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Postmortem analysis of synovial fluid: An alternative method for determining the presence of ethanol

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Abstract

Postmortem toxicological analysis is often an important tool in autopsy in determining the cause of death. There are circumstances however when traditional samples such as blood and vitreous fluid are unobtainable. In such instances, alternative body fluids such as synovial fluid may be useful adjuncts. However, synovial fluid has not been accepted as a widely used alternative. The purpose of this study was to determine the correlation between blood ethanol content and synovial fluid ethanol content. To this end, synovial fluid was obtained from 98 cases presenting to the Office of the Medical Investigator in Albuquerque, New Mexico over a 4 month period. Of these, 20 cases had measurable blood ethanol content at the time of demise, and were selected for use in the present pilot study to determine the viability of using synovial fluid as an alternative specimen for postmortem toxicology screening. Analysis of synovial fluid ethanol content (SEC) was performed using gas chromatography and compared with similarly obtained blood ethanol content (BEC). BEC and SEC were found to have a strongly positive linear relationship, with a Pearson correlation coefficient of 0.96 and a p-value of <0.0001. The present study confirms prior research which indicates synovial fluid is a suitable substitute for the analysis of blood ethanol content particularly when traditional toxicology samples are unavailable.

Introduction

Newspaper articles (Zaragoza, 2005), reports by the Center of Disease Control, and the New Mexico Department of Health (NM 2005) all indicate that New Mexico has one of the highest death rates associated with drugs and ethanol use anywhere in the United States. The Office of the Medical Investigator (OMI) by the decree of state law, maintains jurisdiction over sudden, unexpected, and violent deaths in state of New Mexico. These include deaths that involve common drugs of abuse and ethanol intoxication. Therefore, the OMI is an ideal setting in which to examine new and novel approaches to determining the presence and concentration of drugs of abuse and ethanol in the deceased. The purpose of this pilot study is to demonstrate with a larger sample population, that analysis of synovial fluid to detect ethanol levels is a viable alternative to traditional toxicology screening techniques.

A vital component of postmortem examinations in cases of sudden, unexpected, and violent death is toxicology screening, aimed at detecting numerous substances that may be present in the body at the time of death. There are three major components of toxicology screening: separation of the drug from tissue, purification, and analytical detection and quantification (DiMaio & DiMaio, 2001). Forensic toxicology encompasses not only the identification and quantification of drugs in the postmortem body, but also offers an interpretation as to whether the drug was the primary cause of death, a contributing factor, or had no role in the individual's demise (DiMaio & Dana, 1998). There has been some concern about postmortem metabolism of drugs and diffusion of drugs into body tissues that would potentially invalidate definitive reporting of drug levels at the time of death (Saukko & Knight, 2004). For this reason, sampling from various fluids is the standard of forensic toxicology screening.

Blood from multiple body sites, vitreous fluid, and urine are the established fluids collected at the time of autopsy and subjected to toxicology screening (DiMaio & Dana, 1998). However, there are circumstances when these fluids cannot be used due to excessive trauma, partial remains, postmortem animal activity, and postmortem changes. Postmortem changes result in the potential for postmortem organism growth to alter the level of ethanol. Given that the synovial fluid is within the protected bursa sac, postmortem changes and redistribution may have less effect on ethanol levels in the synovial fluid as compared to other samples. As such, it may be that synovial fluid is one of the most valuable, as yet fully unexplored, samples that can be used by forensic toxicology for determining the presence and quantification of ethanol in the recently deceased.

In the past, little consideration has been given to the analysis of synovial fluid as a viable alternative despite the fact that there have been a few studies of small sample sizes that have indicated its potential relevance. Ohshima *et al*. (1997) and Winek *et al*. (1993) both found a high correlation between synovial ethanol content and blood ethanol content (BEC), showing that ethanol did equilibrate with synovial fluid, as it does with vitreous (more commonly referred to in literature as BAC and SAC for blood alcohol content and synovial fluid alcohol content, respectively). Balabanova (1991, 1993) detected the presence and concentration of nicotine and tetrahydrocannabinol in synovial fluid. More recently, Felscher *et al*. (1998) screened for the presence of a large panel of pharmaceuticals and drugs of abuse, including metabolites, in synovial fluid. Felscher's research was the most comprehensive to date, but focused on establishing the usefulness of fluorescence polarization immunoassays for detection of substances, rather than on the power of synovial fluid as a viable toxicology screening sample.

Winek *et al*. (1993), Ohshima *et al*. (1997), and Felscher *et al*. (1998) all report that synovial fluid demonstrates the necessary properties to be useful in toxicology screening. However, all of these studies used divergent methods for collecting and processing specimens, different analytical methods for interpreting their data, and different markers for comparison. Combined, this leaves open the opportunity to establish a practical and uniform protocol that can be directly applied in the autopsy suite and laboratory to collect and analyze synovial fluid for ethanol, particularly when traditional samples are unavailable.

Constituently, synovial fluid shares many of the same qualities of vitreous fluid such as being an ultrafiltrate and being constrained to an isolated environment devoid of enzymes that can degrade ethanol or other drugs, conditions which generally make vitreous a good candidate for toxicology screening. Synovial fluid is composed primarily of protein constituents derived directly from plasma. The transfer of plasma proteins to synovial fluid is inversely related to the size and shape of the molecule and directly correlated with the degree of inflammation (Sokoloff Volume I, 1980). Combined with these plasma proteins are those constituents secreted by the joint tissues themselves, including a highly viscous hyaluronic acid mixed with collagen, chondroitin sulfate (a glucosaminoglycan), collagenase, and proteinase inhibitors (Sokoloff Volume I, 1980). There is generally a trace quantity of cholesterol present. Synovial fluid has a leukocyte concentration of about two hundred cells per cubic millimeter, a minority of which are neutrophils. No erythrocytes or platelets are present in normal joint fluid (Sokoloff Volume II, 1980). A knowledge of the normal components of synovial fluid is important for identifying those compounds that are anomalous such as ethanol, drugs of abuse (and their metabolites), or pathological conditions (i.e. inflammatory arthritis) that may interfere with the detection of ethanol or drugs.

Reiterated, the value of synovial fluid is the fact that the drugs of interest cross into the joint space or synovial fluid via the ultrafiltrate yet they exist in the absence of ethanol dehydrogenase and other drug-altering metabolic enzymes. This means any confounding effects of drug diffusion within the body's circulation, or postmortem metabolism of ethanol should be limited.

It is apparent from a review of literature that further research is required to determine the strength of the correlation between synovial ethanol content and blood. The potential for using synovial fluid in the analysis of drugs of abuse remains largely untapped. Thus, it is the purpose of this study to determine if synovial fluid can be used as a reliable medium for postmortem toxicology screening of ethanol.

Materials & Methods

Sample Collection

The subjects for this study were recently deceased adolescents or adults, with or without a case history indicating possible ethanol involvement, already scheduled for examination at the OMI during the 4 month research window. In order to collect synovial fluid, verbal consent from the decedent's legal next-of-kin was required. However, not all families agreed to participate in the study. Thus, a total of 98 samples of synovial fluid were collected in combination with traditionally obtained specimens for toxicology screening including: heart blood, femoral blood, vitreous humor, and urine. Of these 98 cases, only 20 cases were incorporated into this pilot study as they were available at the time of analysis and had documented ethanol present in the blood per toxicology screening samples run at either the National Medical Services Lab (NMS) or New Mexico State – Scientific Laboratory Division (SLD).

Synovial fluid from both knees was obtained using a 10ml disposable syringe with a 22-gauge needle inserted into the subpatellar bursa. Prior to attempted extraction, the knee joint was warmed for 10 minutes with warm water to decrease the viscosity of the synovial fluid, in an effort to enhance the volume of the aspirate extracted. Actual collected volumes varied from negligible quantities to 12 ml. The quality of the sample also varied from yellow-clear to murky red-brown. The sample, combined from both knees, was then transferred to a 10 ml plastic vial containing sodium fluoride to prevent/inhibit postmortem organism growth, and tagged with the OMI case number. No other identifiable information (name, date of birth) was included in the labeling. The tubes were kept frozen until the time of analysis by the reference laboratory.

Other toxicology screening samples such as heart and femoral blood, urine, and vitreous fluid were collected and stored per general autopsy/OMI protocols by employees of the facility not involved in this research project.

Analytical Methods

Blood samples were processed according to routine OMI protocol at either the National Medical Services Laboratory or by the New Mexico State Scientific Laboratory Division (depending on lab availability and turn around time). Protocols for gas chromatograph alcohol analysis on blood were similar but not alike between facilities. In all cases, blood samples were preserved in vials containing 2% sodium fluoride until time of analysis. For analysis purposes, blood samples (usually 200 uL) were placed in vials along with 0.08 g/dL of the internal standard N-propanol and 0.04 g/dL to t-butanol in NaCl to facilitate the collection of volatiles in the headspace area. An aliquot of the headspace gas (usually 100uL) was then injected into the isothermal gas chromatograph for detection, identification, and quantification purposes. As both laboratories calibrate their systems to similar commercial standards as well as the internal standard of N-propanol, it was assumed for the purposes of this pilot study that the concentration of ethanol present in case samples was equivalent between laboratories.

Synovial fluid was analyzed at TriCore Reference Laboratories in Albuquerque, New Mexico, using their volatile alcohols by gas chromatography protocol, 2008. This protocol was designed for acceptable specimen types of serum, plasma, and urine, but was extended to include synovial fluid as part of the present experimental study. No prior protocol existed at TriCore for the analysis of synovial fluid, nor was one detailed in the literature. The instrument used was the Agilent 6850 with the Agilent column DB-ALC2.32x30x1.2, set to a column temperature of

40°C, injector temperature of 250°C, detector temperature of 300°C, and operating at a flow rate of 80cm/sec. The gas chromatograph was calibrated against a Cerilliant Multi-component alcohol control standard, a negative control, and during the run by a stock internal standard of N-propanol.

Samples were prepared for analysis by the addition of hyaluronidase when necessary to decrease the viscosity of the synovial fluid. Subsequently, 100.0uL of the synovial fluid specimen was added to an 8ml screw top sample vial containing 100.0uL of an internal standard (N-propanol) and approximately 0.3gm of NaCl. Salt was added to facilitate the concentration of the non-polar volatiles in the vial headspace. A measured aliquot (500uL) of the vapor in the head space was subsequently injected into the gas chromatograph for identification and quantification purposes. (Figure 1)

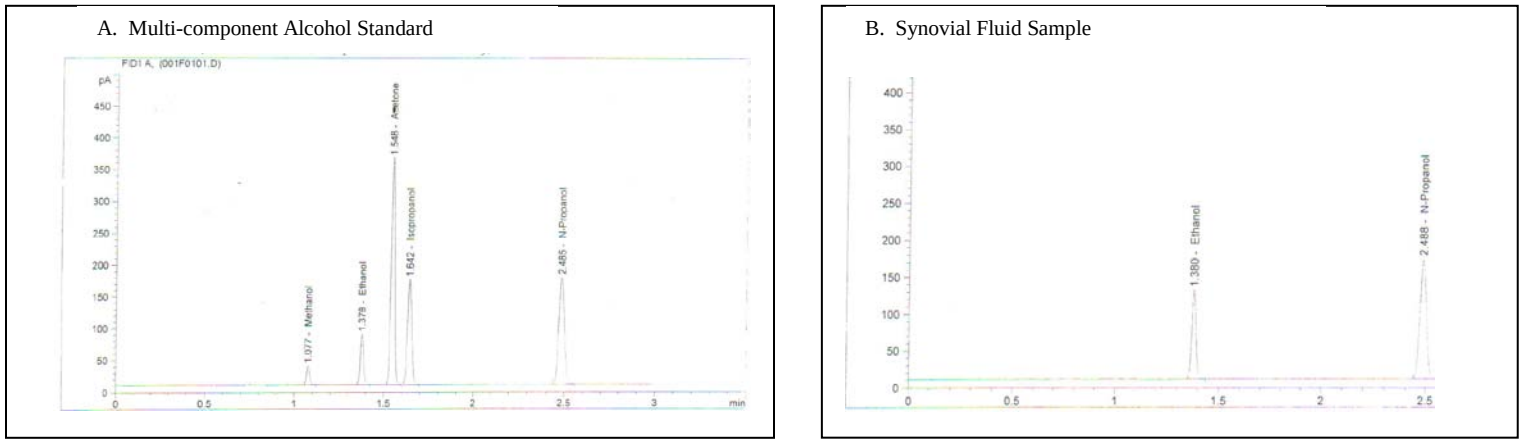

Figure 1. *Alcohol Gas Chromatography. A: Measured peaks of multi-component alcohol standard include methanol (1.077), ethanol (1.378), acetone (1.548), isopropanol (1.642), and N-propanol (2.485). B: Positive synovial fluid specimen demonstrating ethanol peak and internal standard. Area under the curve corresponds to the quantity of alcohol present.*

Statistical Methods

The data was compiled and analyzed using SAS software version 9.1 to determine degree of correlation against existing postmortem screening methods for toxicology. The relationship between blood and synovial concentration was evaluated using a Pearson correlation coefficient, a quantitative measure of the relationship between two variables.

Results

The cases utilized in this pilot study are a convenience sample representing those decedents who presented at the OMI during the research window and for whom authorization for sample collection was obtained. Males were over represented in the population (80% vs. 20%) and tended to be older than females, with median age 49 vs. 35 years, but this difference was not statistically significant. The sample cases demonstrated ethnic diversity including white non-Hispanic, white Hispanic, and Native Americans. However, being a convenience sample, white Hispanics and Native Americans were under represented in the data set when compared to New Mexico demographic statistics (White Hispanic (30% vs. 43%), Native American (5% vs. 10%), White non-Hispanic (65% vs. 44%)(BBER, 2007). See Table 1.

Table 1: Patient Demographics

		Number (%)	Median Age (Range)	Mean BEC (Range)	Mean SEC (Range)
Sex	Males	16 (80%)	49 (19-65)	98.3 (12-237)	63.0 (0-251)
	Females	4 (20%)	35 (15-42)	138.5 (11-289)	168.4 (85-323)
Race/ethnicity	White non-Hispanic	13 (65%)	50 (15-65)	90.1 (11-289)	86.6 (0-323)
	White Hispanic	6 (30%)	30 (19-42)	156.8 (25-220)	83.6 (0-250.7)
	Native American	1 (5%)	41	14	0

The subjects' profiles and causes of death are given in Table 2, along with measured values of blood and synovial fluid ethanol content. The synovial fluid ethanol content values are given as the mean values of both knees. Correlation statistics are provided in Table 3. Through regression analysis, a generally linear relationship with a correlation coefficient of 0.96 was obtained between blood ethanol content and synovial fluid ethanol content (Figure 2).

Table 2
Subjects' profiles, ethanol concentrations, and cause of death

CASE No.	ETHNICITY	Ethanol concentration (mg/dl)		BEC/SEC Ratio	Cause of Death
		Blood	Synovial		
1	White	14	23.3	0.60	CO poisoning
2	White	214	162.97	1.31	Cardiovascular Disease
3	White	13	0		CO poisoning
4	Hispanic	208	Clotted		Blunt Trauma
5	White	74	84.69	0.87	Gun Shot Wound
6	White	237	231.45	1.02	Asphyxia
7	White	20	0		Drug intoxication
8	Hispanic	180	QNS*		Drug intoxication
9	Hispanic	100	0		Drug intoxication
10	Native Am	14	0		CO poisoning
11	White	289	322.78	0.90	Drug intoxication
12	White	11	QNS*		Drug intoxication
13	White	49	0		Drug intoxication
14	White	206	188.06	1.10	Drug intoxication
15	Hispanic	220	250.71	0.88	Drug intoxication
16	Hispanic	208	QNS*		Chronic alcoholism
17	White	17	0		COPD
18	White	15	27.06	0.55	Drug intoxication
19	Hispanic	25	0		Drug intoxication
20	White	12	0		Chronic alcoholism

*QNS (quantity not sufficient)

Table 3

Pearson Correlation Coefficient		
	Blood Ethanol (mg/dl)	
Synovial Ethanol (mg/dl)	0.95651	Pearson Correlation Coefficient
	<.0001	Prob > |r| under H0: Rho=0
	16	Number of Observations

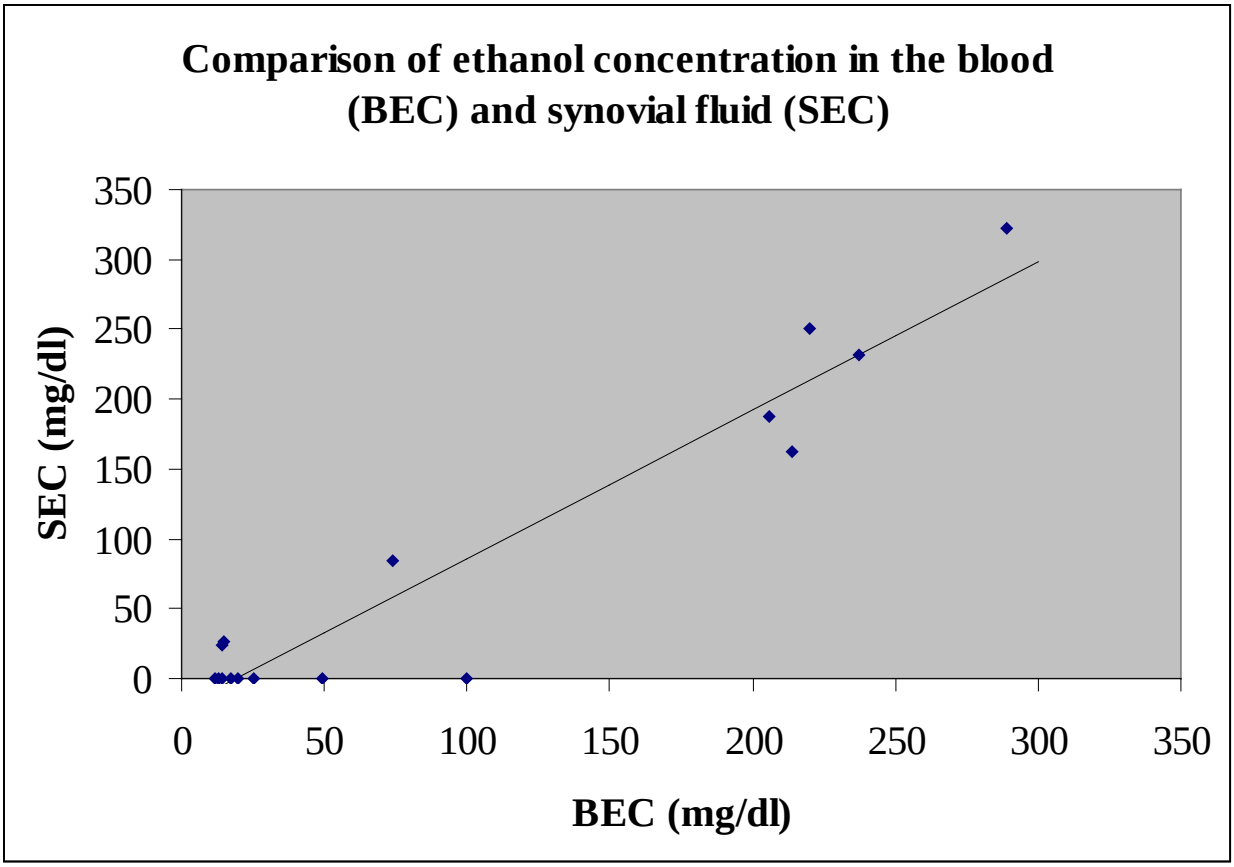

Figure 2. Comparison of ethanol concentrations in the blood (BEC) and synovial fluid (SEC). Regression equation (y=1.06x – 19.95); x, BEC; y, SEC; r=0.96, n=16.

Discussion

Of the 20 cases selected for analysis in the present study, 4 samples of synovial fluid could not be used due to insufficient quantities or sample quality (Table 2). The 16 synovial fluid samples examined in this pilot study demonstrated a linear relationship between blood and synovial fluid ethanol concentration. The data were strongly positively correlated with a Pearson correlation coefficient of 0.96, $p<0.0001$ and was in agreement with the previously published papers of Winek (1993) and Ohshima (1997) as well as with the original studies performed by Aurdlicky in Germany which were published in 1965 and 1971. These original papers were not obtainable here, and thus information regarding Aurdlicky's work is based on the summary done by Ohshima. To date, there are now 5 studies, including this one, that specifically look at the correlation between blood and synovial ethanol content. Despite a continued problem with small sample size, these studies all demonstrate not only statistically significant correlation but remarkable agreement between studies despite different study designs and sample/data analysis (Table 4).

Table 4: Comparison of sample size, ratio of blood ethanol to synovial ethanol concentration, and correlation between four previously published independent studies and this pilot study.

Source	Sample #	BEC/SEC ± SD	Correlation Coefficient
Aurdlicky, 1965	8	0.72 ± 0.18	0.88
Aurdlicky, 1971	14	1.03 ± 0.29	0.93
Winek, 1993	28	0.99 ± 0.29	0.89
Ohshima, 1997	11	0.76 ± 0.12	0.97
Present *	8	0.91 ± 0.25	0.96

*****The present study had a sample size of 20 but detected ethanol in the synovial fluid at a concentration of > 0.0 mg/dL in only 8 of the samples. Correlation coefficient is based on all 16 samples that were analyzed by GC. 4 of the samples of synovial fluid could not be run for technical reasons (table 2). Note: labels were changed from conventions used by Ohshima and Winek (BAC/SAC to BEC/SEC), to specify ethanol as opposed to the more general term "alcohol".

For some reason, the synovial fluid samples in this study seemed to not be particularly sensitive to low blood ethanol concentrations. Eight samples were reported as having no ethanol present despite a median BEC of 18.5 mg/dL ± 30.3. The median BEC in the eight cases that were successful in documenting the presence of ethanol in the synovial fluid was 210 mg/dL ± 107.5. In Ohshima's study, the median BEC was 194.5 mg/dL ± 131.3 with the lowest recorded BEC being 26 mg/dL. As the Ohshima study did not extend as far into the low range of ethanol concentrations as the present study, it is not possible to say whether or not low synovial fluid sensitivity is to be expected when BEC is less than approximately 20 mg/dL. One possible explanation for why ethanol was not detected at these low BEC's, is that the subject died during the absorption phase of ethanol metabolism implying that ethanol consumption first commenced very near to the time of death. As synovial fluid is an ultrafiltrate of the blood plasma, it like vitreous fluid, may reflect ethanol levels in the blood 1-2 hours prior to death (DiMaio & DiMaio, 2001). To fully understand this issue, future research will have to focus on differentiating between the elimination and absorption phases of ethanol metabolism, as well as to elucidate any issues associated with the timing between demise and actual sample collection.

The original intent of this study was ambitious. The aim was to obtain a more robust sample size than had been done in prior studies, with the intention to correlate synovial fluid toxicology with not only blood ethanol, but also with other drugs of abuse which are routinely detected in postmortem blood, vitreous fluid, and urine. Unfortunately, numerous obstacles prevented the realization of the original study proposal.

Sample collection proved to be more difficult than originally anticipated. The need for timely autopsy required that consent be obtained less than twenty-four hours after the arrival of the decedent's body at the Office of the Medical Investigator (OMI). Often, next of kin contact

information was unavailable, or families were unreceptive to the idea of participation in a research study while acutely grieving. In addition, synovial fluid extraction proved difficult as bodies were refrigerated in the OMI prior to scheduled autopsy, thus making the already viscous synovial fluid more so. Over time the extraction protocol evolved to heating the knee joint with warm tap water for 10 minutes to facilitate aspiration and increase the yield. Despite these modifications in protocol and increasing researcher experience in aspiration, the volume of aspirate varied dramatically from body to body, with no apparent relationship between body size or gender.

Laboratory analysis of the samples was delayed far beyond original study timelines. Blood, vitreous fluid, and urine were analyzed by NMS or SLD within one to two months of autopsy. Synovial fluid sample analysis was dependent on TriCore, according to a prearranged agreement with the toxicology department. Due to unforeseen circumstances, the chain of custody of the synovial samples was disrupted, resulting in the misplacement of 33 samples, which were never located. Possible repeated freezing and thawing of samples may have also occurred as the location of the samples moved between the OMI and TriCore, and within divisions at TriCore. As ethanol is a volatile fluid, there was concern that some of the content may have been lost due to desiccation, freeze/thaw cycles, synovial fluid matrix disruption during pilot testing of the gas chromatograph protocol, and bottle leakage. However, per TriCore documentation (Volatile alcohols by GC, 2008), it is stated that specimens collected in fluoride preservative (NaFl-grey top) vials for ethanol testing are stable at 4^{o}C for at least one year so the delay to time of analysis may not have been as problematic as originally thought. In any event, the synovial fluid remained unanalyzed for approximately one year from the date of their original collection.

The study was originally designed to significantly demonstrate the correlation between blood ethanol and synovial fluid ethanol content. The power analysis run using the lowest published correlation coefficient of 0.71 showed that a sample size as low as 16 was adequate to provide a power level of 0.9 with a significance of 0.05 or less. Thus, despite the problems enumerated above, the original power analysis supports that the present study has enough data for statistical significance. Further, the results are surprisingly strong, with a Pearson's correlation coefficient of 0.96, and a $p<0.0001$.

Therefore, though unable to extend past research, the present study supports the linear relationship between blood and synovial fluid ethanol contents. Given this linear relationship, synovial fluid is a viable postmortem toxicology sample when blood is not available. Anecdotally, there was one case during the course of the present study, where the decedent was significantly decomposed, and where the traditional samples of blood and vitreous fluid were unavailable due to body desiccation, yet a fair quantity of clear-yellow synovial fluid was obtained. The present research also dramatically reveals the stability of synovial fluid ethanol content through various adverse conditions over the course of a year's storage.

Though this study was plagued by numerous unforeseen difficulties, synovial fluid analysis still has great potential when applied to the field of forensic toxicology. Given that synovial fluid is an isolated ultrafiltrate, free of metabolic enzymes, it, like vitreous fluid, is in a unique position to potentially be an alternative way to accurately determine an ethanol level at the time of death despite decompositional changes. Future research to specifically examine the time-lag to achieve equilibrium between blood and synovial ethanol content would be useful in helping to differentiate between the absorption and elimination phases of blood ethanol. This in turn, would further help to establish the time-interval between ethanol intake and death and thus

provide further evidence toward a potential cause of death. Secondly, synovial fluid analysis has the potential to be used for the detection of other drugs of abuse and their metabolites such as narcotics, stimulants, hallucinogens, etc. The current protocols for testing for these drugs often require larger samples than could be routinely aspirated from synovial joints. Thus, a goal of future research would be to either develop techniques to more efficiently extract synovial fluid in larger quantities, or establish new gas chromatograph protocols, which can utilize smaller sample sizes. Ohshima in the 1997 study, used a Curie-point pyrolyser connected to the GC apparatus (known as the Py-GC method) to analyze both blood and synovial samples. This technique had the advantage that it required only a sample of 1 uL vs. the 100 uL used in the present study and may be an indication as to how similar samples should be analyzed in the future. Lastly, when adequate blood samples are not available, current toxicology screening protocols will then turn to either urine or vitreous fluid. To further progress the science behind synovial fluid analysis, future studies should look to directly compare synovial fluid findings with those found in simultaneously collected blood, urine and vitreous fluid samples.

References

Balabanova, S., Nachweis von Nicotin in Kniegelenkflüssigkeit und Knorpel [Detection of nicotine in knee joint synovial fluid and cartilage]. Deutsche Apotheker-Zeitung 1991; 131: 1297-1299.

Balabanova, S., Kramer, M., and G. Brandle. Tetrahydrocannabinol-Konzentrationen in der Gelenkflüssigkeit bei Haschischranchern [Tetrahydrocannabinol concentrations in synovial fluid of hashish smokers]. Zietschrift fur Rheumatologie 1993; 52: 38-40.

BBER: Bureau of Business and Economic Research - New Mexico Statistics at a Glance [Online]. 2007 July 1 [cited 2008 Aug 6]; Available from: URL: http://www.unm.edu/~bber/

DiMaio, V.J.M., and S.E. Dana. Handbook of Forensic Pathology. Georgetown, TX: Landes Bioscience, 1998.

DiMaio, V.J., and D. DiMaio. Forensic Pathology. 2nd ed. New York; CRC Press, 2001.

Felscher. D., Gastmeier, G., and J. Dressler. Screening of pharmaceuticals and drugs in synovial fluid of the knee joint and in vitreous humor by fluorescence polarization immunoassay (FPIA). Journal of Forensic Sciences 1998; 43: 619-621.

New Mexico. Department of Health. Drug Abuse Patterns and Trends in New Mexico – September 2004 Proceedings of the New Mexico State Epidemiology Work Group. Santa Fe: Department of Health, Jan. 2005. http://www.health.state.nm.us/pdf/SEWG_NM_2004_FULL_REPORT.pdf

Ohshima, T., Kondo, T., Sato, Y., and T. Takayasu. Postmortem alcohol analysis of the synovial fluid and its availability in medico-legal practices. Forensic Science International 1997; 90: 131-138.

Saukko P., and B. Knight. Knight's Forensic Pathology. 3rd ed. London: Oxford University Press, 2004.

Sokoloff, L. The Joints and Synovial Fluid. Volume I, Chapter 9. Macromolecules of synovial fluid by David A. Swann. Academic Press, Inc., 1980.

Sokoloff, L. The Joints and Synovial Fluid. Volume II, Chapter 7. The physiology of the normal synovium by Daniel J. McCarty. Academic Press, Inc., 1980.

TriCore Reference Laboratories Standard Operating Proceedure, Volatile Alcohols by Gas Chromatography. 2008.

Winek, C.L., Bauer, J., Wahba, W.W., and W.D. Collom. Blood versus synovial fluid ethanol concentrations in humans. J Anal Toxicol 1993; 17: 233-235.

Zaragoza, Krystal. “Duke City is 2nd in U.S. Drug Deaths.” Albuquerque Journal 8 June, 2005.