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Letter to the Editor

Comment on "Determination of testosterone in saliva and blow of bottlenose dolphins (*Tursiops truncatus*) using liquid chromatography–mass spectrometry"

I wish to refer to an article published in 2005 in the Journal of Chromatography B entitled "Determination of testosterone in saliva and blow of bottlenose dolphins (*Tursiops truncatus*) using liquid chromatography–mass spectrometry" by Hogg et al. [1]. One of the major conclusions of this work is that the concentration of testosterone in male dolphin saliva ranges from 10 to 23 ng/mL. The highest level equates to 80 nmol/L which is very high when compared to values quoted for human saliva which are typically in the order of 0.3 nmol/L [2]. A quick review of the literature has shown one paper referring to the concentration of testosterone in the serum of bottlenose dolphins [3]. The values reported ranged from 1 to 54 ng/mL. These values are higher than found in humans but are not high enough to explain the values reported for dolphin saliva unless there is active transport of testosterone from the serum to the saliva. The authors do not make any comments regarding their saliva measurements as to whether they are higher than expected compared to other mammals or compared to previously reported serum measurements in dolphins.

A closer examination of the analytical methodology used in the article may explain these high results. The authors have used liquid chromatography–mass spectrometry to measure testosterone in saliva after SPE extraction. The mode of ionization used was electrospray (ESI). The mass spectrometer used was a single quadrupole and the testosterone appears to have been identified by the presence of the ion 289 ($M+H^+$) at a retention time of 3.6 min. I do not know of any regulatory body that that would consider a single ion at a particular retention time as proof of identity. Most bodies have a three significant ion minimum requirement. The authors make reference to an "acetylated adduct" in Fig. 2 (actually an acetonitrile adduct) but make no further reference as to whether it was used in the analysis. However, even if it was used the presence of such an adduct ion would not be considered as a significant ion for identification. The ion chromatograms in Figs. 3 and 7 clearly show that there are many other compounds present which give the 289 ion response and the peak at 3.6 min which the authors identify as testosterone is a blip on a broad envelope of peaks.

Even if it assumed that the sole contributor to the 289 ion signal observed at a retention time of 3.6 min is testosterone the authors have ignored what has been referred to as the Achilles heel of ESI and that is ionization suppression and enhancement [4]. It is well known that the matrix can have major effects on quantitation in ESI and a paper modelling this was published in 1997 [5]. Numerous papers have been published on matrix effects since then [6–12]. It is generally accepted that if one is to obtain reliable quantitative results from ESI in complex matrices then an appropriate internal standard must be used. The most suitable internal standards are those which are chemically similar to the analyte and which elute at almost the same time as the analyte and are presumed to undergo similar suppression or enhancement. The best internal standards are isotopically labelled analogues of the analyte. The authors have chosen to use Fmoc-glutamine as an internal standard. This compound is chemically unrelated to testosterone, elutes 6 min after testosterone, and is measured using a different mode of ionization (negative not positive). It thus does not meet any of the criteria required of an internal standard for ESI analysis. The authors state on p. 344 that "Radioactive isotopes of testosterone were not considered as an internal standard as the described LC–MS method is to be used in other laboratories where there are no radioactive disposal units". Deuterated testosterone has been readily commercially available for more than a decade and is not radioactive.

On p. 342 the authors state "Ruggedness of the method was ascertained by assaying stock solutions at two different concentrations: 50 and 5 ng/mL, using the same method but with two different 300A C8 columns (Lot no. 02110886-1 and 02110885-1)". When one attempts to determine the ruggedness of a method it is usual to make changes of what are thought to be critical control points. It is not possible to ascertain ruggedness by assaying stock solutions as they have not been through the full method and completely lack matrix effects. Secondly, using a different lot number of the same column type is of little value given the effort manufacturers now make to ensure the uniformity of their products.

On p. 341 the authors describe their sample collection and preparation. The only collections reported are from four male dolphins and the results are given in Table 4. The lowest value reported is 9.7 ng/mL for saliva. However in Table 2 data are shown for spiking trials done at 1 ng/mL. It is obviously not possible to conduct spiking trials at values 10 times lower than the natural concentration. Presumably saliva from female dolphins

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was used. If so this should be stated in the sample collection section.

There are also problems with the results obtained with the method. Table 1 gives inter and intra batch precision for the method. The results in the table are very unusual in that the RSD in all cases gets worse as the concentration of testosterone increases. For example at 1 ng/mL in saliva the RSD is 1.6% whilst at 50 ng/mL it is 14.1%. A precision of 1.6% at 1 ng/mL from a method with a stated LOQ of 0.5 ng/mL is quite extraordinary. It is hard to understand when one looks at the chromatogram in Fig. 7 where a saliva sample containing 8.9 ng/mL is shown. Assuming that the text and not the time axis is correct (the time axis shows the peak eluting at 4.7 min not 3.6 min as stated in the text), then I find it difficult to believe that a peak approximately one tenth the height of that shown could have an RSD of better than 2% given that it would be a small shoulder on the back of a much larger broad series of peaks. One other point relating to this graph is that the text states the sample is from Sirius with a concentration of 8.9 ng/mL and yet Table 4 shows no such result from Sirius with the lowest recorded value being 19.0. The chromatogram also leads me to question the stated limit of detection of 0.2 ng/mL particularly looking at Fig. 7(b). It is difficult to see how a peak one/twenty fifth of that shown could be detected. Was the LOD determined using real samples?

The stability data shown in Table 3 are difficult to explain. When saliva is spiked at 50 ng/mL with no inhibitor at 21 °C and kept for 2 h there is no change and yet after 1 h more the change is highly significant. It is hard to think of an explanation for the results at −80 °C in the presence of $MnCl_2$. After 8 weeks there is no change but after 12 weeks the change is highly significant. Our experience with storing steroids in human body fluids is that they are stable for months if not years at −20 °C. What mechanism is there that breaks down a relatively stable compound such as testosterone at −80 °C? The logic given for the choice of $MnCl_2$ as an inhibitor on p 345 is unusual. I would suggest that method variability is a more likely explanation for some of the observed differences than sample decomposition.

As this paper is apparently the first to report testosterone concentrations in dolphin saliva and blow it is important that the results are as accurate as possible. Unfortunately, the analytical approach employed in this article does not appear to meet the normal method criteria expected for measuring analytes such as testosterone in biological fluids. It appears that the critical review of this paper prior to publication did not provide appropriate feedback to the authors.

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