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High-Performance Liquid Chromatography with Column Switching for the Determination of Cocaine and Benzoylecgonine Concentrations in Vitreous Humor

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ABSTRACT: A sample concentration technique was adapted for the determination of cocaine and benzoylecgonine (BE) concentrations in vitreous humor. Vitreous humor (0.5 mL) was diluted 1:1 with water and applied through a filter onto a 3-cm preconcentration column. Following a simple wash step, the analytes were flushed directly onto a reversed-phase analytical high-performance liquid chromatography (HPLC) system. Absolute recoveries were high (above 90%) and the chromatograms were free from interference. Analysis for the drug and its breakdown product was performed using ultraviolet (UV) visible photodiode array detection, which allowed confirmation of peak identity. Recognizable UV spectra could be measured with as little as 20 ng on column. Comparison of the drug levels in 27 blood and vitreous humor samples showed that, while there was only a low correlation between the blood and vitreous concentrations ($R = 0.70$), vitreous cocaine and BE determinations were good indicators of antemortem cocaine use. In almost all cases, the vitreous BE concentrations were higher than the cocaine concentrations. The technique was easy to perform and the vitreous samples were especially compatible with this low-labor analytical procedure.

KEYWORDS: toxicology, vitreous humor, benzoylecgonine, cocaine, HPLC, postmortem drug distribution

Most drug determinations in forensic toxicology are performed on urine, blood, or homogenized tissue, the latter two of which are particularly susceptible to sample-handling problems during extraction. These problems arise from the large degree of variability in sample quality, which can lead to pipetting errors, problems with emulsification, and the need for multiple-step extraction procedures to obtain a clean extract suitable for instrumental analysis. The combination of these factors can result in a loss of sensitivity and poor reproducibility in an assay.

Another postmortem material, which is available in most cases but is often ignored, is vitreous humor. It is a relatively simple matrix in comparison with postmortem blood

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or urine (Table 1) and is not as susceptible to major postmortem changes [*1*]. Compartmentalized and isolated, it is also a suitable toxicological material even after a cadaver has undergone serious burning or embalming, when blood may be unavailable.

Although the vitreous is not heavily vascularized, it is a partitioned fluid with transport across the blood/vitreous barrier limited by the lipid solubility of the drug and its charge at physiological pH [*1*]. Most existing data on this partitioning concern antibiotics used in treatment of ocular infections or after surgery. For example, increasing the lipophilicity of *p*-aminohippuric acid or penicillin through esterification leads to their accumulation in the eye [*2*].

Most studies concerning the forensic analysis of vitreous humor have been concerned with time-since-death determinations [*3*]. Quantitative postmortem determination of vitreous drug levels is reported only sporadically [*4–7*] and is probably a reflection of the limited volume available and the difficulty of interpreting the levels. We recently reported the use of a pseudo direct-injection high-performance liquid chromatography (HPLC) procedure for analysis of acid/neutral antiepileptic drugs in vitreous humor [*8*]. This procedure was modified to allow the detection and measurement of cocaine and its major breakdown product, benzoylecgonine (BE), in vitreous humor samples.

The increase in the number of postmortem cases in which cocaine involvement is suspected (accidental, suicidal, and homicidal) [*9*] led to the investigation of the use of a rapid vitreous humor analysis as an indicator of recent antemortem cocaine use.

The HPLC method was relatively specific for cocaine and BE on the basis of retention time alone; however, additional selectivity was provided by the use of diode array detection (DAD), which allowed the collection of a full UV spectrum for all peaks eluting from the column [*10,11*].

Method

HPLC analysis was performed on a conventional HPLC instrument with a minor modification in the plumbing of the injection valve, whereby the sample loop was replaced with a 3-cm precolumn containing 40 μm C-18 packing material, as described elsewhere [*8*]. The analytical column was a 250 by 4.6-mm CH-8 Lichrospher column (E. Merck); the mobile phase was 30% acetonitrile in 0.05*M*, pH 3 phosphate buffer, and it was pumped at a flow rate of 1.5 mL/min.

The vitreous sample (0.5 mL) was mixed with water (1 mL) and applied onto the

TABLE 1—*Comparison of the composition of three postmortem body fluids, showing the relative simplicity and stability of vitreous humor.*

Putrified Blood (pH 4–9)	Urine (pH 4–9)	Vitreous Humor (pH 7–7.8)
Water (20–70%)	water (96–99.9%)	water (98.0–99.7%)
Few cells + debris	glucose	glucose
Lysate	protein	hyaluronic acid
Clots	ammonia	simple anions/cations
Denatured clots	creatinine	collagen
Bacterial debris	urea	
Denatured protein	uric acid	
Fat droplets	steroids	
Steroids	pigments	
Putrefactive bases	amino acids	
Protein microagglutinates		
Enzymes		

precolumn through a glass wool filter with the injection valve in the LOAD position. Application was followed by a wash with 2 mL of HPLC-grade water to remove any residual matrix material. The injection valve was then switched to INJECT, which diverted the flow of the mobile phase through the precolumn, carrying the analyte onto the analytical column. The analysis time from sampling to completion of the chromatogram was 8 to 10 min.

Vitreous humor samples were analyzed using this procedure in 28 cases in which blood cocaine analysis had been performed.

Results

The chromatograms were relatively clean and were free from interference from matrix contaminants (Fig. 1). The linearity of the method was determined for five concentrations over the range 0.02 to 1.0 μg/mL and was found to be linear for both cocaine and BE (R = 0.999 and 0.998, respectively).

The detection limit was determined as 0.01 μg/mL cocaine (S/N = 5), although the lowest level at which the UV spectrum of cocaine could be recognized with greater than 95% certainty by the system was 0.02 μg on-column, that is, 40 ng/mL in the vitreous sample. A sample chromatogram with the corresponding UV spectrum at this level is shown in Fig. 2.

Recoveries were determined by peak height comparison and were 90% (COV = 4.8%, n = 8) for BE and 92% (COV = 4.2%, n = 8) for cocaine. This high recovery, coupled with the fact that the on-line sample preparation method allows the application of all the recovered analyte onto the column, gives this technique a clear advantage over liquid/

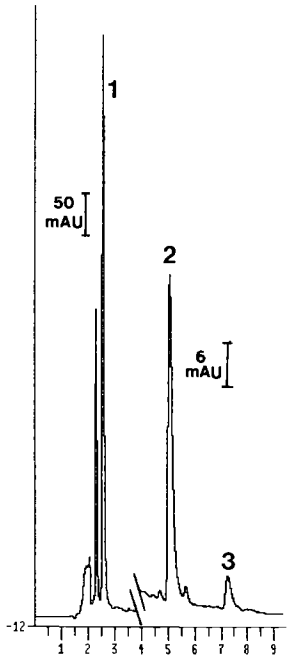

FIG. 1—*On-line analysis of a postmortem vitreous humor sample containing* (1) *BE (2.8 μg/mL),* (2) *cocaine (0.9 μg/mL), and* (3) *ethyl BE. The blood-alcohol concentration was 0.150 mg/100 mL.*

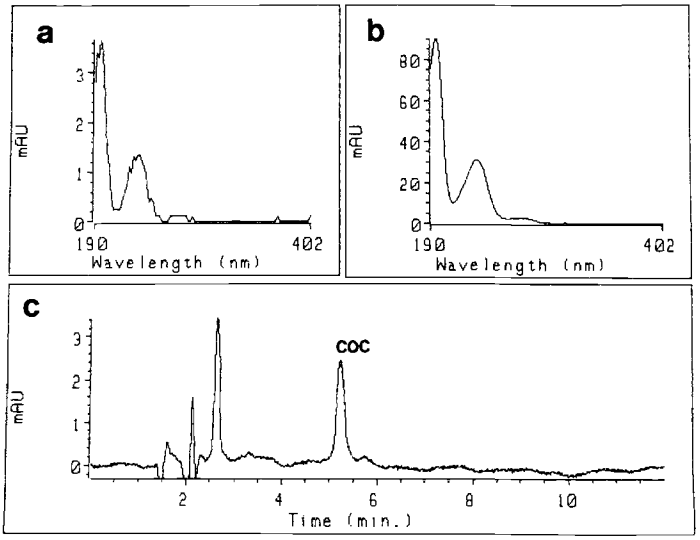

FIG. 2—*Analysis of 20 ng of on-column aqueous cocaine standard showing* (a) *the UV-visible spectrum and* (c) *a chromatogram. The reference cocaine spectrum* (b) *corresponds to 500 ng on a column.*

liquid and off-line solid-phase extraction techniques. BE often eluted on the shoulder of the injection front; however, accurate quantitation was not seriously affected.

The use of cinchocaine (RT = 6.4 min) was initially investigated as an internal standard; however, its recovery (90%) was similar to that of the analytes and its use improved neither the precision nor the accuracy of the method.

Other compounds identified in vitreous samples during this study included tripelennamine, pentazocine, quinine, lidocaine, caffeine, atropine, meperidine, and ephedrine/pseudoephedrine. None of these compounds interfered with the identification or quantitation of cocaine or BE.

Application

Twenty-eight vitreous humor samples for which blood cocaine levels had been determined were analyzed by the above method. Blood BE concentrations were available for only five of these cases. Some early data (which gave a poorer correlation) were discarded because the blood samples had not been preserved with sodium fluoride, which is generally agreed to be the best method for preventing bacterial or enzymatic degradation of cocaine postmortem [*12*].

The results of the blood and vitreous analyses are given in Table 2. BE concentrations in the vitreous humor ranged from less than 0.01 to 5.19 μg/mL. Cocaine concentrations ranged from less than 0.01 to 0.79 μg/mL. An examination of these data showed that in almost all cases (96%) BE concentrations were higher (Table 2). Furthermore, in several cases BE was detected in the vitreous in the absence of cocaine in either vitreous humor or blood. This suggests that vitreous BE determination should be a more sensitive indicator of recent antemortem cocaine use than either blood or vitreous cocaine measurement. In addition, although the limited data available for blood BE levels did not allow the assessment of any quantitative relationship, BE levels in the vitreous appear to be of the same order as the blood concentrations. With this technique, however, the

TABLE 2—*Concentrations of the cocaine and BE in blood and vitreous samples from postmortem cases.*

Vitreous humor, μg/mL[a]		Blood, μg/mL[b]	
Cocaine	Benzoylecgonine	Cocaine	Benzoylecgonine
0	trace	0	. . .[c]
0	2.37	0	. . .
0	2.96	0	. . .
0	3.67	0	. . .
0	2.75	0	. . .
0	3.12	0	. . .
0.01	. . .	0.10	. . .
0.03	. . .	0.10	. . .
0.05	0.02	0	. . .
0.05	0.21	0	. . .
0.05	0.31	0	. . .
0.07	0	0	. . .
0.09	0.57	0.08	0.91
0.11	1.61	0.20	0.95
0.16	. . .	0	. . .
0.18	0.89	0.20	. . .
0.21	2.88	0.10	. . .
0.22	0.94	0.19	. . .
0.22	5.19	0.50	1.7
0.23	. . .	0.10	. . .
0.30	0.86	0.05	. . .
0.30	1.03	0.20	. . .
0.32	0.66	0.10	. . .
0.47	4.80	0.50	. . .
0.48	0.41	0.20	. . .
0.60	2.22	0.33	. . .
0.63	4.28	0.41	0.13
0.79	3.52	0.30	2.81

[a] 0 = less than 0.01 μg/mL.
[b] 0 = less than 0.05 μ/mL.
[c] . . . = not determined.

determination of BE in the vitreous was considerably more simple to perform than a blood determination [*13*].

A comparison of blood and vitreous cocaine concentrations showed a correlation of $R = 0.70$, with a considerable spread in the data (Fig. 3). The mean vitreous-to-blood cocaine ratio was 1.61 (range, 2.6 to 0.1). This wide range and poor correlation limits the use of vitreous cocaine concentrations in assessing the corresponding blood levels. The apparent wide spread of vitreous/blood ratios must be due in part to decomposition taking place in the blood prior to preservation. This is supported by the higher correlation observed between blood and vitreous levels for the chemically more stable compounds, phenobarbital and phenytoin, observed elsewhere [*8*].

The extent to which cocaine degradation occurs in the blood in the interval between death and the collection of the postmortem sample is extremely difficult to assess, although the half-life for cocaine in plasma, *in vitro*, at 30°C has been measured as 34 min [*14*]. Although the absence of cholinesterase activity in vitreous humor will prevent the breakdown of cocaine through enzymatic hydrolysis to ecgonine methyl ester, there is likely to be some postmortem degradation through chemical hydrolysis to BE. This can best be prevented by maintaining the samples at pH 5 and analyzing them rapidly after collection [*12*].

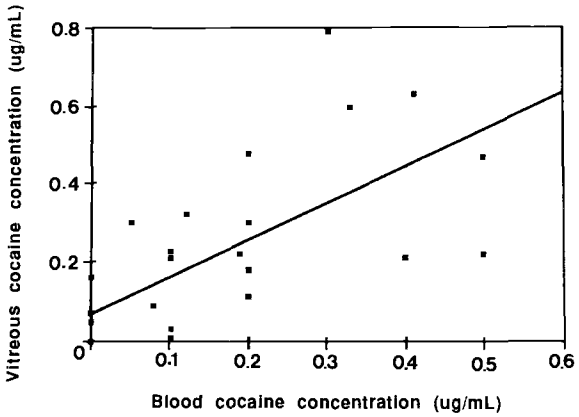

FIG. 3—*Comparison of cocaine and BE concentrations in vitreous humor. The BE concentrations were higher in all but one case.*

In general terms, high blood cocaine concentrations were accompanied by high vitreous cocaine concentrations, and more importantly, in all cases where cocaine was present in the blood, it was also detected in the vitreous samples, confirming the validity of vitreous cocaine determination as an indicator of recent antemortem cocaine use.

In a number of cases, a peak eluting at 7.4 min (Fig. 1) was noted having a UV spectrum similar to that of cocaine and BE. Gas chromatography/mass spectrometry (GC/MS) analysis of the collected fractions confirmed the identity of this peak as ethyl BE (homocaine, ethylcocaine). This material has been reported as a biotransformation product of cocaine in urine, although its method of formation is not known [*15*]. It was noted that in all cases, the appearance of ethyl BE was accompanied by moderate to high (above 0.05 mg%) blood-alcohol concentrations.

Solid-phase extraction methods for cocaine and BE in other body fluids have been described elsewhere [*16*]. The advantage of this technique is in its direct application of the extracted drug onto the HPLC column, without an evaporation and reconstitution step. To date, successful analyses have been achieved on vitreous humor, cerebrospinal fluid, and plasma samples. The lifetime of the preconcentration column is severely compromised, however, by the use of plasma, which allows only 10 to 15 injections, in comparison with the 50 to 60 samples possible with vitreous humor and spinal fluid.

Conclusions

1. HPLC was an ideal technique for this application because of its ability to measure cocaine and BE simultaneously without derivatization. It separated not only cocaine and its major breakdown component, but also a number of other compounds of toxicological significance.

2. Diode array detection has been found to be an extremely significant development in HPLC as far as the forensic sciences are concerned, due to the additional information it provides for peak identification.

3. On-line sample preparation was simple to perform, being extremely compatible with vitreous humor and, to a lesser extent, with some other biological fluids. The procedure also allowed efficient recovery of both the drug and its metabolite, as well as the transfer of all the recovered analyte directly onto the HPLC system, thus increasing the sensitivity.

4. The development of similar techniques for other sample types and analytes is also of great interest and is currently under investigation.

5. The data showed that vitreous cocaine and, particularly, vitreous BE determinations were good indicators of cocaine use prior to death.

6. The short analysis time and the limited sample preparation make vitreous cocaine and BE analysis an ideal method for rapid screening for recent antemortem cocaine use.

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