration of liquid blood. By understanding the limitations and uncertainty in this process the forensic toxicologist will be better equipped to interpret the results of such an investigation.

Phase I – Determination of Mass to Volume Conversion for Dried Blood
Specimen selection:
The authors randomly selected fifty specimens submitted to the clinical laboratory of the Veterans Affairs North Texas Health Care System at Dallas for CBC analysis. Specimens submitted for CBC analysis were chosen for two reasons; the fact that they are whole blood collected in EDTA tubes (approximately 7 mg) and are thus unpreserved, and the fact that CBC analysis is a rather routine assay for all patients and would not overly represent any one disease state, as would specimens submitted for hemoglobin A1C, for example. Each tube contained 3.5 – 4 mL of whole blood. Since the specimens were existing diagnostic specimens and the information was recorded in a fashion such that subjects cannot be identified, no IRB approval was necessary. The demographics of the selected specimens are shown in **Figure 1.**

Figure 1.

Demographics		
Gender:	Males: 48	Females: 2
Age:	Mean: 63	Range: 33 – 90
Race:	Caucasian: 28 African American: 13 Hispanic: 3 Unknown: 6	

Experimental:
The specimens were mixed on a rotary mixer for at least 15 minutes. A weighing boat was pre-weighed and the weight recorded. Five hundred microliters of blood was quantitatively pipetted into the weighing boat and the weight was immediately recorded. Specimens were dried at room temperature (71±1°C, Relative humidity: 42±8%) for a minimum of 72 hours. Specimens were then re-weighed and the weight recorded. The weight of dried blood per milliliter of blood was calculated. The percent water and blood density were also calculated for the purpose of comparison to related studies.

In an effort to achieve the best accuracy and precision, one analyst performed all of the pipetting. Additionally, the weight of the dried blood was corrected for the weight of the EDTA anticoagulant at a ratio of 2 mg/mL.

Results:
The results of the calculations and their comparisons to related studies are shown in **Figures 2 and 3**, respectively. (Below)

Figure 2.

Results	
Dried blood to liquid blood mass:volume ratio	
Mean:	0.215 ± 0.025 g/mL
Range:	0.161 – 0.259 g/mL
Median:	0.220 g/mL

Figure 3.

Comparison to other studies	
Blood density:	
1.052 ± 0.020 g/mL	Present study
1.055 g/mL	Karch, Forensic Issues in Alcohol Testing, CRC Press, 2007
1.06 g/mL	Cutnell, et al. Physics, 4th edition, Wiley, 1998
1.025 – 1.125 g/ml	Benson, K. MCAT Review, Emory University, 1999
1.043 – 1.057 g/mL	Hinghofer-Szalkay, et al., Continuous Monitoring of blood volume changes in Humans, Journal of Applied Physiology, Vol 63, 1987
Percent water:	
79.6 ± 2.4 % g/g	Present Study
76.9 – 82.0 % g/g	
80.5 % g/g	Females
78.9 % g/g	Males

Lijnema, et al., Gravimetric determination of the water concentration in whole blood, plasma and erythrocytes and correlations with hematological and clinicochemical parameters. Clinica Chimica Acta, 214, 1993

Discussion:
Preliminary observations would indicate that it may be possible to retroactively determine the volume from which a sample of dried blood was derived within approximately 23% with a confidence of 95%.

Limitations of Study:
The limitations of this study lie primarily in the small sample size of 50 participants comprised of mostly males with a mean age of 63 as compared to the mean age in the United States of 35 – 38 years. An additional confounding parameter may be that the sample, due to the age of the participants and hospital setting, may over represent individuals with underlying disease states. It must also be recognized that specimens containing EDTA as an anticoagulant may not adequately represent dried blood collected from a crime scene do to possible in-situ clotting

Phase II – Drug Recovery and Stability in Dried Blood
Experimental:
Randomly selected specimens submitted to the clinical laboratory of the Veterans Affairs North Texas Health Care System at Dallas for CBC analysis, as previously described, were pooled and the mass of dried blood to volume for the pooled blood was determined as previously described. The pooled blood was screened by GC/MS for amphetamine, methamphetamine, MDA, MDMA, codeine, morphine, hydrocodone, hydromorphone, cocaine, ecgonine methyl ester, and benzoylecgonine. After determining that the pooled blood contained none of these drugs at or above the laboratory LOD, three separate aliquots of the pooled blood were spiked for determination of drug recovery and stability. One specimen was spiked at 100 ng/mL with amphetamine, methamphetamine, MDA, and MDMA, another specimen was spiked at 100 ng/mL with codeine, morphine, hydrocodone, and hydromorphone, and yet another with 100 ng/mL with cocaine, ecgonine methyl ester, and benzoylecgonine. To preserve the integrity of the mass to volume conversion, the pooled blood specimens were spiked by first evaporating the drug containing solvent in a container then adding the blood to the dried drug residue.

A brief description of the analytical method is as follows: Dried blood specimen was transferred into a disposable 16 x 100 mm culture tube and ground to a powder using the rounded end of a 10 mm diameter glass stir rod as a pestle. Powdered specimen was transferred to a tared tube and the weight of the specimen was recorded. Buffer of the appropriate pH for the planned extraction was added to each tube. Tubes were capped and rotated on laboratory rotator for 10 minutes then shaken vigorously. Tubes were centrifuged, if employing a subsequent SPE extraction. Extraction proceeded from this point as if the specimen were whole blood.

Recovery:
Percent recovery of each analyte from dried blood was calculated by converting the mass/mass concentration of each analyte recovered from the dried blood specimen to a mass/volume concentration using the mass to volume conversion factor previously calculated for the pooled blood, then dividing by the concentration of the corresponding analyte in the original liquid blood specimen. Percent recovery was calculated on the first day after drying was complete. Due to its expected lability, drying of cocaine spiked specimens for recovery calculation was expedited by a stream of nitrogen and a short period of time in a 60°C oven.

Drug Stability:
Stability of each analyte in dried blood was determined by pipetting 1 mL of blood spiked as previously described into a weighing boat and drying as previously described. Randomly selected dried specimens were assayed over a period of days, weeks and months to determine residual mass/volume concentration after converting from mass/mass concentration using the previously calculated mass to volume conversion factor for the pooled blood. Due to expected degradation of cocaine and metabolites in the original spiked pooled liquid blood specimen, cocaine stability for both the dried and original spiked specimen was calculated against a freshly spiked blood standard using the original spiking solution which was stored at -46°C.

Results:
The results of the recovery study are shown in Table 1.
The results of the stability studies are shown in **Figures 4, 5,** and **6.**

Table 1.

Recovery	
Amphetamine	95 %
Methamphetamine	93 %
MDA	94 %
MDMA	85 %
Codeine	96 %
Morphine	99 %
Hydrocodone	95 %
Hydromorphone	94 %
Cocaine	84 % *
Ecgonine methyl ester	103 % *
Benzoylecgonine	98%

*These values may reflect some breakdown of cocaine to EME

Discussion:
Drug recovery from the dried blood matrix was quite satisfactory with recovery of most of the analytes approaching 100%. A notable exception is MDMA at 85%. Of interest is cocaine at 84% and ecgonine methyl ester at 103%. These values may reflect degradation of cocaine to EME during the drying of the blood.

As can be seen from Figures 4 and 5, amphetamine, methamphetamine, MDA, MDMA, codeine, morphine, hydrocodone, and hydromorphone are all quite stable for a period of approximately 6 months, losing only 20 – 25% of the original concentration during that time. The erratic results for MDMA, are likely related to erratic recovery, as previously indicated.

The stability of cocaine and its metabolites, as shown in Figure 6, require a more detailed discussion. Due to the expected lability of these analytes and for direct comparison purposes, stability in both the unpreserved liquid (stored at 4°C) and dried blood was determined and plotted on the same graph.

Cocaine
Cocaine in refrigerated unpreserved blood, as expected, dropped to undetectable levels in a matter of a few days. Cocaine in the dried blood specimens showed a loss of 44% in 14 days (the first determination), but appeared to be reasonably stable beyond that point. This phenomenon may be due to a rapid loss of cocaine at room temperature in the unpreserved blood prior to becoming fully dry. A future study is planned that will include more early points. It is interesting to note, however, that Alfazil and Anderson (JAT, 32:511; 2008) reported a cocaine loss in dried blood spots of only 19.9% in one month which would indicate that in this present study cocaine degraded at four times the rate determined by these investigators. This is likely due to Alfazil and Anderson's choice of specimen matrix. Alfazil and Anderson prepared a "blood" matrix by diluting packed cells in isotonic saline. This process, for the most part, replaced the plasma in the whole blood and probably resulted in a greatly reduced amount of pseudocholinesterase being present, as compared to authentic whole blood, thus greatly reducing cocaine degradation. (Baselt, Journal of Chromatography, 268,1983,502-505)

Benzoylecgonine
Benzoylecgonine appears to be more stable in dried blood vs. unpreserved liquid blood. However, this observation may be misleading due to an apparent increased tendency for cocaine to degrade to benzoylecgonine rather than EME in dried blood as evidenced by a slightly increasing concentration of BE in the dried blood matrix. In short, what appears to be stability may simply be the result of competing processes of production and degradation of benzoylecgonine.

Ecgonine Methyl Ester
As expected, EME rapidly rises as a result of the degradation of cocaine in unpreserved liquid blood, prior to dropping due to degradation. In contrast, EME in dried blood rises only slightly before dropping at a less rapid rate. This tends to demonstrate that cocaine in dried blood degrades more rapidly to benzoylecgonine than EME.

Limitations of Study:
Whereas this study was conducted under controlled laboratory conditions, actual crime scene blood may not be accurately represented. Actual crime scene blood would be subjected to in-situ clotting, protein binding of drugs, differing substrates from which it must be recovered, and a wide variety of weather conditions.
Future Studies:
The present study is admittedly preliminary and warrants further investigations. Among areas of investigation that should be pursued are: evaluating a larger and more diverse population in regard to the conversion of the mass of dried blood to a volume of liquid blood, evaluating the stability of cocaine and its metabolites in more detail, evaluate the recovery and stability of more drugs, and validating the study in authentic individuals.

Figure 4.

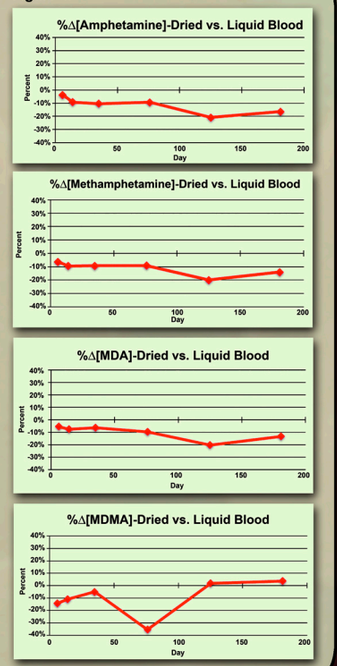


Figure 5.

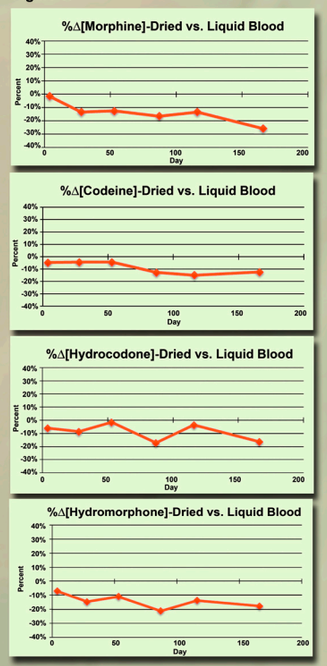
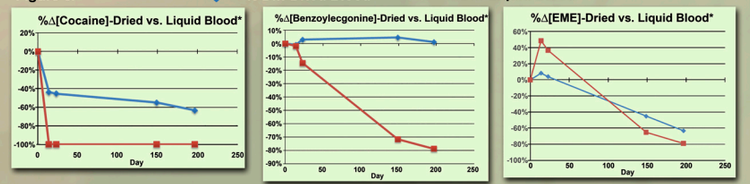


Figure 6. % Diff Dried Blood % Diff Liquid Blood



* Concentrations relative to freshly spiked blood standard.

Conclusions:
The current investigation demonstrates that it is feasible, after further study and evaluation, to detect and quantitate drugs in dried crime scene blood, convert the mass/mass concentration to standard mass/volume concentration, and interpretthe results with a known degree of uncertainty.