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

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Abstract

A rapid, accurate and reproducible assay utilising high performance liquid chromatography–mass spectrometry (LC–MS) has been developed and validated for determining testosterone concentrations in saliva and blow of bottlenose dolphins. Sample preparation used solid phase extraction with specific preconditioning of cartridges. Analytes were eluted with 100% acetonitrile, dried under nitrogen and stored at −80 °C. Samples were reconstituted in 60% acetonitrile for LC–MS analysis. Chromatographic separation was achieved with an Alltech Macrosphere C8 stainless steel analytical column (2.1 mm × 150 mm i.d., 5 μm particle size, 300 Å pore size) using a 55% mobile phase B isocratic method (mobile phase A = 0.5% acetic acid; mobile phase B = 0.5% acetic acid, 90% acetonitrile). Samples were analysed in SIM at *m/z* 289.20 (testosterone mw 288.40) and a positive ion ESI. The limit of quantification was 0.5 ng/ml with a limit of detection of 0.2 ng/ml. The concentration curve was linear from 0.5 to 50 ng/ml ($y = 0.01x + 0.0045$, $r^2 = 0.959$, $r = 0.979$, $p < 0.001$). The R.S.D.s of intra- and inter-batch precision were less than 15% for saliva and 11% blow. Recovery of the assay for saliva was 93.0 ± 7.9% (50 ng/ml) and 91.5 ± 3.72% (1 ng/ml), and for blow was 83.3 ± 6.8% (50 ng/ml) and 85.8 ± 4.6% (1 ng/ml). Recovery of the internal standard in saliva was 73.0 ± 14.2% and in blow was 78.63 ± 4.29. The described assay was used to determine the presence of endogenous testosterone in saliva (9.73–23 ng/ml, $n = 10$) and blow (14.71–86.20 ng/ml, $n = 11$) samples of captive bottlenose dolphins.

Keywords: Liquid chromatography; Mass spectrometry; Dolphin; Testosterone; Saliva; Blow exudate

1. Introduction

Testosterone is an androgenic steroid hormone produced by the gonads (Fig. 1) and plays a key role in the growth, differentiation and maintenance of sexual tissues, and the development of secondary sexual characteristics [1]. In mammalian males, testosterone is the principal androgen and is secreted by the testes and in females testosterone is produced in limited quantities by the ovarian follicle [1]. Human testosterone research has been well documented; however there is less known about testosterone concentrations in wildlife and with the present focus on captive breeding programs, there is a priority to understand wildlife reproductive hormone cycles. This leads to better animal management and research of potentially threatened species. The internal physiology of many marine mammals has not been studied due to the difficulties of working in the marine environment so a major advantage of working with trained captive animals is that they mirror animals in the wild. Currently, there are a number of biological sampling techniques that are used to assess hormones in captive cetaceans (whales, dolphins and porpoises). These include blood sampling [2–4], faecal sampling and urine collection [5–8]. Blood sampling is invasive and potentially painful which can cause stress to animals and in turn affect reproduction and reproductive hormone concen-

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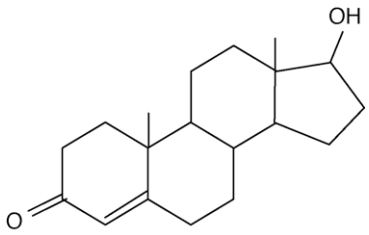

Fig. 1. Chemical structure of testosterone.

trations. Faecal and urine samples are pooled over a period of time and can be easily contaminated.

Saliva has been used in a number of human [9–13] and non-human [14,15] studies to determine a wide range of substances, including biological substances and pharmaceutical drugs. Previously, measurement of biological analytes in saliva has used radioimmunoassay (RIA) [11,12] and enzyme immunoassay (EIA) [10,16]. More recently, high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) has been used to determine reproductive hormones in a number of human biological matrices: blood [17,18], urine [19,20] and saliva [21]. There are several distinct advantages of using LC–MS compared with immunoassay in biological matrices. Smaller sample volumes can be assayed (minimum sample volume: RIA > 200 μl; LC–MS > 50 μl), cross-reactivity is not problematic and multiple compounds and their metabolites can be measured within one sample. This can provide information on the biological/biochemical pathways. In addition, data is archived for future detection of unknown compounds.

This study describes a novel non-invasive sampling technique and a validated LC–MS method for testosterone using saliva and blow samples. Unlike terrestrial mammals, marine mammals use a greater portion of their lungs with each breath. The purpose of analysing blow was to assess if biological fluids in the lungs were present in dolphin blow (exhalation) and if the blow could be used to determine hormone concentrations. The aim of this ongoing wildlife research program is to develop non-invasive techniques that would allow for determination of testosterone and other reproductive hormones in both saliva and blow of captive dolphins, with similar potential applications in great whale species. In addition, the study reports a preliminary biological range of salivary and blow testosterone in captive bottlenose dolphins (*Tursiops truncatus*).

2. Experimental

2.1. Chemicals and solutions

All chemical reagents were of HPLC grade: acetonitrile (BioLab Scientific, Melbourne, VIC, Australia), acetic acid 17.5 M (BDH Laboratory Supplies, Poole, England), water was purified by a Milli-Q system (Millipore®, Sydney, NSW, Australia) and nitrogen gas (5.0, BOC, Sydney, NSW, Australia) was of ultra-high purity.

Crystalline testosterone (Sigma–Aldrich, Sydney, NSW, Australia) was >99% purity as tested by thin layer chromatography by the manufacturer. Stock solutions were made fortnightly by dissolving testosterone in 60% acetonitrile (1 mg/ml) and stored at 4 °C away from light. From the stock solution, working concentrations of testosterone at 100, 10, 1 μg/ml and 100 ng/ml, were prepared volumetrically by serial dilution with 60% acetonitrile. Stock solutions of the internal standard (Fmoc-L-Gln(Trt)-OH; Auspep, Melbourne, Australia) were prepared in 60% acetonitrile at a concentration of 10 μg/ml. To maintain consistency with testosterone analysis in samples, 100 mM manganese chloride ($MnCl_2$; Clyde Industries, NSW, Australia), stabilising agent for testosterone, was also added to each working concentration. Thus, each working concentration was 60% acetonitrile in 100 mM $MnCl_2$.

2.2. LC analysis

LC separation was performed on a Shimadzu LC-10AD liquid chromatograph (Shimadzu, Japan) and Shimadzu SIL-10Axl autoinjector. Samples were analysed using an Alltech Macrosphere C8 stainless steel analytical column (2.1 mm × 150 mm i.d., 5 μm particle size, 300 Å pore size) operating at room temperature (21 °C). The LC elution conditions for the analysis were as follows: mobile phase A, 0.5% acetic acid and mobile phase B, 90% acetonitrile, 0.5% acetic acid, at isocratic conditions of 55% B and a flow rate of 0.2 ml/min. The HPLC column was equilibrated for 30 min prior to a series of runs and maintained its performance for over 500 injections.

2.3. LC–MS methods

LC–MS analysis was performed on Shimadzu LC directly connected to a quadrapole Shimadzu LCMS-2010 equipped with an electrospray interface operating in positive ion mode. The MS instrumental parameters were: detector gain, 2 kV; capillary voltage, 4.5 kV; drying gas was nitrogen at 4.5 l/min; drying gas temperature, 250 °C; nebulizer pressure, 6.89 MPa; ionization source at 200 °C. Conditions for the internal standard were identical but set for negative ion mode. The LC–MS system was controlled by Shimadzu LCMS solution 1.0 software. Data acquisition was set for 11 min using a *m*/*z* 289.20 $[M + H]^+$ (Fig. 2) for testosterone with zero variability of detector range. A second *m*/*z* 330.25 $[M + H + CH_2CN]^+$ was the acetylated adduct of testosterone and was used to further confirm the retention time (RT) [22]. The acetylated adduct of 330.25 was present in both biological samples and testosterone standard with a ratio of 30.9% (Fig. 2) compared to the *m*/*z* 289.2 peak. The Fmoc-Glutamine *m*/*z* was 609.15 $[M - H]^+$.

The data acquisition time was lengthened to 27 min when analysing biological samples due to an interfering peak at

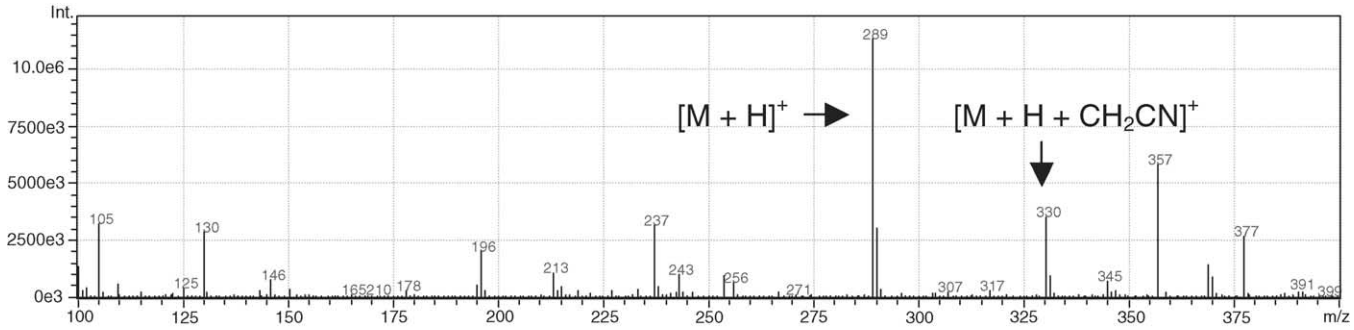

Fig. 2. Determination of testosterone *m/z* by direct infusion into the mass spectrometer (5 μl injection of 100 μg/ml in 60% acetonitrile), testosterone (*m/z* 289.20, 100% relative intensity), acetylated adduct (*m/z* 330.25, 30.9% relative intensity).

16–24 min RT (Fig. 3). Isocratic conditions were used as gradient conditions and equilibration of the column were found to have a longer run time.

2.4. Sample collection and preparation

Saliva and blow samples were collected just prior to the breeding season from four male captive bottlenose dolphins, two adults: Sirius (23 years) and Delbert (36 years); and two sub-adults: Zac, (10 years) and Buck (4.5 years). The dolphins were trained to open their mouths on cue and the saliva was collected from the oral cavity by wiping fresh cotton gauze (Smith & Nephew, Sydney, Australia; 7.5 cm × 7.5 cm × 4ply) along the roof of the mouth. Saliva was then expressed from the gauze by placing it inside a 6 ml syringe and applying pressure to the plunger. Blow samples were collected using 50 ml polypropylene tubes (Sigma–Aldrich, Sydney, Australia). The tube was held inverted over the animal's blowhole at a distance of 2–3 cm and the dolphins were trained to exhale on cue. Two exhalations were made per tube. Importantly, there was no apparent stress to the dolphins observed by the dolphin trainers from either collection method.

To prevent degradation of testosterone in saliva samples, 200 μl of 100 mM $MnCl_2$ was added to each sample. For blow samples, 500 μl of $MnCl_2$ was added and the tube shaken vigorously to ensure that all blow mucus was collected in the solution. Saliva samples were stored in cryo-storage tubes (1.8 ml round bottom, Sigma–Aldrich, Sydney, NSW, Australia) and blow samples were stored in 50 ml polypropylene tubes. Samples collected at the dolphin's location were stored at −20 °C for two days (equipment at location) before transport on dry ice to −80 °C freezer at the analytical laboratory.

Testosterone was extracted from unspiked dolphin saliva and blow using a 'Envi-Chrom P' solid phase extraction (SPE) cartridge (Sigma–Aldrich, Sydney, NSW, Australia) and was adapted from the method described by Vickers et al. [13] for SPE of human saliva. The SPE procedure was as follows: (1) 5 ml Milli-Q water added to 200 μl of sample (saliva or blow) in a 10 ml polypropylene centrifuge tubes (Crown Scientific, Sydney, NSW, Australia), vortexed for 30 s and then centrifuged for 10 min at 3000 rpm to separate particulate matter before being loaded onto the SPE cartridge; (2) SPE cartridges were preconditioned using 20 ml acetonitrile and then 5 ml Milli-Q water (higher than the manufacturer's recommendations, to completely remove interfering extra-

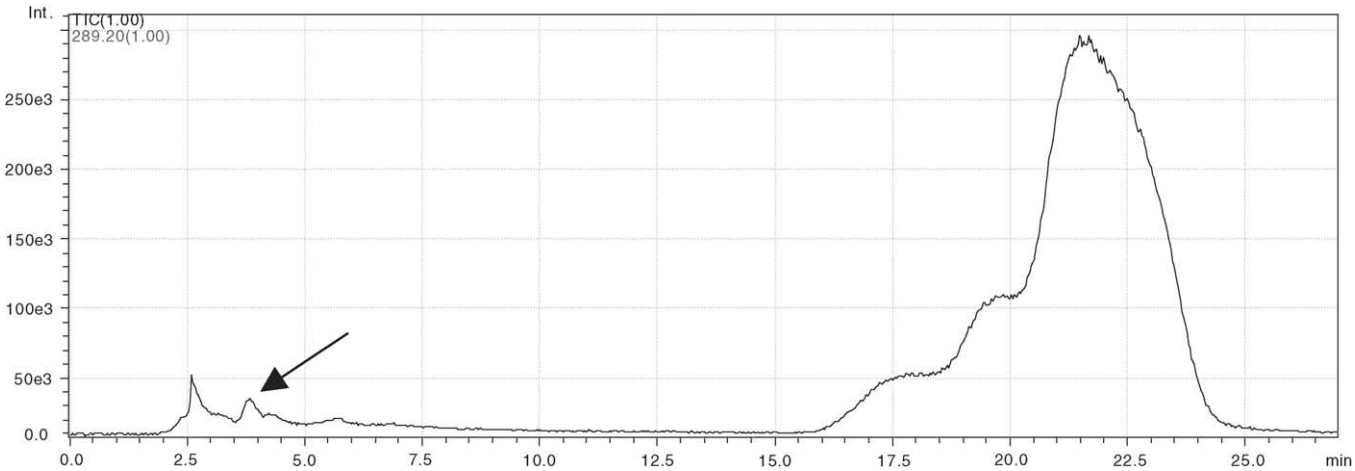

Fig. 3. MS chromatogram (*m/z* 289.20) of saliva sample from dolphin Sirius showing the native testosterone peak at 3.6 min and peak at 16–24 min requiring duration of isocratic conditions to have a 27-min assay time.

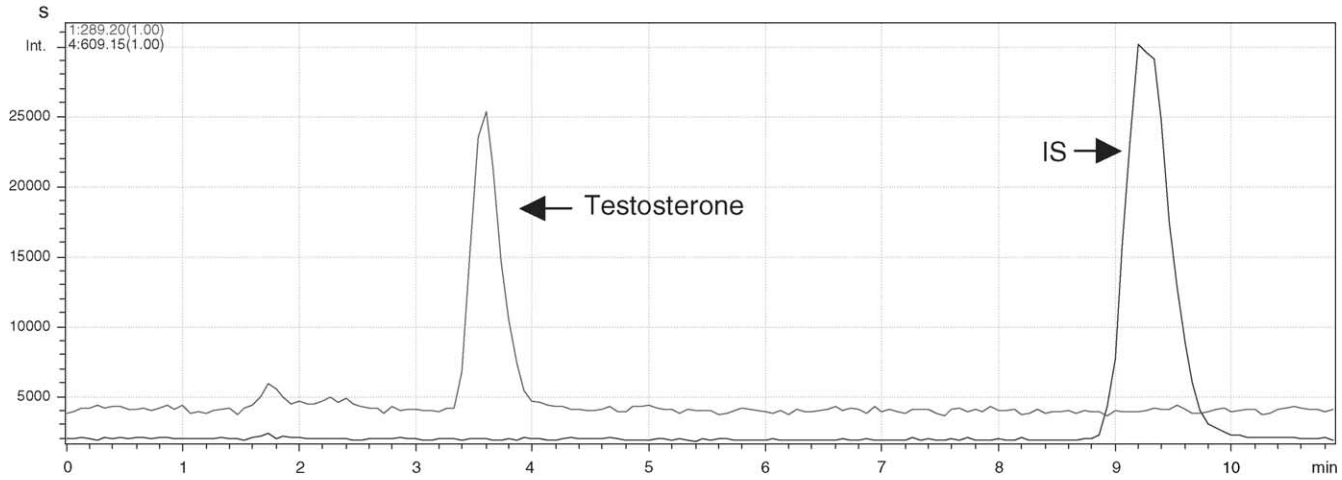

Fig. 4. Retention times of testosterone 3.59 min (20 ng) and the internal standard Fmoc-Gln 9.22 min (20 ng). HPLC conditions: 55% isocratic B, mobile phase A 0.5% acetic acid, mobile phase B 0.5% acetic acid 90% acetonitrile, 0.2 ml/min.

neous material) [13]; (3) samples were loaded onto the cartridges at 5 ml/min; (4) cartridges were then washed with 7.5 ml of Milli-Q water to remove salts and the eluant discarded; (5) elution of testosterone was carried out with 5 ml acetonitrile and dried under N_2; (6) reconstituted in 50 μl 60% acetonitrile (to prevent testosterone precipitation) for LC–MS analysis.

2.5. Validation

The method was validated for specificity, linearity, accuracy, precision, limits of quantification and detection, and stability. A calibration curve was calculated for testosterone in 60% acetonitrile for: 50, 20, 10, 5, 1 and 0.5 ng/ml, four curves were run on four separate days. The calibration curves were calculated with unweighted least-squares regression method [22]. Three different runs of six repeats on separate days were performed to evaluate intra- and inter-batch accuracy and precision, respectively, using 1, 20 and 50 ng/ml spiked saliva and blow. 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 300 Å C8 columns (Lot no.: 02110886-1 and 02110885-1). Recovery of the SPE extraction was determined by spiking samples with high (50 ng/ml) and low (1 ng/ml) concentrations of testosterone. Samples were separated into 2 × 200 μl aliquots, one 200 μl aliquot was used for the pre-extraction assay ($n = 6$). The other was extracted and reconstituted in 200 μl 60% acetonitrile, and used for the post-extraction assay ($n = 6$).

2.6. Stability

Biological samples of saliva and blow were spiked with 50 and 5 ng/ml testosterone standard and stored at 21, −20 and −80 °C. To determine if oral/lung bacteria interfered with

Table 1
Intra-batch and inter-batch precision and accuracy for testosterone in dolphin saliva and blow

Sample type	Concentration (ng/ml)	*n*	Mean (ng/ml)	Precision (R.S.D., %)	Accuracy (R.E., %)
Intra-batch					
Saliva	1	6	0.9	1.6	4.4
	20	6	20.0	5.3	0.1
	50	6	48.7	14.1	2.7
Blow	1	6	1.0	2.5	2.6
	20	6	20.0	5.3	−0.1
	50	6	49.4	10.9	−1.2
Inter-batch					
Saliva	1	18	1.0	4.5	0.2
	20	18	20.2	5.5	1.2
	50	18	49.5	12.0	1.0
Blow	1	18	1.0	4.9	0.8
	20	18	20.2	7.3	1.1
	50	18	49.7	9.8	0.7

Table 2
Freeze–thaw results of saliva and blow from spiked samples immediately assayed compared with freeze–thaw samples

Spiked testosterone concentration (ng/ml)	Saliva			Blow		
	F-statistic	d.f.	*p*	*F*-statistic	d.f.	*p*
1	15.4	3	0.001	12.1	3	<0.001
20	5.1	3	0.009	23.0*	3	<0.001
50	4.9	3	0.015	42.8	3	<0.001

All tests were ANOVA except non-parametric Kruskal–Wallis test was used (*).

testosterone stability in unextracted samples ($n = 6$ for each concentration at each temperature), were spiked with a broad-spectrum antibiotic, amoxycillin with potassium clavulanate (Augmentin®, SmithKline, Beecham, Australia) or $MnCl_2$ and their stability assessed.

A series of freeze–thaw experiments were conducted to determine testosterone degradation from multiple freeze–thaw cycles. Samples ($n = 6$) were spiked with 50, 20, or 1 ng/ml of testosterone and measured before and after freezing at −20 °C over three freeze–thaw cycles.

2.7. Statistical analysis

Analysis of variance (ANOVA) was used to determine any significant differences in the freeze–thaw and stability studies. If the data did not meet the assumptions of ANOVA, Mann–Whitney U or Kruskal–Wallis tests were used where appropriate. Pearson's *r* correlation and residual analysis were used to assess linearity of the calibration curves.

3. Results and discussion

3.1. Validation

Retention time for testosterone was 3.6 min and the internal standard was 9.2 min (Fig. 4). Retention times for column

Table 3
Testosterone stability in saliva and blow samples

Storage temperature (°C)	Hormone concentration (ng/ml)	Inhibitor	Storage time	Statistical test, statistic	d.f.	*p*
Saliva						
21	50	No inhibitor	2 h	ANOVA $F = 2.7$	2	NS
21	50	No inhibitor	3 h	ANOVA $F = 16.6$	3	<0.001
21	5	No inhibitor	1 h	Mann–Whitney U: $Z = 2.3$		0.021
21	50	$MnCl_2$	15 h	ANOVA $F = 2.2$	4	NS
21	50	$MnCl_2$	18 h	ANOVA $F = 7.8$	5	<0.001
21	5	$MnCl_2$	15 h	K–W: chi-square = 2.6	4	NS
21	50	Augmentin®	15 h	ANOVA $F = 1.2$	4	NS
21	50	Augmentin®	18 h	ANOVA $F = 6.3$	5	0.005
21	5	Augmentin®	6 h	ANOVA $F = 0.2$	3	NS
21	5	Augmentin®	12 h	ANOVA $F = 7.6$	3	0.004
−20	50	$MnCl_2$	3 weeks	ANOVA $F = 2.6$	3	NS
−20	50	$MnCl_2$	4 weeks	ANOVA $F = 3.1$	4	0.025
−20	5	$MnCl_2$	1 week	Mann–Whitney U: $Z = 3.5$		<0.001
−80	50	$MnCl_2$	8 weeks	ANOVA $F = 3.1$	2	NS
−80	50	$MnCl_2$	12 weeks	ANOVA $F = 138.3$	3	<0.001
−80	5	$MnCl_2$	4 weeks	Mann–Whitney U: $Z = -3.4$		<0.001
Blow						
21	50	No inhibitor	1 h	Mann–Whitney U: $Z = -2.6$		0.009
21	5	No inhibitor	3 h	ANOVA $F = 0.9$	3	NS
21	5	No inhibitor	4 h	ANOVA $F = 5.5$	4	0.003
21	50	$MnCl_2$	15 h	ANOVA $F = 2.2$	4	NS
21	50	$MnCl_2$	18 h	ANOVA $F = 4.2$	5	0.007
21	5	$MnCl_2$	9 h	ANOVA $F = 2.7$	3	0.08
21	5	$MnCl_2$	13 h	ANOVA $F = 13.5$	4	<0.001
21	50	Augmentin®	15 h	ANOVA $F = 1.2$	4	NS
21	50	Augmentin®	18 h	ANOVA $F = 6.3$	5	<0.001
21	5	Augmentin®	9 h	K–W: chi-square = 4.0	3	NS
21	5	Augmentin®	13 h	ANOVA $F = 86.8$	4	<0.001
−20	50	$MnCl_2$	1 week	Mann–Whitney U: $Z = 1.4$		NS
−20	50	$MnCl_2$	2 weeks	K–W: chi-square = 9.3	2	0.009
−20	5	$MnCl_2$	1 week	Mann–Whitney U: $Z = 2.9$		0.004
−80	50	$MnCl_2$	8 weeks	ANOVA $F = 2.4$	4	NS
−80	5	$MnCl_2$	4 weeks	ANOVA $F = 0.3$	2	NS
−80	5	$MnCl_2$	6 weeks	ANOVA $F = 5.5$	3	0.007

A (Lot no. 02110886-1) and B (Lot no. 02110885-1) were 3.62 min and 3.71 min, respectively. The RSD between the results of the two columns was 8.4% ($n = 12$) at 5 ng/ml and 10.1% ($n = 12$) at 50 ng/ml. The equation for the calibration curve for testosterone (0.5–50 ng/ml) was $y = 0.01x + 0.0045$ and was linear, ($r^2 = 0.959$, $r = 0.979$, $p < 0.001$). The residuals were normally distributed and centred around zero. The LOD (S/N 3:1, ±5% retention time) was 0.2 ng/ml, and the LOQ (S/N 5:1, ±5% retention time) was 0.5 ng/ml. Table 1 shows intra-batch and inter-batch precision and accuracy data. Precision was determined by calculating the relative standard deviation (R.S.D., %) and accuracy by calculating the difference between the nominal and spiked values for the spiked saliva and blow samples (R.E., %) [18].

Recovery of the SPE for saliva at 50 ng/ml testosterone was 93 ± 7.9% and at 1 ng/ml was 91.5 ± 3.72%. Recovery of 50 ng/ml of testosterone in blow was 83.3 ± 6.8% and for 1 ng/ml, 85.8 ± 4.6%. Recovery of the internal standard in saliva was 73.0 ± 14.2% and in blow was 78.63 ± 4.29%. As the metabolic pathways of testosterone in dolphin saliva and blow are unknown, Fmoc-glutamine was selected as the internal standard to prevent potential interferences. 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.

In the saliva and blow freeze–thaw experiments, there were significant differences in the testosterone concentrations between spiked samples undergoing immediate analysis and those that had been frozen at −20 °C (Table 2). There were no significant differences in concentrations between saliva or blow samples that had been frozen and thawed (1–3 cycles).

Table 3 shows the stability of testosterone in dolphin saliva and blow samples for the different stability experiments at 21, −20 and −80 °C. Saliva and blow samples with no inhibitor ($MnCl_2$ or amoxycillin/potassium clavulanate) added were not stable at 21 °C as the testosterone concentration increased significantly after 3 h (Table 3, Fig. 5). Stability of testosterone in saliva and blow improved with the addition of an inhibitor, such as amoxycillin/potassium clavulanate or 100 mM $MnCl_2$ (Table 3, Fig. 6). $MnCl_2$ was determined to be the superior stabiliser for testosterone in both saliva and blow at 21 °C (Fig. 6) as it stabilised both high and low concentrations of testosterone for 12 h. It was for this reason that $MnCl_2$ was used as the inhibitor for the −20 and −80 °C stability experiments. In summary, biological samples with $MnCl_2$ added (but prior to SPE) should be analysed within 12 h if stored at 21 °C; within 1 week at −20 °C; and within 4 weeks at −80 °C (Table 3). Saliva and blow samples in dry extract from the SPE were stable for 4 months at −80 °C.

Review of the literature and results of this study, indicate a number of issues regarding the stability of testosterone in dolphin samples. Previous work, using RIA has shown that testosterone is stable in human saliva [10,23,24]. However,

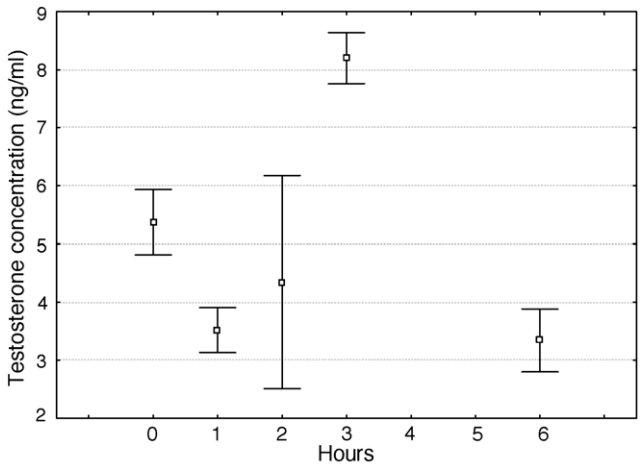

Fig. 5. Stability over 6 h of saliva spiked with testosterone (5 ng/ml injection) with no inhibitors added (mean ± S.D.).

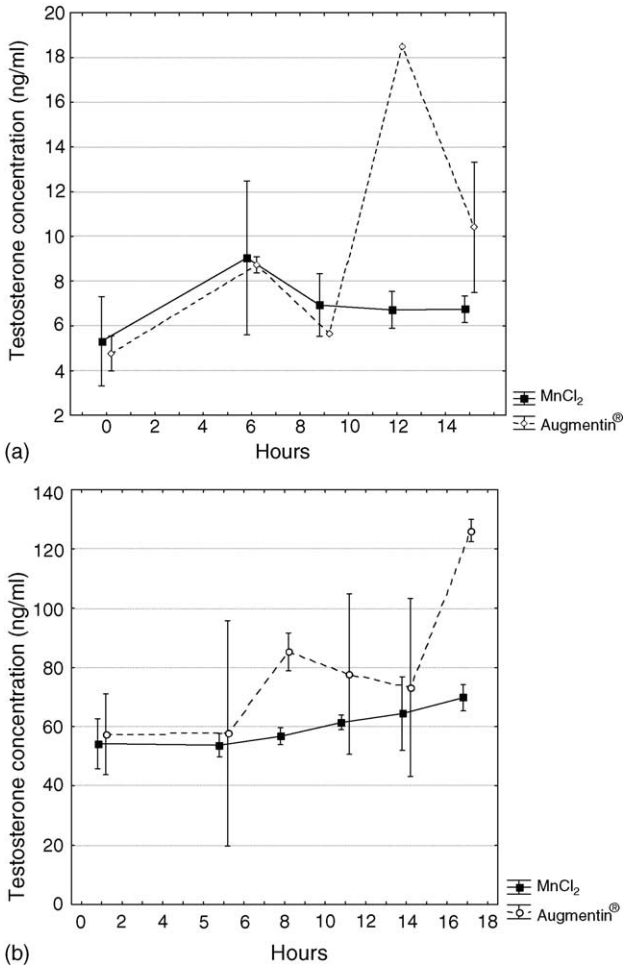

Fig. 6. (a) Stability of saliva spiked with testosterone (5 ng/ml) at 21 °C with amoxycillin/potassium clavulanate (Augmentin®) and $MnCl_2$ over 18 h (mean ± S.D.). (b) Stability of blow spiked with testosterone (50 ng/ml) at 21 °C with amoxycillin/potassium clavulanate (Augmentin®) and $MnCl_2$ over 18 h (mean ± S.D.).

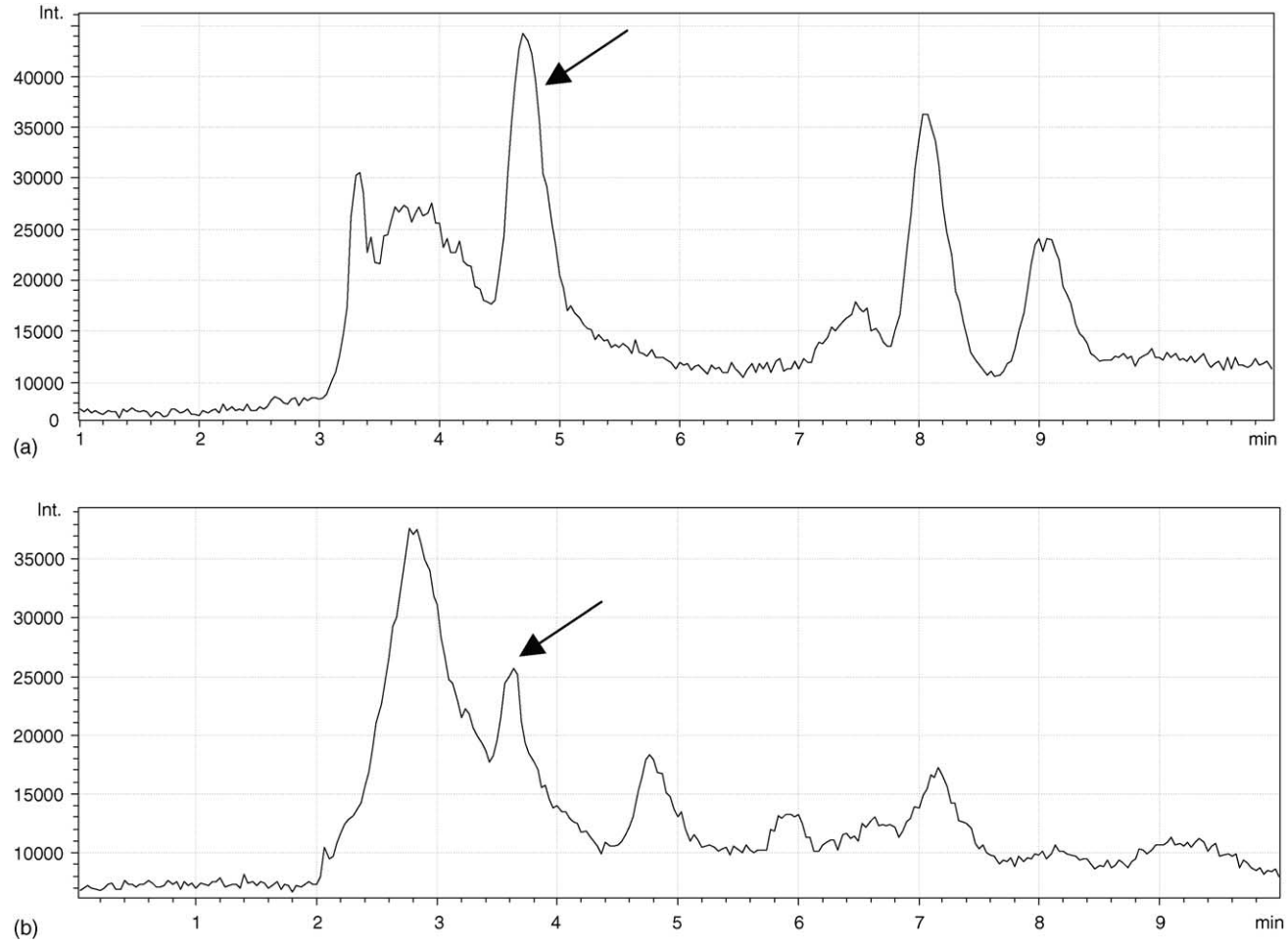

Fig. 7. (a) MS Chromatogram of endogenous testosterone (8.9 ng/ml) in dolphin (Sirius) saliva (arrowed), (*m*/*z* 289.20, RT = 3.6 min). (b) MS chromatogram of endogenous testosterone (4.9 ng/ml) in dolphin (Sirius) blow (arrowed).

the stability of testosterone in dolphin saliva in this study showed it fluctuates (increases then decreases) within the first 6 h of the sample being spiked (Fig. 5). Saliva and blow samples from dolphins require the addition of a suitable pathway inhibitor. Sodium azide has been used previously to stabilise testosterone in human saliva [24], however it is a toxic substance especially for marine wildlife, and hence, it was not considered in this study. $MnCl_2$ specifically inhibits the production of testosterone in the Leydig cells of a rat [25]. $MnCl_2$, compared with amoxicillin/potassium clavulanate, was the superior inhibitor (Fig. 6).

3.2. Dolphin samples (endogenous testosterone concentrations)

Testosterone could be measured by LC–MS in all male saliva samples (Fig. 7a) ranging from 9.7 to 23 ng/ml ($n = 10$) (Table 4). The two adult dolphins had a mean testosterone concentration of 15.2 ± 5.7 ng/ml ($n = 5$) and the two sub-adult dolphins had a mean testosterone concentration of 13.5 ± 3.5 ng/ml ($n = 5$). Testosterone in blow samples (Fig. 7b) ranged from 14.7 to 86.2 ng/ml ($n = 11$) (Table 4). The two adult dolphins had a mean testosterone concentration of 42.5 ± 21.8 ng/ml ($n = 6$) and the two sub-adult dolphins had a mean testosterone concentration of 59.9 ± 23.9 ng/ml ($n = 5$) in the blow.

Although sample sizes for this study were small, expected trends were observed in saliva where the two adult male dolphins had higher concentrations of testosterone than the two sub-adult males. The variability of both adult and sub-adult samples may be explained due to age. One of the adult males (Delbert) had lower concentrations of testosterone in both saliva and blow samples. Delbert at 36 years old may have fluctuating testosterone concentrations, as male and female bottlenose dolphins are known to have slowing reproductive rates from mid-thirties onwards [26]. One of the sub-adult males (Zac) had lower testosterone concentrations due to the presence of a contraceptive implant. Testosterone blow concentrations were higher than saliva. As blow sampling has not been trialled previously a comprehensive and longitudinal study of this species using the method described will provide a greater understanding of the fluctuations in testos-

Table 4
Endogenous testosterone concentrations of male bottlenose dolphins

	Saliva testosterone (ng/ml)	Blow testosterone (ng/ml)
Adults		
Sirius	19.0	48.2
	23.0	59.9
	Not obtained	30.5
Delbert	9.9	14.7
	14.4	73.1
	9.9	28.6
Mean ± S.D.	15.2 ± 5.7	42.5 ± 21.8
Sub-adults		
Buck	17.2	86.2
	16.2	Not obtained
	14.4	84.7
Zac	9.9	48.1
	Not obtained	34.1
	9.7	46.7
Mean ± S.D.	13.5 ± 3.5	59.9 ± 23.9

terone concentrations in bottlenose dolphins. The measurement of hormone concentrations in captive dolphins using a non-invasive and easily trainable technique provides an outstanding opportunity to further understanding of the physiology of wild marine mammals. Saliva and blow samples allow for multiple daily sampling, and provide no observable stress to the animals, allowing for diurnal and seasonal data measurements.

Studies have shown [23,27] that biological samples collected with cotton gauze or cotton salivettes have elevated concentrations of reproductive hormones when using immunoassays. To observe if this was also the case with LC–MS, a series of direct infusions with the gauze were conducted to identify possible interfering *m*/*z* values. Direct infusions were conducted in scanning mode, scan range *m*/*z* 100–500. There were no *m*/*z* ratios in the cotton gauze that interfered with testosterone identification. 'Cotton-based' sampling materials, such as gauze, which can alter the results of hormonal RIAs [23,27] does not appear to affect 'male' hormonal results with LC–MS.

4. Conclusion

An accurate, reproducible and rapid LC–MS method has been developed to determine testosterone concentrations in obtainable biological matrices (saliva and blow) of bottlenose dolphins. A major advantage of LC–MS over RIA is multiple analyte measurement from the same sample particularly when blow sample volumes are low (typically <50 μl per animal). Work is currently in progress for determining female sex hormones in dolphins using LC–MS with application for other marine mammals.

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