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TECHNICAL NOTE

Interpretation of drug concentrations in an alternative matrix: the case of meprobamate in vitreous humor

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Abstract The use of vitreous humor (VH) as an alternative matrix to blood in the field of forensic toxicology has been described for numerous drugs. Interpretation of drug concentrations measured in VH, as in other matrices, requires statistical analysis of a data set obtained on a significant series. In the present study, two diagnostic tests interpreting postmortem VH concentrations of meprobamate in 117 sets of autopsy data are reported. (1) A VH meprobamate concentration threshold of 28 mg/l was statistically equivalent to that of blood meprobamate concentration threshold of 50 mg/l distinguishing overdose from therapeutic use in blood. The intrinsic qualities of the test were good, with sensitivity of 0.95 and absolute specificity of 1. (2) A novel interpretation tool is proposed, allowing blood concentration range to be evaluated, with a known probability, from VH concentration.

Keywords Meprobamate · Vitreous humor · Forensic toxicology · Intoxication · Autopsy

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Introduction

In most cases, blood remains the reference matrix to provide an optimal toxicological victim profile at the time of death [1]. When no blood is available, alternative matrices are used with empirical interpretation, with cases found in the literature as benchmarks. This approach makes therapeutic, toxic, and lethal intoxication levels difficult to distinguish. Comparison of concentrations between blood and alternative matrices on statistically significant series is required to provide reliable tools for interpretation. The interest of postmortem analysis of the vitreous humor (VH) is well known. VH has been recommended in case of absence of blood (exsanguination or mutilated cadaver), postmortem redistribution [2, 3], embalmment [4, 5], or putrefaction and bacterial contamination [6]. In addition, VH is easy to collect. Toxicological analysis of VH has a qualitative interest, shown by the large number of different molecules that have been detected [7]. The quantitative interest, due mainly to the correlation between blood and VH concentrations, was studied for various drugs: e.g., ethanol [8], cocaine [9], opiates [10], and benzodiazepines [11–13]. There was great variability in results according to the molecule considered and to experimental conditions. Despite that meprobamate has been replaced by other sedative agents in some countries, it stays a drug frequently detected on postmortem toxicological investigation [14, 15]. It is a carbamate derivative, prescribed as an anxiolytic but with well-established abuse potential [16]. It is also the active metabolite of carisoprodol. Although VH meprobamate concentrations were described in some case reports [17, 18], no investigation of the correlation between blood and VH concentrations has

Table 1 Test performance observed for various threshold values

VH concentration threshold (mg/l)	Specificity (Sp)	Sensitivity (Se)	Total risk $\alpha + \beta$
0	0.00	1.00	1.00
10	0.71	1.00	0.29
20	0.95	1.00	0.05
30	0.99	0.95	0.06
40	0.99	0.90	0.11
50	1.00	0.78	0.23
60	1.00	0.70	0.30
70	1.00	0.58	0.43
80	1.00	0.45	0.55
90	1.00	0.38	0.63
100	1.00	0.28	0.73
110	1.00	0.23	0.78
120	1.00	0.18	0.83
130	1.00	0.18	0.83
140	1.00	0.13	0.88
150	1.00	0.08	0.93

α is the risk of a false positive in a subject without a toxic blood meprobamate level and β that of a false negative where the blood concentration is toxic

been performed on a statistically significant series. Previously, the interpretation of meprobamate concentrations in bile was reported [14]; the present study looked at VH meprobamate concentrations, with the same objective and a similar approach.

Material and methods

Study population

A retrospective study was performed in the Lyon (France) Forensic Institute between July 1st 2005 and May 31st 2010. Sampling respected the Council of Europe Circular R99 [19]. All cases in which meprobamate had been detected in at least one matrix (blood, stomach contents and/or urine) and from which a VH sample from at least one of the two eyes had been taken were included. Meprobamate was detected in 5.34% (n=177) of the 3,313 autopsies performed over the study period. Both femoral blood and VH samples were taken in 66.1% (n=117) of the positive cases. In 93 cases, left and right VH (LVH, RVH) were both collected, in six cases only RVH, in 11 only LVH, and in seven, a mixture of LVH and RVH. For the 117 cases included in the statistical analysis, postmortem times varied from a few hours to several days. Seventy-seven of the 117 cases were considered non-overdosed (meprobamate blood concentration<50 mg/l) and 40 overdosed (>50 mg/l). The mean age of the population was 49.7 years. The male to female ratio was 0.98. For men, the mean age was 46 years (range 18–83 years, five unknown), and for women, 51 years (range 24–80 years, five unknown).

Analytical methods

Blood samples were analyzed using a technique previously described [14], adapted and validated for meprobamate assay in VH as follows; 20 µl methanol solution at 1 mg/ml in carisoprodol (internal standard (IS)) were added to 200 µl VH. The mixtures were diluted in 1 ml ammoniac buffer (pH=9.5, 0.07 M) and extracted with 1.5 ml of a chloroform-isopropanol (9:1, *v*:*v*) mixture. After 20 min agitation and 10 min centrifugation at 1,400×*g*, the organic phase was collected and evaporated at 40°C under nitrogen. The dry residue was dissolved in 100 µl methanol. Aliquots of 1 µl were injected into the GC/MS system, using Gaillard's method [15], modified as follows: the gas chromatograph was a Hewlett Packard 6890 series (Les Ulis, France), with HP 7683 automatic injector and HP 5973 detector. The analytic column was an HP-5MS capillary column (30 m×0.25 mm i.d.; 0.25 µm film thickness). Helium was used as carrier gas at a constant flow rate of 1.4 ml/min. A splitless injection mode was adopted at a temperature of 193°C. The initial oven temperature of 150°C was held for 1 min then increased to 200°C by 25°C/min and held for 4 min and finally increased then to 295°C by 30°C/min and held for 5,83 min. The retention times of meprobamate and carisoprodol (IS) were 5.26 and 6.15 min, respectively. Data were recorded in full scan; the ions monitored were: *m*/*z* 83-114-144 and 97-158-184 for meprobamate and carisoprodol, respectively (ions *m*/*z* 83 and 158 were used for quantification).

Statistics

Statistics were analyzed using R language, version 2.11.1, available at http://cran.r-project.org/.

Differences between right and left VH concentrations

Right and left VH concentrations were compared by paired Wilcoxon signed rank test with continuity correction because of the non-normality of the distributions. Depending on the case, further computations were performed using the mean of the LVH and RVH concentrations when available, or else the LVH or RVH concentration only or a mixture of the two.

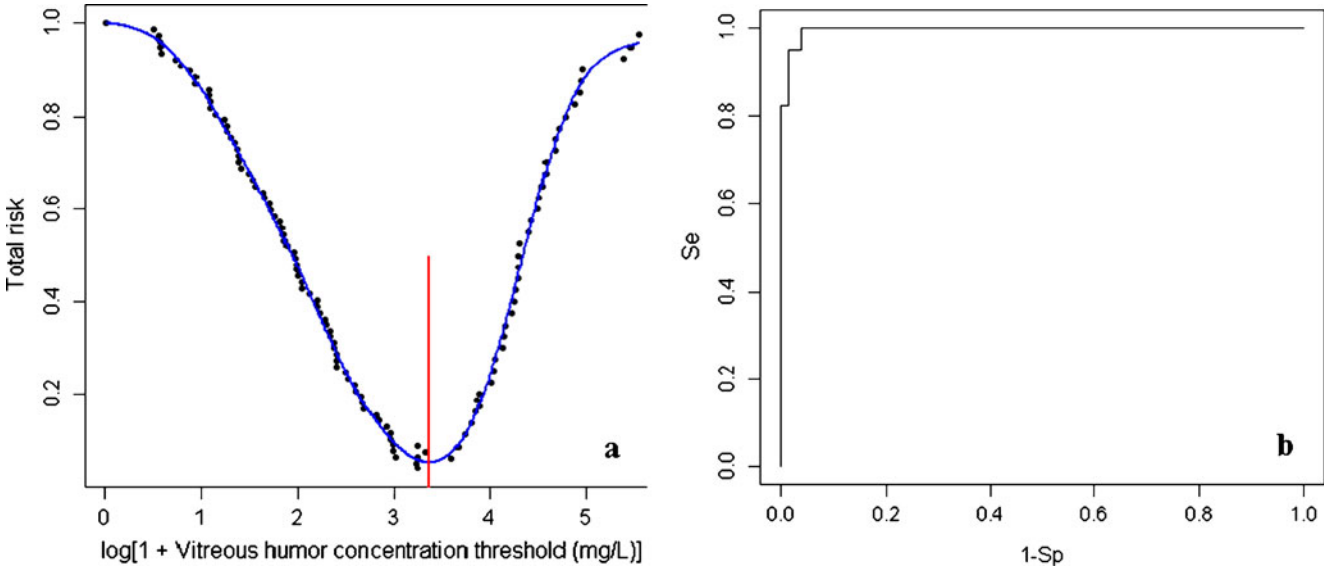

Fig. 1 **a** Total $\alpha + \beta$ error risks per threshold value; **b** receiver operating characteristics (ROC) curve (n=117)

Meprobamate overdose test

The approach was similar to that described by Fanton et al. [14]. We sought to determine the VH meprobamate concentration corresponding to the 50 mg/l blood concentration threshold above which overdose is generally agreed to be suspected [20, 21]. Risk α (probability of wrongly diagnosing overdose) and risk β (probability of wrongly considering the dose to be therapeutic) were computed for each VH concentration. The curve showing the total risk ($\alpha + \beta$) against VH concentration was smoothed using a generalized additive model (function gam of the R package, called mgcv). The chosen threshold for forensic use is the VH concentration for which the total risk given by the model is minimum. The widely used receiver operating characteristics (ROC) curve is given by the rocdemo.sca function of the ROC package.

Correlation between VH and blood concentrations

To normalize the data (n=117) and linearize the relationship between the concentrations in the two matrices (VH and blood), the concentrations were transformed into natural logarithms of the concentration value, incremented by one. The linear fit of the blood concentration against the VH concentration was studied using the lm function of the R language. Then, for different hypothetical observed VH concentrations, the probability of the predicted blood concentration falling within intervals defined as therapeutic (≤30 mg/l), supra-therapeutic (]30,50]), low toxic (]50;100]) and high toxic (>100) concentrations, was computed.

Results

Calibration curve and validation

Calibration curves were linear for blood (r^2=0.997; 6 calibration points; 12.5, 25.0, 50.0, 100.0, 200.0, and 400.0 mg/l; in triplicate) and for VH (r^2=0.996; 6 calibration points; 4.0, 20.0, 40.0, 60.0, 80.0, and 120.0 mg/l; in triplicate). For each curve, the intercept did not significantly differ from zero (Student's t test). Homogeneity of variances was confirmed on Cochran's

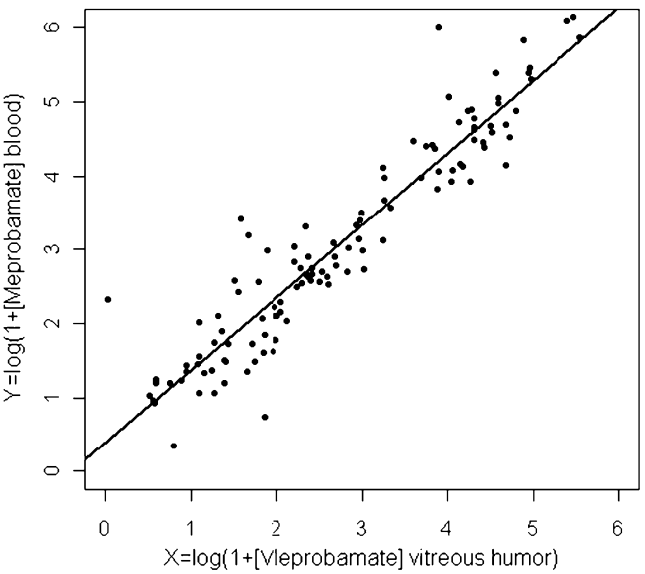

Fig. 2 Correlation curve between VH and blood log-concentrations (n=117)

Table 2 Probability of various blood concentration intervals given various observed VH concentrations

[VH meprobamate] (mg/l)	[Blood meprobamate] (mg/l)			
	[0;30]	]30;50]	]50;100]	>100
0	1.000	0.000	0.000	0.000
5	0.995	0.005	0.000	0.000
10	0.920	0.072	0.008	0.000
11	0.891	0.096	0.012	0.000
12	0.858	0.123	0.018	0.000
13	0.822	0.151	0.026	0.001
14	0.784	0.180	0.035	0.001
15	0.744	0.208	0.046	0.001
16	0.704	0.236	0.059	0.002
17	0.663	0.262	0.073	0.003
18	0.623	0.286	0.088	0.003
19	0.583	0.308	0.105	0.005
20	0.544	0.327	0.123	0.006
21	0.507	0.344	0.142	0.008
22	0.471	0.358	0.161	0.010
23	0.437	0.369	0.181	0.012
24	0.405	0.378	0.202	0.015
25	0.375	0.384	0.222	0.018
26	0.347	0.389	0.243	0.022
27	0.320	0.391	0.263	0.026
28	0.295	0.391	0.283	0.030
29	0.272	0.390	0.303	0.035
30	0.251	0.386	0.322	0.040
31	0.231	0.382	0.341	0.046
32	0.213	0.377	0.358	0.052
33	0.196	0.370	0.375	0.059
34	0.180	0.363	0.391	0.066
35	0.166	0.355	0.406	0.074
40	0.109	0.307	0.466	0.117
45	0.072	0.257	0.502	0.168
50	0.048	0.210	0.516	0.225
60	0.022	0.135	0.497	0.346
70	0.010	0.085	0.441	0.464
80	0.005	0.053	0.373	0.569
90	0.003	0.033	0.306	0.658
100	0.001	0.021	0.246	0.731
120	0.000	0.009	0.155	0.836
140	0.000	0.004	0.096	0.900
160	0.000	0.002	0.059	0.939
200	0.000	0.000	0.023	0.976

In practice, if, for example, a VH concentration of 13.4 mg/l was observed, the nearest VH concentration line was read—in this example, 13 mg/l. It was deduced that the most likely blood concentration range was between 0 and 30 mg/l (probability of about 82.2%)

test over the whole test range. The dilution of the sample was validated to analyze samples initially falling outside the validated calibration curve (>120 mg/l). Repeatability, reproducibility, and recovery were tested at low (20 mg/l) and high (80 mg/l) concentration levels. The repeatability study (each of the two concentration levels analyzed 10 times) gave variation coefficients of 2.4% and for 3.6% for the low and high levels, respectively. The reproducibility study (each of the two concentration levels analyzed 10 times for 3 days consecutively) gave variation coefficients of 5.3% and of 4.3% for the low and high levels, respectively. Mean recovery was 93% for the low and high levels. The limit of detection (LOD, three standard deviations from the mean concentration measured on ten blank samples) was 0.2 mg/l. The lower limit of quantification (LOQ, ten standard deviations from the mean concentration measured on ten blank samples) was 0.4 mg/l.

Statistics

Meprobamate concentrations in blood and VH samples were determined for 117 cases. Blood concentrations ranged from 0.41 to 464.4 mg/l, mean 56.3 mg/l. VH concentrations ranged from 0.02 to 255.6 mg/l, mean 35.6 mg/l.

Differences between right and left VH concentrations

There was no significant difference between right and left VH concentrations (n=92, p=0.87). The mean of the two concentrations was therefore computed when available in both eyes.

Meprobamate overdose test

Table 1 gives the sensitivity (1-α), specificity (1-β), and summed $\alpha + \beta$ error risks observed for VH thresholds

Table 3 Blood and VH meprobamate concentrations reported in literature

Case number	[Blood meprobamate] (mg/l)	[VH meprobamate] (mg/l)	Reference
1	9.8	8.1	[17]
2	22.0	20.6	
3	5.3	5.0	
4	134	93	[18]
5	98	68	
6	160	136	
7	32.3	28.3	
8	8.8	10	
9	26.4	19.8	

ranging between 0 and 200 mg/l by 10 mg/l steps. Estimates were based on 117 subjects with concentrations above (n=40) or below (n=77) the blood concentration overdose threshold of 50 mg/l. Figure 1a shows total $\alpha + \beta$ error risk per threshold value. The threshold associated with the minimum total risk given by the smoothing model was 28 mg/l and therefore taken as the test detection threshold, with an associated sensitivity of 0.95 and absolute specificity of 1. The ROC curve showed an area under curve close to 1 (0.996) (Fig. 1b). Applying the test to the parent population from which the sample was drawn and where the rate for subjects presenting blood meprobamate concentrations> 50 mg/l was 34%, the positive and negative predictive values were 100% and 97%, respectively.

Correlation between VH and blood concentrations

Figure 2 shows the highly significant linear relationship between blood and VH concentrations (n=117, r=0.94, $p< 10^{-70}$). The following model was adopted:

$$\log_e(1 + [\text{Blood}]) = 0.40 + 0.98 \log_e(1 + [\text{VH}])$$

Using this model, Table 2 was constructed, giving the probability of blood concentration being in one of the four intervals (≤30,]30,50],]50;100] and >100 mg/l) for a given VH concentration.

Discussion

Like its companion paper on bile [14], the present study is based on a significant correlation established between blood and VH meprobamate concentrations in a representative sample (n=117). A toxic threshold in VH (28 mg/l) was found to be statistically equivalent to the blood concentration threshold of 50 mg/l distinguishing overdose from therapeutic use. The test showed very good intrinsic qualities of sensitivity and specificity and excellent predictive values, better than the meprobamate bile test [14]. This is confirmed by the successful use of this threshold in 8 of the 9 published cases of death implicating meprobamate (Table 3). The interpretation of case 7, with a VH meprobamate concentration close to the threshold value, must be done cautiously. According to Cox et al. [22], a good correlation between VH and blood concentrations could be the consequence of a transport process across the blood–VH barrier primarily by diffusion. The small molecular weight (218.25 g/mol) of meprobamate and its low fraction bound to protein (fb=20%) [23] reinforce this hypothesis. Furthermore, a novel interpretation tool is proposed to evaluate blood concentration range from VH concentration, with a known probability. Both tools can be used even if only one of the two VH can be collected: as previously established for certain biochemical constituents [24] and for barbiturates [17], there was no significant difference in meprobamate concentration between the right and left eyes of a given subject in the present series. Since the model was constructed using a non-selected autopsy population with various postmortem intervals, causes of death, intervals between absorption and death and absorbed doses, it can be used in all cases where VH is available and recommended where blood sampling is not feasible or for confirmation when postmortem redistribution is suspected. As the model failed in case of meprobamate resulting from carisoprodol intake [25], the present procedure should not be performed when carisoprodol is detected during general unknown screening step.

To complete this descriptive study and to confirm the hypothesis concerning the transport mechanism of meprobamate through the blood–VH barrier, it could be interesting to conduct experimental studies as it has already been performed for ophthalmic drugs [26, 27]. Since each drug has its own pharmacological and physicochemical properties, these results may not be transposable: other molecules should be studied with the same objectives before their VH concentrations can be interpreted.

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