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Study of chemiluminescence measured by luminometry and its application in the estimation of postmortem interval of bone remains

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ABSTRACT

A substantial challenge faced by forensic medicine is determining the postmortem interval (PMI) of skeletonized remains. Currently, the luminol method is of limited forensic usefulness, since it uses qualitative and subjective methods to estimate PMI by the naked eye assessing the degree of chemiluminescence (CL) emitted by bone remains, a technique which is not sensitive enough to distinguish between historical or forensically significant time intervals. The aim of the present study was to use a direct and accurate measurement of the CL by luminol technique in relative light units (RLU) using a luminometer to establish this method as a possible complementary and low cost tool for the determination of the PMI for distinguishing between remains of medical-legal (< 20 years) and historical (≥ 20 years) interest in 102 femur remains with a range of PMI between 15 and 64 years. The results suggest that, under favorable conditions, the luminol technique can detect haemoglobin in the bone in a PMI range of 0–65 years, finding significant differences in the CL intensity among samples with PMI < 20 years and PMI ≥ 20 years. In addition, the intensities of CL measured at 10 s, 15 s and 20 s after reaction with luminol show a statistically significant inverse relationship with PMI in the bone studied, following a decreasing logarithmic model. The conclusion is that this quantitative, objective and contrastable technique could be very useful for determining the PMI in bone remains, since it allows a good degree of precision and eliminates the subjectivity introduced by qualitative techniques.

1. Introduction

Determination of the PMI in bones is of great medical-legal importance but, due to the absence of precise methods, it continues to have no clear solution [1].

Many methods and criteria have been used to determine the PMI in skeletal remains, such as morphological criteria, which are reliable but not objective [2] and where the experience of the researcher plays a significant role [3], estimating deterioration of other items recovered in the place of discovery (clothes, personal items, etc.), or the use of chemical and physical methods [4].

Chemical criteria are based on the study of modifications that bones suffer after death due to putrefaction phenomena and the main changes that occur during the diagenesis of bone the loss of organic material, increase in crystallinity of bone mineral, changes in bone porosity, and changes in trace element composition [5–8]. The result of all these changes is the formation of increasingly more elemental molecules, analysis of which is a valid tool for the determination of the PMI of the studied skeletal remains.

There are several studies on the use of physical-chemical methods for the determination of the time elapsing since death, including research into the lixiviation rates of fatty tissue and other organic matter [9], the variation of biochemical parameters such as collagen type I proteins and nitrogenous bases [10], proteins, nitrogen, amino acids and fluoride [11–16], the inorganic components present in bone tissue [11], the citrate content of bone matter [17] or the evaluation of radioisotope levels in tissue (bone) [18–22].

In addition, several studies have reported the luminol test as a promising chemical technique, albeit with some limitations, for the assessment of skeletal material of possible forensic interest. The forensic use of luminol has been extended to the estimation of PMI of skeletal remains by correlating the time elapsed since death and the persistence of haemoglobin traces within the bone tissue [23–27].

Introna et al. [27] studied the CL in bones in contact with luminol reagent, and obtained promising results concerning the PMI of bone remains. They showed that when skeletal remains are subjected to luminol reagent, the CL decreases as the PMI of the bone remains increases. Recent bones (from 1 month to 3 years old) showed a high CL

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in 100% of cases, which was easily detectable with the naked eye. The oldest bone samples (from 25 to 35 years old) showed a lower CL in 35% of cases, while the oldest (80 years) did not show any CL. Both the positivity and negativity of the reaction is classified by measuring the presence or absence of the CL by a scale of greys after filming the reaction.

In their studies, Ramsthaler et al. [24,26] studied the CL of luminol and its use for ascertaining the PMI of bone remains. The first investigation used eighty bone samples with a PMI ranging from 6 to 1500 years in a random design, which used direct observation of the CL produced. The conclusion was that this technique is susceptible to significant classification error according to the observer. Subsequently, with the objective of verifying the practical usefulness of this technique, the authors evaluated the efficacy in thirty nine samples of long bones and five samples of animal bones with a PMI ranging from 1 to 2000 years. The results showed that the lack of luminescence and decrease in UV fluorescence were of greater significance for estimating the PMI than the presence of luminescence.

Ermida et al. [23] evaluated the technique based on luminol, measuring the CL in fifty samples of skeletonized remains as an indicator of the PMI that ranged, in this case, from 0 to 20 years and conclude that this technique was of use as a presumptive test in the forensic field since the results showed a high degree of inter and intra-observer agreement.

A major problem of methods used in forensic science is reproducibility and the possibility of contrasting the results objectively way. In the case of luminol, the above mentioned studies used techniques with a subjective component, when not using semiquantitative indirect techniques. The objective of the present study was to make a direct and accurate measurement of the CL technique using relative light units (RLU) measured by a luminometer, which allowed the precise and reproducible quantification of data, thus establishing this method as a possible additional, useful, simple, accessible, low cost tool for the determination of PMI.

2. Material and methods

In our study, bones from 102 individuals of both sexes (61.8% males (n = 63) and 38.2% females (n = 29)), with an average age of 66.52 (S.D = 20.14, range 13–97 years), with different and known postmortem intervals were tested. For the purpose of this study, samples from 102 femur were used: skeletonized remains from the Cemetery of Murcia (southeastern Spain) with a PMI ranging between 15 and 64 years, with an average of 23.85 (S.D = 11.00).

Following the description of several authors and according to the consulted literature, the portion of femur that was used for the analysis was from the inner part of the compact diaphysis. The bone powder consisted primarily of endosteal bone of compact bone because it is more likely to be recovered during site excavation and is far less susceptible to diagenetic effects, being considerably more durable in the burial environment [25,27–29].

All procedures were performed in compliance with two laws and one regional regulation, law of biomedical investigation Law 14/2007 of 3 July [30], Organic Law 15/1999 of 13 December on the Protection of Personal Data [31] and Regulation of Mortuary Sanitary Police 1991 of Murcia Region [32]. This study was approved by the Ethics Committee of the University of Murcia.

For analysis purposes, the samples were classified into two groups depending on the postmortem interval (PMI $<$ 20 years (n = 59) and PMI $\geq$ 20 years (n = 43)) and in accordance with the statutes of limitations for the crimes committed, as mentioned in the relevant Spanish legislation [33].

In order to standardize the sample, a single type of bone was selected, the femur, with no known or visible bone pathology. The differences between sexes and age were not taken into account for the statistical analysis [24–27].

The specimens (small fragments of a similar size of about 15 × 15 mm) were taken from the 102 selected bone elements using a powered professional tool. Then, the bone samples were pulverised by a planetary micro mill (Pulverisette 7®, Fritsch, Idar-Oberstein, Germany) for 10 min at 3000 rpm.

Finally, 30 mg of each of the 102 samples of bone powder were placed into 96-well plates Nunclon® Δ Multidishes (D7039-1CS, Sigma Aldrich, St. Louis, MO, USA) and the luminescence intensity, obtained in RLU in a spectral range of 240 and 740 nm, was measured using a FLUOstar Galaxy plate reader (BMG Lab Technology, GmbH, Offenburg, Germany) for 300 s, making partial measures every 5 s.

The luminol solution (123072-25G, Sigma Aldrich, St. Louis, MO, USA) was prepared according to Weber [19], and the test was conducted by adding 0.1 ml of the fresh luminol solution to a 30 mg sample of the bone powder in a darkened room. After luminol was added to the bone powder, the well plates were gently shaken for a few seconds and the positive response was recorded after 15 s.

Descriptive and explorative statistics were carried out using the software packages SPSS1 vers. 20.0.

3. Results

The luminol chemiluminescence (CL) intensities of femur samples of PMI $<$ 20 years and samples of PMI $\geq$ 20 years pointed to a significant decrease ($p < 0.001$) with time (Table 1). These results suggest that, under favorable conditions, the luminol technique can detect haemoglobin in bone samples in a range of PMI of 0–65 years, since significant differences were found in the intensity of CL between samples.

Table 2 shows a correlation analysis of PMI and the different intensities of CL studied. The results presented a statistically significant inverse relationship between all intensities and the PMI, but it was found that CL10s ($r = -0.361$, $p < 0.01$) CL15s ($r = -0.367$, $p < 0.01$) and CL20s ($r = -0.366$, $p < 0.01$) presented the highest correlation with the PMI.

A curvilinear estimation was carried out to determine the mathematical model of the formula that best fits the graphic representation of a dependent variable regression. Table 3 shows that 16.1% of the variable PMI could be explained by CL15s and CL20s intensities and that 15.3% of the variable PMI could be explained by CL10s intensity.

In the discriminant analysis (Table 4) applied to the variables CL10s, CL15s and CL20s as a function of PMI when two groups were established, PMI $<$ 20 years (45.8%) and PMI $\geq$ 20 years (88.4%), 63.8% of the cases were correctly assigned to their group. When the samples were analysed as a function of all the CL intensities the

Table 1
Comparison of the mean RLU of CL studied as a function of two postmortem intervals (PMI) of the bone studied and *p* Lambda Wilks test for differences between groups.

	$<$ 20 years (N = 59) Mean ± SD	≥20 years (N = 43) Mean ± SD	*p*
CLorigen	15534.88 ± 12847.53	9509.39 ± 10012.70	0.012
CL05s	33640.20 ± 26476.22	16709.53 ± 20485.95	0.001
CL10s	29483.31 ± 26300.22	11318.58 ± 16298.92	0.000
CL15s	26091.71 ± 25506.46	8029.93 ± 12005.14	0.000
CL20s	22838.41 ± 23689.65	6054.54 ± 9106.91	0.000
CL25s	20188.32 ± 22122.06	4814.77 ± 7176.10	0.000
CL30s	17842.14 ± 20390.85	3968.72 ± 5960.29	0.000
CL35s	15886.97 ± 18844.83	3365.12 ± 5109.64	0.000
CL40s	14228.56 ± 17400.79	2873.37 ± 4374.79	0.000
CL45s	12780.56 ± 15924.54	2497.60 ± 3814.92	0.000
CL50s	11581.86 ± 14680.62	2220.26 ± 3445.42	0.000
CL55s	10589.15 ± 13684.93	1991.58 ± 3145.54	0.000
CL60s	9761.14 ± 12809.33	1802.18 ± 2904.01	0.000
CL65s	9017.44 ± 11851.01	1645.84 ± 2702.52	0.000
CL70s	8413.00 ± 11114.39	1509.40 ± 2528.49	0.000