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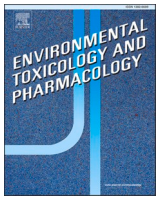

Validation of the Lead Care II System in Cape vultures (*Gyps coprotheres*) in comparison to ICP-MS using pure standards

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ABSTRACT

Lead toxicosis remains a concern in raptors, especially following feeding on carcasses sourced from hunting. Rapid diagnosis of lead exposure and easy field monitoring is desirable. The LeadCareII analytical system, validated for rapid diagnoses of lead toxicity in humans, has been described as a useful evaluation system in various species. For this study we attempt to validate the LeadCareII system in the Cape Vulture (CV) (*Gyps coprotheres*). Blood samples from CV housed under captive conditions and low background lead exposure, were pooled and spiked with known concentrations of a lead standard (0–60 μg/dL). Samples were analyzed by the LeadCareII system and by ICP-MS. The final results showed that despite good linearity the LeadCareII system underestimated lead concentrations by up to 50 %. While the results can be corrected by the derived equation, this is not supported due to the large underestimations evident. The reason for the underestimation is presently unknown.

1. Introduction

Lead poisoning in vultures remains a major problem in Africa, especially in South Africa where a number of causes of lead exposure have been identified. These include exposure to lead bullets and sinkers in the environment; lead in the soil and the legacy impacts of lead in fuel and paints (Naidoo et al., 2012, 2017; Koeppel and Kemp, 2015; Krüger and Amar, 2018; Van den Heever et al., 2019). Following exposure to lead, animals can develop subclinical signs which can impact on reproductive health, embryonic health and red cell production, while in more severe conditions clinical signs include neurological signs, inability to feed, depression and seizures. Of the two forms of exposure the subclinical effects tend to be more insidious with progression from subclinical to clinical disease linked to the extent and time to exposure. In terms of disease progression, subclinical toxicity may not necessarily proceed to clinical toxicity if the exposure insult does not continue (Naidoo et al., 2012), while acute toxicity conversely can result quickly from a single large exposure typically associated with ingestion of a lead source such a lead bullet, pellet or sinker (Koeppel and Kemp, 2015).

Despite the clinical signs being well recognized such as developmental abnormalities in chicks, subclinically exposed vultures are more difficult to diagnose as this is dependent on laboratory testing of whole blood lead concentrations. At present, concentrations in the region of 10–20 μg/dL are said to be indicative of background exposure, concentrations between 20 and 50 suggestive of subclinical exposure, while concentrations above 50 are suggestive of clinical exposure (Naidoo et al., 2012). A further complication with determination of blood lead concentrations, is the need to submit samples of blood to a laboratory that has the correct equipment, typically Induction coupled plasma -mass spectrometry (ICP-MS) or Graphite Furnace Atomic Absorption Spectrometry (AAS). In addition to the expense of the equipment, laboratory-based analysis is not useful for immediate monitoring in the field. As a result, alternate methods or mechanisms are needed, such as a field testing using a rapid analyzer, of which the LeadCare® diagnostic system marketed by Magellan Diagnostics, which uses anodic stripping voltammetry, offers a potential solution.

While the LeadCare analytic system has been used in many bird and wild mammalian species including vultures, none of these publications have validated the system for use in Cape vultures (*Gyps coprotheres*) (CV), an endemic vulture species in South Africa. The importance of validation has been highlighted in a study by Herring et al. (2018), in which a number of publications using the LeadCare system were identified. In this review, the authors raised concerns on conclusions being drawn in literature in the absence of proper validation. Further they

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were able to demonstrate that a degree of correction of the results were required as the LeadCare results tended to be lower than that quantified by laboratory methods.

As a first step we evaluated one blood sample from a healthy Cape vulture on the LeadCareII (LCII) system and ICP-MS with results of at 12.2 and 18 μg/m respectively, suggested that the LCII analyser was detecting lower concentrations than detectable with a gold standard method. The latter results were not too dissimilar to an unpublished report using the same two systems, from 27 CVs evaluated in the field with a reported correlation of only 13.7 % (L van den Heerver, Pers comm 2023). This poor accuracy poses numerous concerns as it makes proper monitoring of birds in the field difficult with potential misdiagnoses of birds with lead toxicity. It also creates concerns with long-term monitoring as results over time may be non-comparable if the results are not standardized. For the following study, we attempt to validate the LeadCareII system in comparison to an ICP- MS using Cape vulture whole blood spiked with known concentrations of a lead standard.

2. Materials and method

2.1. Animals

The study was approved by the Animal Ethics Committee of the University of Pretoria (REC087-22). Blood was collected from five adult CVs, by means of venipuncture by needle and syringe from the tarsal vein and immediately transferred into EDTA evacuated tubes. For the transfer the tubes were opened, the vacuum broken and samples transferred by syringing the samples into the opened tube as previously recommended by Herring et al. (2018) to allow sufficient time for any remnant sulfur-based curing agent from the rubber lid to dissipate. In contrast to Herring due to limitations in blood volume, all the blood samples were pooled into a glass container and allowed to stand.

2.2. Sample preparation

A lead standard stock solution was prepared as per the Magellan instructions for external calibration. For this a 10^{-3} g/mL the Lead reference standard [$Pb(NO_3)_2$ in HNO_3 0.5 mol/l 1000 mg/l; Merck South Africa] was diluted to a concentration of 10^{-5} g/mL in deionized water and 70 % nitric acid. Subsequently the pooled blood samples were evenly split into six aliquots and a predetermined concentration of a diluted lead standard solution or water added in to bring each aliquot to a concentration of 0, 10, 20, 45 or 60 μg/dL. After allowing a period to stabilize, samples were split into four. A pure set of standards at the same six concentration was also made up in deionized water.

2.3. LeadCare II System

For the LeadCare analysis, we made use of the LCII system with an analytical range of 3.3–65 μg/dL. The analyzer was calibrated with the key provided with the analytical kit (2230M-05). All samples were analyzed as per the manufacturer's instructions by the same person to minimize variation in results. Analysis was started by calibrating the analyzer with the supplied 10.8 and 30 μg/dL calibrants. For the spiked blood samples or pure samples, samples were transferred into the acid lysis buffer using the manufacturer's vial, and thereafter transferred onto a fresh test strip for every sample and the results read off the screen and captured. Samples were analyzed in quadruplicate.

2.4. ICP-MS method

Blood sample per concentration were analyzed in quadruplicate by an ICP-MS method at a commercial accredited chemical pathology laboratory in South Africa (Ampath) according to published methods (Choe and Gajek, 2016; Gajek et al., 2013). In short, specimens were diluted 25 times with water prior to direct injection. The matrix matched method had a calibration range of the method of 0.1–50 μg/dL, with calibration being prepared in synthetic matrix and linearity (R^2) of 0.9991. The method has a limit of detection (LOD) and quantification (LOQ) of 0.025 μg/dL and 0.1 μg/dL respectively. To ensure quality, the laboratory used Seronorm Trace Elements Whole Blood Controls and participate in the UK NEQAS External Quality Assessment/Proficiency Testing scheme monthly.

2.5. Statistical analysis

With the blood samples having detectable lead levels as expected for background exposure, the lead concentration for the unspiked sample were deleted from the samples with spiked lead concentrations (10, 20, 45 or 60 μg/dL). For each method the accuracy and precision were determined and evaluated as per the VICH guidelines for method validation for chemicals in animal matrices with the acceptable range for accuracy being 80–110 % and a %CV of less than 15 % for precision (VICH GL 49, 2015). Results from the ICP-MS results were also plotted against the expected concentration or the LCII quantifiable concentrations to ascertain linearity and the confidence interval of the difference determined. Bland-Altman evaluation of the differences between the two methods were also determined.

3. Results

The lead concentrations from the pure standards and calibrants for the LCII are presented in Fig. 1. The 10.8 calibrant sample tested within the range, while the two samples of 30 μg/dL tested lower than expected, with the first sample out of the manufacturer's range (28 ± 4). In comparison to an ideal calibration curve ($y = x$), the calibrants tended towards under-estimation. The pure standard curve showed good linearity with a range of 4.4–51.9 μg/dL. The pure diluent with zero lead added tested at 4.4 μg/dL despite being lower than the range of the analyzer. In general concentrations of lead below 30 μg/dL were slightly over-estimated, and the those above were underestimated by the LCII system. The accuracy of the samples was 132, 116, 105, 85 and 86 % for the 5, 10, 20, 30, 45 and 60 μg/dL samples respectively.

The pooled vulture blood samples without any spiked lead tested at a concentration of 4.9 ± 0.42 and 12.52 ± 0.26 μg/dL for the LCII and ICP-MS methods respectively as background lead concentrations. The corresponding lead concentrations measured in spiked blood samples by ICP-MS and the LCII analyzer are presented in Table 1, following baseline correct (deletion of the zero-sample concentration) and presented in Fig. 2. When the measured concentrations were plotted against the expected concentrations, both methods showed good linearity. However only the ICP-MS was both accurate (108–112 %) and precise (< 6.77 %) with evaluated concentrations measuring slightly higher than the expected concentrations. In contrast the LC II method while showing good precision (< 10.48 %) had poor accuracy with samples varying from 53 % to 72 % of the expected concentrations, with the lower concentrations having the better accuracy. When the LC II results in blood were compared to pure sample, the blood samples were 60 ± 7.8 % underestimated.

When the two methods were compared for the spiked blood samples, there was good correlation (97.57 %) that was defined by good linearity with a coefficient of determination of 95 %. As expected from the difference in the results, the Bland-Altman plots supported the LCII method producing lower concentrations than the ICP-MS gold standard (Fig. 3). More importantly the extent of the difference grew larger at the higher concentrations (Table 2), to such an extent that the 60 μg/dL concentration tested at 50 % lower on the LCII method. The mean bias was 14.93 ± 10.26 μg/dL (95 % confidence 10.95–18.98 ud/dL), and had the most marked difference for the 60 μg/dL sample at 33.55 ± 2.92 μg/dL.

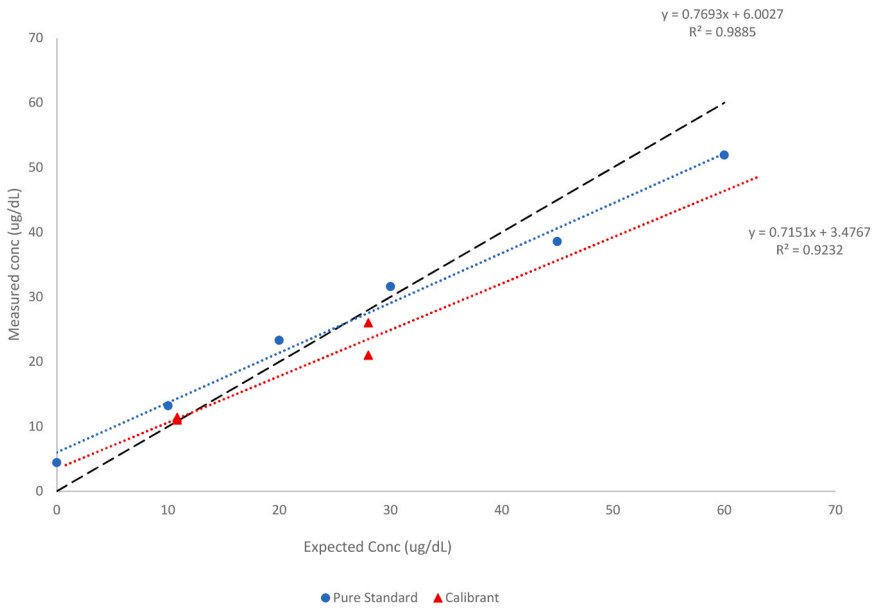

Fig. 1. Lead concentrations of the pure samples and sample calibrant plotted against the expected concentration on the LCII machine, corrected the presence of background lead concentration of 4.4 μg/dL. The best fit equation was ($y = 0.7693x + 1.60027$ with $R^2 = 0.9885$). The dashed line, represent the ideal scenario of a zero intercept and a gradient of 1 ($y = x$).

Table 1
Concentration of lead (μg/dL) in spiked vulture blood samples following LCII or ICP-MS analysis.

Method	Expected Conc	Run				Mean	SD	Mean Accuracy (%)	RSD (%)
		1	2	3	4				
LCII	10	8.3	7.8	7	8.5	7.90	0.67	70.38	8.46
	20	13.2	14.7	11.5	12.4	12.95	1.36	59.40	10.48
	30	19.1	16	18.4	16.3	17.45	1.53	53.57	8.78
	45	28.7	29	27.2	25.9	27.70	1.44	55.23	5.18
	60	30.6	30.2	31.2	36.1	32.03	2.75	48.99	8.58
ICP-MS	10	10.7	12.4	10.8	11	11.23	0.79	112.25	6.77
	20	22	22.6	20.7	21.9	21.8	0.80	109	2.63
	30	31.4	32.8	31.4	34.7	32.58	1.56	108.58	4.70
	45	48.2	49.8	48.3	54.3	50.15	2.86	111.44	5.66
	60	66	64.9	65.5	65.1	65.38	0.49	108.96	0.82

SD – Standard deviation; RSD – Relative Standard Deviation.

4. Discussion

The following study was primarily undertaken to ascertain if the LeadCare II point of care system could be validated for use in the Cape vulture following an earlier discrepancy evident following clinical evaluation. A secondary objective, if validation was not possible, was to ascertain if a correction factor could be applied to the obtained results to improve their accuracy. Further, while previous studies compared lead concentrations in blood samples from animals in the field by the LeadCare system and ICP-MS/AAS, for this study we undertook a more direct analysis by spiking blood with known concentrations to be better quantify the accuracy and precision of the method. However, since we were unable to obtain lead-free blood, due to the persistence of background lead concentration in wild animals, we relied on pooled samples from five vultures to standardize the baseline. The latter was subsequently subtracted from all spiked sample in order to obtain baseline corrected values. From this result, the spiked blood concentrations evaluated by ICP-MS method was both accurate and precise in comparison to the matrix matched calibration curve used by the laboratory. In contrast, for the LeadCare method we were able to conclude that the method was not accurate, even though the method was linear and precise with an RSD (%CV) of 5–10 %. While correlation between the two methods was possible, with high concentration measured by LC II being 50 % lower than the ICP-MS analysis, it is questionable if a factor should be applied to the LC II obtained results.

A major finding for this study that the LCII system in CGVs resulted in a large mean negative biases of 14 μg/dL over all the tested concentrations, leading to the conclusion that the LCII system was not adequate for field assessment of lead exposure in Cape vultures. This finding was markedly different to studies undertaken in Cinereous (*Aegypius monachus*) and Griffon vultures (*Gyps fulvus*) where a negative bias of 0.637 and 1.094 μg/dL respectively were identified. The results are not too different from that reported by Herring et al. (2018) where the results for the Common raven (*Corvus corax*), Golden Eagle (*Aquila chrysaetos*), Red-tailed Hawk (*Buteo jamaicensis*), Swainsons' hawk (*Buteo swainsoni*) and Turkey vultures (*Cathartes aura*) were 30 % different. In Ravens, Craighead and Bedrosian (2008) also described a negative bias with the LeadCare results reading 30 % lower. In contrast to studies in humans using a large sample size found a positive bias of 19.15 ± 8.26 mg/dL and 29.25 ± 14.04 mg/dL for the LC system for the high concentrations of 45 and 65 μg/dL, while a lower bias of 0.3 μg/dL was seen for the concentrations less than 10 mg/dL. In the Brown bear (*Ursus arctos*), a positive bias of 22 % was noted (Boesen et al., 2019). Evident from these comparisons is that large differences exist between birds and mammalian, indicating that validation is needed per species, and cannot be extrapolated between species as differences between species and

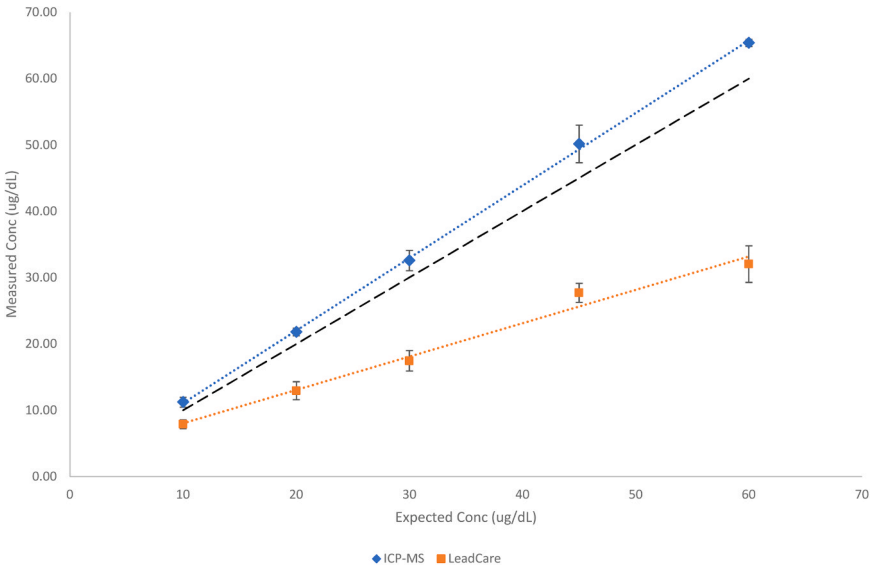

Fig. 2. Concentration of lead in spiked vulture blood samples, analysed with either ICP-MS or the LCII machine, corrected for mean baseline concentrations. The dashed line, represent the ideal scenario of a zero intercept and a gradient of 1 (y = x). The ICP-MS concentration was defined by the equation y = 1.0934x + 0.1417 (R^2 = 0.005) and the LCII was defined by the equation y = 0.503x + 3.0074 (R^2 = 0.9851).

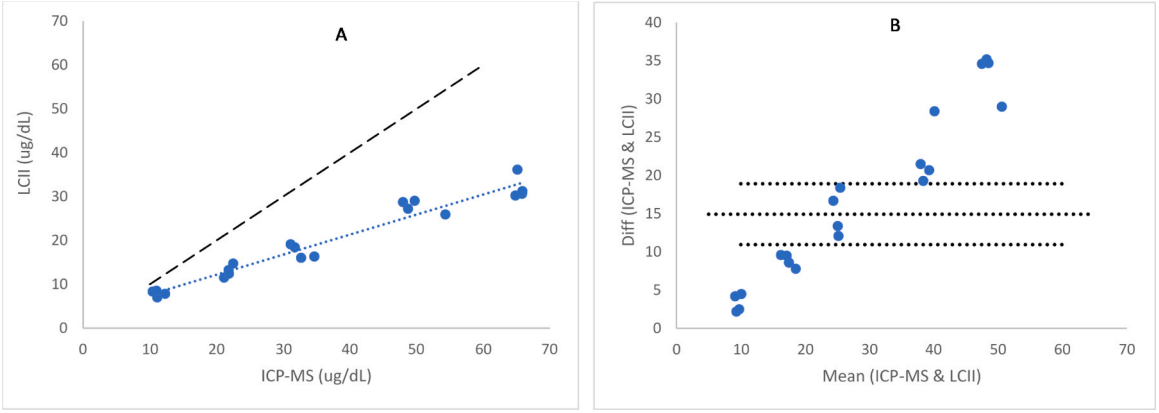

Fig. 3. Comparison of the mean lead concentrations of the same samples of the LCII machine plotted against the concentrations obtained by ICP-MS analysis by linear regression (A) or by Bland-Altman plots (B). The dashed line, represent the ideal scenario of a zero intercept and a gradient of 1 (y = x). The dotted lines on the Bland-Altman plot indicates the mean differences, and the upper and lower 95 % confidence interval of the mean difference. The regression plot was defined by the equation y = 0.4569x + 3.052 (R^2 = 0.9521).

Table 2
Comparison of the mean lead concentration (μg/dL) between ICP-MS and LCII and the 95 % confidence interval of difference between the two methods.

Conc	ICP-MS		LCII		Confidence Interval of the Diff (95 %)			
	Mean	SD	Mean	SD	Mean Diff	SD	Lower	Upper
10	11.23	0.76	7.90	0.67	3.33	1.17	2.18	4.47
20	21.80	5.18	12.95	2.78	8.85	0.85	8.02	9.68
30	32.58	6.39	17.45	3.68	15.13	2.91	12.28	17.97
45	50.15	5.43	27.70	2.66	22.45	4.05	18.48	26.42
60	65.38	0.57	32.03	1.36	33.35	2.93	30.48	36.22

analyzers in use.

At this point, while the reason for the poor accuracy of the method in unknown, the use of pure samples in water (in the absence of red cells, blood proteins, antibodies, and other metals) suggests that the interference was resulting from a blood constituent rather than the methodology. Thus far for the voltage stripping method in use, factors that have been described to interfere with lead readings are copper concentrations, glutathione concentrations and thiarum containing compounds (Thiarum are sulfur-based compounds that are remnants from the rubber vulcanization manufacturing process) (FDA (US Food and Drug Administration), 2018).

We cannot rule out the presence of another heavy metal in the blood which will require further evaluation. With regard to the presence of thiarum, BD vacutainers are contraindicated for use for lead analysis using an anode stripping method (FDA (US Food and Drug Administration), 2018), as the rubber stopper is reported to release thiarum gas

which binds with lead in a stable reaction, rendering it unavailable for analysis by the LCII analyzer. We however doubt that this was of significance in this study as we followed a published method that indicated that opening the tubes prior to sample (and breaking the vacuum) mitigates the reaction by releasing the gas. In a study with four raptor species in Spain using the same collection methodology, no major differences were present between a LCII and ICP-MS method. Further it was also reported by Herring et al. (2018), that the effects of sulfur-containing compounds can be mitigated by immediately transferring the samples into a second propylene tube. For this study, we transferred the sample to glass bottles, and allowed it to stand prior to addition of the lead standards.

Glutathione and concentrations in the blood is thus the most likely reasons (Herring et al., 2018). From published literature and the manufacturers patent glutathione (an organosulfur thiol), which is present in whole blood, can bind to the strips reducing the ability of the strips to measure lead concentrations accurately. As a result, the manufacturer provides correction to their result by accounting for the average glutathione present in humans (0.849 ± 0.163 mM, equivalent to 26.12 ± 5 mg/dL) (Michelet et al., 1995). More importantly they indicate that for every 0.85 mM of glutathione, the lead concentrations can be reduced by 10–15 %. At present the glutathione concentration in Cape vultures has not been measured. From literature values as high as 4.56 ± 0.99 uMol/g (equivalent to 4.56 mM) have been reported in the Griffon vultures in the presence of blood lead concentrations of 15.32 ± 8.28 μg/dL to lower concentrations of 1.11 μg/dL (equivalent to 0.036 μM)(Glutathione = 307.33 g/Mol) in Cinereous vultures (Espín et al., 2014; Pikula et al., 2013). If the CV glutathione levels are similarly high at the Griffon vulture, this would explain the interference. Considering that the analyzer corrects for the presence of 0.85 mM of glutathione and that each 0.85 mM of glutathione reduces the detectable lead concentration by 10–15 %, the presence of an additional 3.71 mM of glutathione above the 0.85 mM correction level, could decrease concentrations by 43–60 %, which is margin of error evident in this study. As a next step it is suggested that glutathione concentrations in Cape vulture be investigated to determine their impact on the LCII analyzer.

5. Conclusion

Based on the result, it thus becomes evident that the LCII could potentially be useful confirm lead exposure in the CV when very high concentrations are present. However, at intermediate lead concentration the system is unlikely to be very accurate, as these concentrations read as lower concentrations without correction. While the linear regression of the LCII results suggests that a correction factor may be applied, the major negative bias seen with the highest concentration would suggest that this correction factor should not be applied.

CRediT authorship contribution statement

Vinny Naidoo: Writing – review & editing, Writing – original draft, Validation, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Kerri Wolter:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Author Declaration

The authors have no competing interests to declare

Author contributions

All authors contributed equally to the design of the study, data collection, data analysis and writing of the manuscript

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: V Naidoo reports financial support was provided by National Research Foundation (South Africa). V Naidoo reports a relationship with VulPro that includes: board membership. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is available in the publication.

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