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Rapid noninvasive measurement of hormones in transdermal exudate and saliva

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Abstract

Experiments were conducted in both sheep and humans to evaluate techniques for rapid sampling and measurement of testosterone, insulin, 17-β estradiol, cortisol and glucose collected in saliva or transdermal exudate. Ultrasound and an electric current facilitated the latter collection. All but insulin were successfully measured in saliva, under resting conditions, and the measured hormones correlated best with blood levels 20–40 min prior to the saliva collection. With imposition of, and recovery from, an exercise stress, this correlation was weakened irrespective of considering the time lag between blood measures during this period and subsequent changes in saliva values. Provided an initial transdermal flux was established, all the hormones and glucose were successfully measured in the transdermal exudate at levels correlating with blood measures at the time of collection, and this held across stressor application and recovery. The transdermal exudate sampling and measurement apparatus is relatively portable, enabling noninvasive collection and analyte measurement, rapidly, at the site where the experiment is being conducted with minimal interference to subjects. This potentially offers a tool of considerable value to endocrine studies.

Keywords: Hormones; Noninvasive measurement; Saliva; Transdermal exudate

1. Introduction

Sampling blood-related analytes painlessly has received considerable research effort [16,19] for some time, reflecting the potential usefulness of noninvasively obtained measures for clinical and laboratory use.

Saliva shows a good correlation to blood for some analytes, including hormones, and is easily collected from humans and from ruminant livestock [3,8,12–14]. However, there is a time lag between changes in the composition of the saliva and events in the blood, and partitioning between the two body fluid compartments is often not simple, making correlation difficult and at times not predictable. Although saliva samples are relatively low in needed compliance, they are still not easy to collect in mid- to large-size animals without considerable restraint. In small animals, collecting sufficient saliva volume is an issue, and in all sized animals, intensive sample collection over relatively short periods can be difficult.

Transdermal exudate, sampling analytes that have 'migrated' from blood vessels to skin surface, has shown promise in the collection of a number of analytes particularly if enhanced by ultrasound [16,18,19]. Blood glucose levels have been successfully tracked using this sonophoresis technique [16]. This method appears best with hydrophilic molecules, from published literature. Patent literature [17], however, suggests that combining an electric field with ultrasound (electrosonophoresis) may further improve the movement of analytes from blood to skin surface, particularly those that are hydrophobic or complex in carrier charge. These methods, however, are often time consuming in sample methodology, and vacuum extraction [16] from the skin surface appears needed to concentrate the analyte for measurement, making the method a little cumbersome for noncompliant subjects or nonhuman ambulatory animals. Concentration of analyte is also needed for saliva measurement of some substances, and indeed a traditional problem in all noninvasive measures relative to blood analytes is obtaining sufficient quantities of the analyte in a short time span, or small volume, of sampling.

Irrespective of collection technique, most sample obtained is analysed off-line following purification. Having a

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measure in relative real time during the course of experimentation is extremely useful. For animal and human experimentation, mindful of welfare and experimental ease, a painless and low-invasive method of sampling combined with the ability to measure changes relative to blood-borne levels would be an excellent addition to currently available experimental tools.

Implantable immunosensors have been used successfully in vivo to measure, in real time, hormonal changes in brain tissue and circulation of conscious behaving animals [4–6]. These immunosensors are enclosed within a dialysate membrane, which affords a preliminary sample separation, and use osmotic equilibration principles to maximise sample collection [1] within a very small volume of fluid. Within the immunosensor itself, when an antibody detection of analyte is used, there is a large antibody surface area available relative to the volume of circulating analyte, with the antibody binding and detection performed on the measuring electrode. This arrangement facilitates capture and measurement [11] at low concentrations (fg/ml). This consequently allows collection over very short sample times. The immunosensor itself is very small allowing it to be positioned in a relatively nonobtrusive fashion. Combining such technology with noninvasive sampling may allow an on-line monitoring, noninvasive, device to be constructed.

The purpose of this study was to combine two techniques of noninvasive sampling: transdermal exudate facilitated by electrosonophoresis and saliva collection by bulb suction, with rapid measurement using appropriate immunosensors and compare their use. In the case of the electrosonophoresis, the sampling and measurement components were constructed as one hand-held device. For saliva, the collected volume was added to the measurement part of this device. Testosterone, cortisol, estradiol and insulin were chosen as the analytes because of their broad endocrinological roles and interest. The hormones were followed across an exercise stress as this changes their levels in circulation in a short period. Glucose was chosen as a hydrophilic marker, again of broad relevance.

2. Methods

2.1. Subjects and stressors

Twenty sheep (Romney cross ewes, 36–44 kg live weights) were farmed together as a flock for 14 days prior to experimentation. On each of these days, they were rounded up, penned, a patch on their backs (approximately 5 cm in diameter) shaved and a mixture of sodium lauryl sulfate (40%) and either a commercial ultrasound conductive gel (Aquasonic, Parker Lab., NJ 07004, USA) or a 10% ethanol gel (the two gels were alternated in use) rubbed onto this patch. Two minutes later the electrosonophoresis device was placed briefly on this patch (not turned on). At the end of 1 h of penning saliva was collected using a plastic suction bulb positioned manually into the mouth of the sheep. In this manner the sheep were familiarized to the experimental procedure. Collected saliva was analysed for cortisol concentration as previously described [3,8].

Ten of these animals were used to develop the transdermal exudate collection technique. These 10 animals were penned as above, and a catheter inserted into the jugular vein as has been described [4]. A mixture of sodium lauryl sulfate and either the ultrasound gel (SLS–UG) or the 10% ethanol gel (SLS–EG) was positioned onto the shaved area on the back of each animal. The gel remained on this area for 1 h; replenished occasionally (the ethanol containing mix tended to dry out over a 15–20-min time span), during which time three blood samples were also taken. At the end of the hour, the remaining mix on the back was collected. The entire procedure was repeated three times. This mix was subject to assay (see below) for hormones and glucose content.

On the following day, one of the two gel mixes was rubbed on and, after 10 min, ultrasound (20 kHz, 10 W/cm^2, pulsed 5 s on/5 s off using an ITO Physiotherapy and rehabilitation unit, Tokyo 176-8605, Japan) applied for 1 min directly onto the mix. Ten minutes later, the mix was again collected and measured. This was then repeated with 2 min of the ultrasound application, and on a following day, the entire procedure repeated using the alternative gel mix on the shaved patch on the animal. Times greater than 2 min were not attempted due to potential risk of skin damage. Blood samples were collected at time of ultrasound application and again at time when the gel mix was also collected. The entire ultrasound procedure was then repeated on subsequent days with the addition of an application of a 9-V electric field either side of the ultrasound head (in contact with skin).

On a separate day, in a further repeat of the above procedure, the collecting head (see below for description) was positioned onto the ultrasound device. The ultrasound device was modified to ensure good coupling for use with the collecting head and delivery of needed parameters to the skin. The output of the device was reset to achieve the same energy and pulse delivery to the skin's surface as when the collecting head was not present, and the ultrasound device had been placed directly in contact with the gels (as described above). Three fluid flow rates into the collection chamber of the head were compared: 150, 300 and 600 μl/min. The effluent fluid was then assayed. On a final day, this was repeated with the immunosensors positioned in the exit line from the chamber, with immunosensor measurement of the hormones made as has been described [4–7].

From these experiments, parameters for transdermal collection were set (see below). These were used in the subjects undergoing the exercise stress, both human and sheep.

In the remaining 10 sheep, experimentation took place on three separate days, each 3 days apart. On days of experimentation, animals were penned as above and the jugular vein of the animal was catheterized. Animals were left to

rest for 50 min and then a single ultrasound application applied for 1 min as above. This initial ultrasound application was necessary to start the transdermal flux (data not shown). Provided the application was given 10 min prior to the start of further experimentation, it allowed subsequent transdermal collections to be made concurrently with blood sampling (i.e. no time lag between the two samples). A series of electrosonophoresis collections were made concurrently with blood collection from the jugular vein, every 5 min for a further 1 h. Saliva samples were collected on every second blood sample (i.e. every 10 min). At completion of this time, a human shepherd, intermittently for 30 min, then ran the animals around a paddock. During this 30 min, each animal was briefly restrained manually and sampled on all three parameters every 10 min (i.e. three samples repeated three times). At the end of this period, animals were repenned and rested for 10 min, and a further saliva collection was made. Following this saliva collection for a further 1-h period, collection as above, prior to exercise, was repeated. In this manner for each animal, in each session, 27 blood and transdermal samples and 16 saliva samples were collected.

Ten human volunteers (males aged 19–23, body weights 85–103 kg) were seated at 09:30 h. During the experiment, they were held at a room temperature of 20–22 °C. An initial 1 min burst of ultrasound, as for the sheep, was made on the volar surface of the forearm, following application of the SLS–EG mix. Ten minutes later, transdermal (electrosonophoresis) collection began from this site on this forearm, simultaneous to a venepuncture blood collection being made from the other. A saliva collection was then made, followed by a second, third and fourth saliva collection 20, 30 and 40 min after the blood sample. These measurements were repeated three times over 3 h (i.e. a total of three blood and transdermal samples and 12 saliva samples). In between each sampling series, subjects were allowed to walk around, eat lightly and drink water. Following this collection, the five volunteers exercised at moderate intensities, for 40 min (heart rates maintained in the range of 130–160 bpm) on a rowing machine, stopping briefly at 10, 20 and 30 min to give blood, transdermal and saliva samples. The subjects gave another set of samples at 40 min upon completion of rowing, then warmed down lightly with stretching exercises for a further 10 min and gave a further saliva sample at the end of this time. Two more saliva samples were collected at 60 and 70 min after exercise started. At the end of this time, sampling was repeated as above, prior to exercise, for a further 3 h. The entire procedure was repeated twice a week for 3 weeks (a total of six times). The distance rowed (*m*) represented the total work rate during the exercise.

In a separate series of experiments on these human subjects, the transdermal and blood samplings were repeated as for the nonexercising period, however, on each transdermal sample, a different site on the volar forearm was used, ranging from wrist to elbow level. This was then repeated with the room temperature increased to 28–30 °C.

2.2. Sample analysis

Blood samples were centrifuged, separated and stored at −20 °C until assay, as previously described [3,4,8]. Insulin, 17-β estradiol, testosterone and cortisol concentrations, both free and total in serum, were assayed by ELISA or RIA (Elisa Kits, DRG Instruments, D-35039 Marburg, Germany, RIA constituents, Sigma, St. Louis, MO 63178, USA) and glucose by a spectrophotometer kit (Sigma). Collected SLS–UG and SLS–EG were assayed as for blood. Saliva was divided into two samples. The first sample was stored at −20 °C and assayed as for blood. The second sample was assayed at the time of experimentation using the apparatus and immunosensors described below.

2.3. Measurement head for transdermal exudate and saliva

A solid block of acetal polyformaldehyde plastic was hollowed out towards one end where 0.5 cm was left solid. The hollowed out end was designed to fit tightly over an adapted commercial hand-held ultrasound device (ITO). At the solid end of this head, a small chamber (300 μl volume) was constructed and the end piece then further hollowed out another 0.2 cm around this chamber, leaving only a minimum layer of solid support. The chamber had an entry and exit port feed by polyethylene tubing allowing for a constant perfusion flow. An adapted semipermeable dialysis membrane (Spectra/Por, Spectrum Lab., CA 90220-6435, USA), which was sealed down making the chamber watertight by a rubber O ring, covered the chamber. This end of the head made contact with the skin during sampling. On either side of the head were electrodes providing a 9-V driven electric field. The other end of the sample head fitted tightly onto the modified ultrasound device. Four immunosensors were lined up in series at the exit point of the sampling head chamber. For saliva sample analysis, this head was not connected to the ultrasound device, and saliva was fed straight into the collection chamber to flow through to the immunosensors. Fig. 1 depicts the head and ultrasound device.

2.4. Operation of the transdermal collection

A small amount of the SLS–EG was rubbed into the skin area of collection 10 min prior to the procedure, and an initial application of ultrasound for 1 min was given to start the transdermal flux. Applying standard ultrasound gel to the end of the adapted commercial ultrasound gun before positioning the collecting head increased ultrasound coupling to the collecting head. The chamber in the head was filled with 10% ethanol and the head then positioned against the skin surface of the subject and 1 min of ultrasound (output 20 kHz, 10 W/cm^2 calculated at skin surface, pulsed 5 s on/5 s off) applied. At the completion of 1 min, fluid was allowed to flow through the chamber at a rate of 300 μl/min for a further min while the device remained in contact with the skin. During the full 2 min

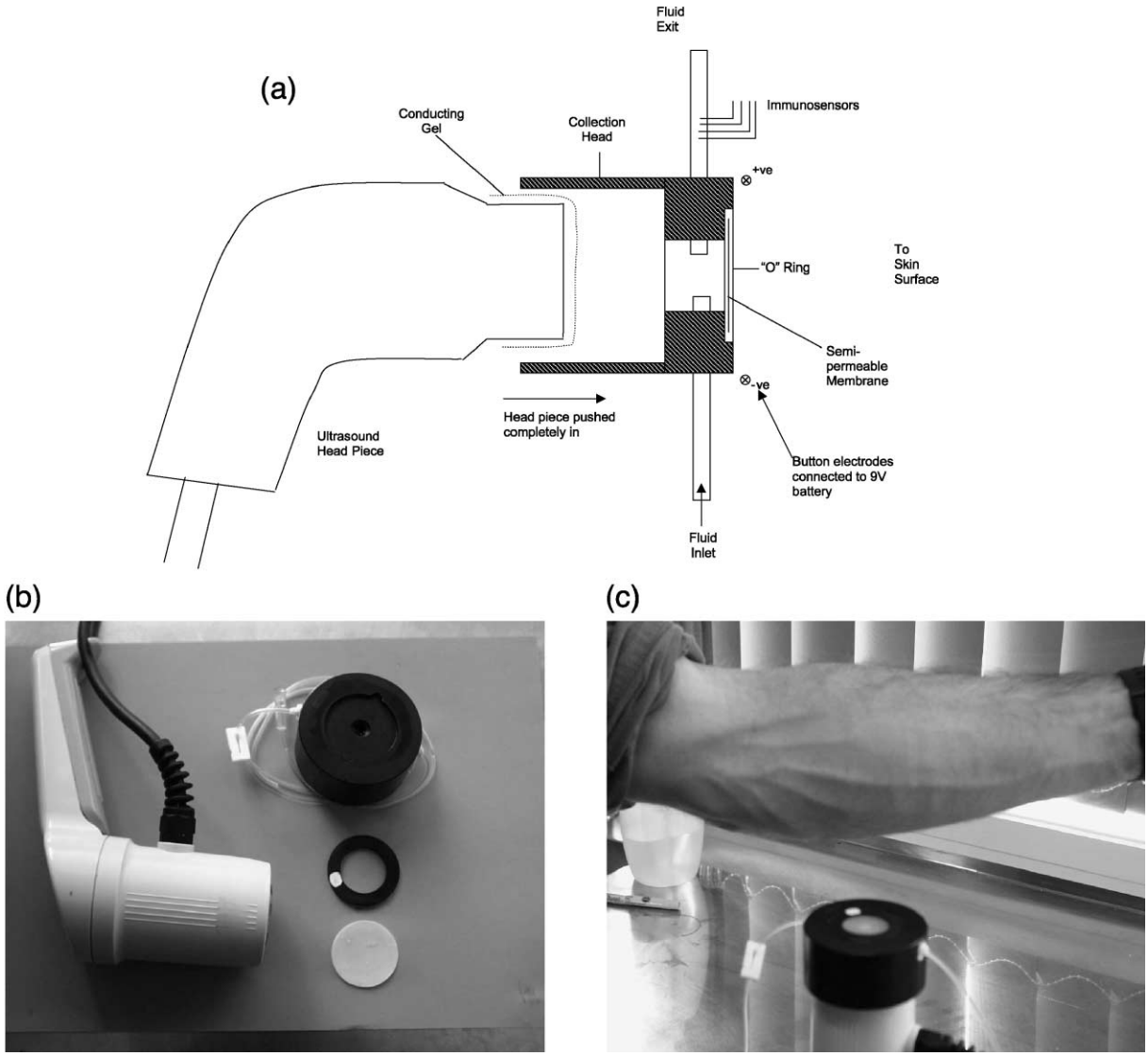

Fig. 1. Depicts the transdermal collection device: (a) a schematic of the device; (b) a picture of the adapted ultrasound delivery device, the collecting chamber, membrane and O ring; and (c) a picture of the device in use on human subjects.

contact with the skin, a 9-V electric field was passed across the head, at the skin surface.

Immunosensor measurement took a further 1 min, and then both the chamber and immunosensors were flushed taking another 1 min. Theoretically, repeat measures could be obtained every 4 min using this cycle. The semi-permeable membrane was, however, changed manually if used on separate subjects to avoid any crosscontamination. This added time to the actual cycle. Subjects were staggered in timing to allow measurements on a 5-min basis when necessary and to maintain a constancy of time across the experiment.

2.5. Immunosensor measurement

Immunosensors were designed as described previously [4–6]. With this technique, antibodies to the analyte of interest (for example cortisol) are fixed within a dialysate probe, or in the effluent of the probe. The detection system is based upon a competitive reaction of endogenous and peroxide (HRP) labeled exogenous analyte with specific, to the analyte, antiserum immobilized on the platinum electrode housed within the dialysis membrane. After binding, a peroxidase reaction is initiated and substrate oxidation is monitored by the change in current between the electrodes in the probe, which have been set to a voltage sufficient to ensure oxidation. The bound HRP is activated by aminosalicylic acid, and the resultant electrical output of this activation is monitored. This output is proportional to the bound HRP and is therefore inversely proportional to the bound analyte of interest. The platinum electrode acts simultaneously as immunoreagent and electrochemical detector, a combination producing increased assay sensitivity [11]. Surrounding the

electrodes with a dialysis membrane, adapted for use, allows continuous measurement in vivo without the need for sample withdrawal. Active constituents diffuse across the dialysis membrane in the direction of osmotic equilibration drive [1].

Briefly, each immunosensor consisted of an adapted outer dialysis membrane (100 K MW cutoff), 4 mm in length, used to enclose a central platinum electrode (working electrode) and two further electrodes situated further up the probe, a reference (Ag/AgCl) and counter (Ag) electrode. Polyclonal antibodies for cortisol, testosterone, 17-β estradiol and insulin (Sigma) were absorbed onto the surface of the platinum electrode by cathodic copolymerisation, as previously described, of four different probes, respectively. In vitro and pilot in vivo studies (data not shown) suggested antibody dilutions of 1:20 cortisol, 1:20 testosterone, 1:10 17-β estradiol and 1:10 insulin for human subjects, and 1:20, 1:15, 1:5, 1:10, respectively, for sheep gave the appropriate sensitivities over the range of measurement.

The electrode configuration was set at 750 mV versus the Ag/AgCl reference and probes were perfused at 2 μl/min 10% ethanol using a syringe pump as previously des-cribed [4–8]. For humans and sheep, calibration was performed over the ranges of 0.1–100 ng/ml cortisol, for testosterone 25–1000 pg/ml, for 17-β estradiol 0.1–20 pg/ml and for insulin 0.1–5 ng/ml for sheep and 0.1–25 μU/ml for humans. Immunosensors were also calibrated for interference, crossreactivity, changes with pH and oxygen content and washout of measured analytes as described.

Immunosensors were then positioned into the exit line of the collection head in series, and fluid containing varying concentrations of testosterone, 17-β estradiol, insulin and cortisol pumped through the collecting head to ensure probe calibration was maintained in vivo. This was alternated with effluent collection, while immunosensors were off, which was analysed off-line as above. In this manner, immunosensors were calibrated before and after each day of experimentation.

Glucose was not measured by immunosensor but was instead monitored in the effluent fluid as described above.

2.6. Statistical analysis

Student *t* tests, ANOVA or Wilcoxon test for paired samples were made as appropriate. Statistical significance was taken at $P \leq .05$ level. For analysis across the exercise stress, data were grouped into pre, during and post exercise values as actual sample times relative to the exercise for transdermal and blood obtained values. For pre, during and post exercise grouping of saliva samples, a 20-min lag was allowed relative to blood and transdermal values in sheep and a 30-min lag in human subjects.

3. Results

3.1. Immunosensor performance

The combination of the adapted semipermeable membranes showed in vitro recoveries between 23% and 35% for the hormones. Washout curves gave no evidence of any of the hormones being retained on the dialysis membranes when the working electrode and electrosonophoresis had been active. There was a slight retention of hormones (between 1% and 7% of total recovery) when immunosensors/electrosonophoresis were not active. However, this retention when it occurred was constant across measurements and did not affect the linearity of response.

The immunosensor showed linear responses to the ranges of hormones outlined in the Methods. In the immunosensors designed for the sheep, sensitivities of 1–2 nA/ng/ml cortisol, 1–2 nA/pg/ml testosterone, 1–2 nA/10 pg/ml insulin and 1–2 nA/0.1 pg/ml 17-β estradiol were seen. Limits of detection were 0.5 ng/ml, 1 pg/ml, 0.1 pg/ml and 0.1 pg/ml, respectively. Greater than 90% of response was seen in less than 30 s (27.3 ± 4.9, mean ± S.E.M.) for the hormones as a group. A pH below 6.0 decreased sensitivity but did not affect linear responses. Changing oxygen levels had no effect, and increasing K^+ increased the background slightly (approximately 0.2 nA for each additional 100 mmol/l K^+). Ascorbate had no effect as the immunosensors were coated with phenylenediamine (excludes electrointerference from ascorbate).

Immunosensors designed for the human subject showed similar performance characteristics, however, sensitivities were 1–2 nA/0.1 ng/ml cortisol, 1–2 nA/10 pg/ml testosterone, 1–2 nA/0.5 μU/ml insulin and 1–2 nA/pg/ml 17-β estradiol.

Immunosensors showed stability at room temperature for >48 h or at least 800 measurements.

3.2. Transdermal collection

In the mix of SLS–UG, or SLS–EG, alone (without electrosonophoresis), only glucose was measurable between 0.05% and 0.09% blood serum levels. The addition of ultrasound application increased the glucose recovery considerably (to approximately 4%), and both cortisol and testosterone were reliably detectable at 5% and 2% serum levels. Insulin and 17-β estradiol were detected (2% and 1%, respectively, of serum levels) but were not always reproducible at this level in each animal. The three hormones, when detected, were in higher concentrations in the SLS–EG mix than in the SLS–UG mix, although the mix had no effect upon detectable glucose. In each case, a short burst of ultrasound (1 min) was needed to start the transdermal flux, and a delay of 10 min seen before full concentration was achieved. Once the flux was established, there was no lag in time between blood and transdermal samples within the measurement cycle of

Table 1
Concentrations of hormones and glucose in transdermal exudate collected by different formats in sheep

	Glucose		Testosterone		Estradiol		Insulin		Cortisol	
	Serum, mg/l	Exudate, mg/l	Serum, ng/ml	Exudate, ng/ml	Serum, pg/ml	Exudate, pg/ml	Serum, ng/ml	Exudate, ng/ml	Serum, ng/ml	Exudate, ng/ml
SLS + UG	844.6 ± 81.2	0.61 ± 0.11 (0.07%)	1.1 ± 0.6	ND	2.3 ± 1.9	ND	0.2 ± 0.6	ND	10.9 ± 1.6	ND
SLS + 10% E	751.4 ± 110.9	0.59 ± 0.87 (0.08%)	0.9 ± 0.4	ND	1.4 ± 1.6	ND	0.18 ± 0.09	ND	14.3 ± 2.8	ND
SLS + 10% E + US 1 min	910.8 ± 120.4	36.4 ± 5.6 (4%)	0.9 ± 0.3	0.02 ± 0.005 (2%)	1.9 ± 1.1	0.05 ± 0.04 (2%)	0.4 ± 0.15	0.004 ± 0.002 (1%)	11.7 ± 2.4	0.55 ± 0.13 (5%)
SLS + 10% E + US 1 min + 9V	808.5 ± 80.9	59.7 ± 10.4 (7%)	0.7 ± 0.6	0.05 ± 0.02 (7%)	2.0 ± 0.8	0.09 ± 0.05 (4%)	0.21 ± 0.08	0.0064 ± 0.0041 (3%)	12.5 ± 2.8	1.1 ± 1.0 (9%)
SLS + 10% E + US 1 min + 9V + Collection Head	890.3 ± 99.7	80.1 ± 11.5 (9%)	0.8 ± 0.6	0.11 ± 0.06 (14%)	1.3 ± 1.0	0.11 ± 0.05 (8%)	0.19 ± 0.11	0.013 ± 0.006 (7%)	13.1 ± 2.9	1.9 ± 0.93 (14%)

SLS: sodium lauryl sulfate, UG: ultrasound gel, E: ethanol, US: ultrasound, 9V: 9-V electric field, ND: not detectable.
Means ± S.E. are displayed.
(%) Relative recovery rounded to the nearest whole number, except for glucose exudate collected without ultrasound.

4 min, however, application of ultrasound was needed at least every 30 min (data not shown) to maintain this. In practice, ultrasound was applied on each measurement.

The addition of an electric field to the ultrasound improved recovery of glucose concentration slightly (7%) and greatly increased the concentration measured of all four hormones (Table 1), allowing reproducible detection. This was further enhanced by the use of the collecting head containing the adapted membrane (Table 1).

Immunosensors on-line with the collection head gave hormonal measurements that are strongly correlated ($r^2 \geq .94$) with the off-line analysis of effluent. However, the immunosensors were able to detect the hormonal concentrations in a smaller volume of sample fluid than when using off-line analysis (150 μl perfusion of chamber compared to 300 μl needed for effluent detection). Correspondingly, this meant that a smaller time span of each sample collection could be used (1.5 min versus 2 min). For the course of the experiment, however, to ensure maximum recovery, 300 μl (2 min) was used.

3.3. Application of stressors

3.3.1. Sheep

During the familiarization period, salivary cortisol values fell from 6.4 ± 1.2 ng/ml on the first day to 1.1 ± 0.9 ng/ml on Day 14. By Day 14, animals showed no subjective aversion to penning and handling.

On experimental days, the animals did, however, show some mild flinching behaviour with the jugular catheter-

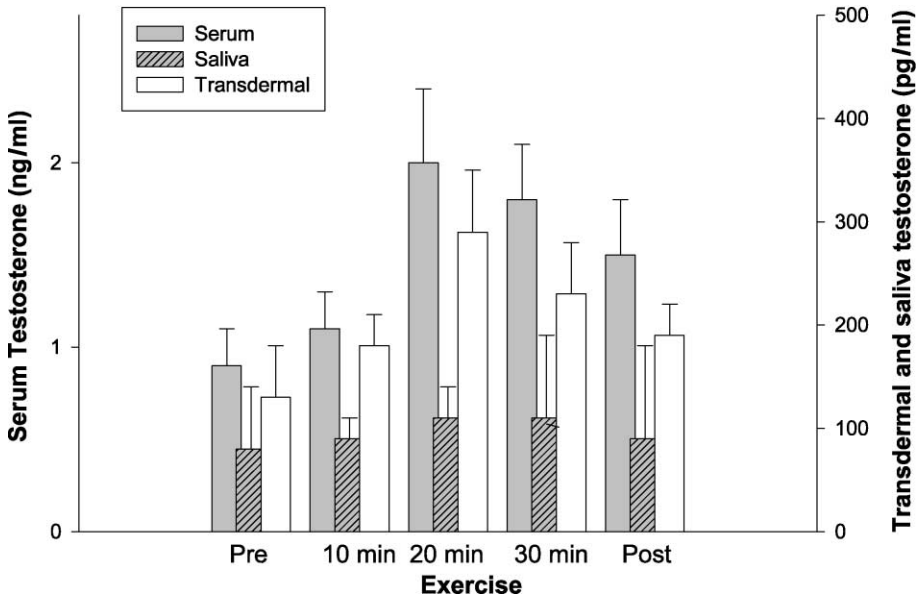

Fig. 2. Changes in concentration of testosterone as measured in serum, saliva and transdermal samples obtained from sheep before, during and after exercise. Means ± S.E. are presented for serum (ng/ml), transdermal (pg/ml) and saliva (pg/ml). Saliva values represent those obtained at or up to 20 min after both serum and transdermal samples both for pooled values (pre and post) and exercise time points.

Table 2
Concentration of hormones and glucose in serum, transdermal and saliva samples collected before, during and after exercise in sheep

	Glucose			Insulin			Estradiol			Cortisol		
	Serum, mg/l	Saliva, mg/l	Exudate, mg/l	Serum, ng/ml	Saliva, ng/ml	Exudate, ng/ml	Serum, ng/ml	Saliva, ng/ml	Exudate, ng/ml	Serum, ng/ml	Saliva, ng/ml	Exudate, ng/ml
Preexercise	734.8 ± 89.7	31.7 ± 3.9 (0.76) (4%)	70.5 ± 91.3 (0.87) (9%)	0.39 ± 0.08	ND	0.029 ± 0.009 (0.79) (7.5%)	1.4 ± 1.2	0.05 ± 0.03 (0.67) (5%)	0.12 ± 0.06 (0.82) (9%)	11.9 ± 4.7	1.07 ± 1.03 (0.81) (9%)	1.71 ± 0.85 (0.91) (14.5%)
10 min Exercise	911.3 ± 96.8	32.9 ± 7.3 (0.52) (3.5%)	77.9 ± 11.6 (0.85) (8.5%)	0.21 ± 0.07	ND	0.016 ± 0.005 (0.81) (7.5%)	1.5 ± 1.1	0.038 ± 0.011 (0.49) (2.5%)	0.14 ± 0.08 (0.79) (9%)	19.6 ± 5.1	1.51 ± 0.97 (0.61) (8%)	2.91 ± 0.83 (0.89) (15%)
20 min Exercise	1205.6 ± 101.3	34.5 ± 7.4 (0.47) (3.0%)	107.4 ± 14.6 (0.88) (9%)	0.12 ± 0.05	ND	0.009 ± 0.003 (0.80) (7.5%)	1.3 ± 1.1	0.046 ± 0.009 (0.45) (3.5%)	0.12 ± 0.07 (0.80) (9%)	27.8 ± 4.6	2.35 ± 2.19 (0.55) (8.5%)	3.92 ± 1.01 (0.88) (14%)
30 min Exercise	1458.1 ± 112.7	36.0 ± 9.1 (0.55) (2.5%)	130.3 ± 13.4 (0.85) (9%)	0.11 ± 0.05	ND	0.008 ± 0.004 (0.79) (7.0%)	1.5 ± 1.3	0.03 ± 0.012 (0.51) (2%)	0.14 ± 0.09 (0.82) (9%)	39.3 ± 7.4	2.44 ± 1.76 (0.62) (6%)	5.74 ± 0.96 (0.90) (15%)
Postexercise	1179.8 ± 105.8	32.3 ± 8.41 (0.67) (2.5%)	102.6 ± 14.3 (0.86) (9%)	0.19 ± 0.08	ND	0.014 ± 0.008 (0.83) (7.5%)	2.2 ± 1.1	0.052 ± 0.021 (0.59) (4%)	0.20 ± 0.06 (0.79) (9%)	18.3 ± 4.9	1.34 ± 1.81 (0.72) (7%)	2.71 ± 0.55 (0.89) (15%)

Means ± S.E. are displayed.
( ) Indicates r^2 value for that period.
(%) Relative recovery rounded to the nearest 0.5% based on mean values.
Preexercise, exercise and postexercise values for transdermal and blood samples represent pooled.
Measurements for the periods are indicated.
For saliva samples, preexercise includes saliva samples at 10 and 20 min into exercise, exercise values for saliva samples are 30, 40 and 50 min from the start of exercise.
This allows a 20-min lag between values in the blood and their reflection in saliva.

Table 3
Concentration of hormones and glucose in serum, transdermal and saliva samples collected before, during and after exercise in human subjects

	Glucose			Insulin			Estradiol			Cortisol		
	Serum, mg/l	Saliva, mg/l	Exudate, mg/l	Serum, μIU/ml	Saliva, μIU/ml	Exudate, μIU/ml	Serum, ng/ml	Saliva, ng/ml	Exudate, ng/ml	Serum, ng/ml	Saliva, ng/ml	Exudate, ng/ml
Preexercise	814.9 ± 112.6	28.5 ± 7.9 (0.78) (3.5%)	79.0 ± 13.4 (0.87) (10%)	16.3 ± 5.4	ND	1.35 ± 0.90 (0.83) (8%)	15.6 ± 5.7	0.71 ± 0.25 (0.69) (4.5%)	1.23 ± 0.46 (0.82) (8%)	22.7 ± 8.1	2.25 ± 1.04 (0.89) (10%)	2.79 ± 0.85 (0.93) (12%)
0 min Exercise	905.4 ± 89.7	31.3 ± 9.2 (0.51) (3.5%)	86.1 ± 9.3 (0.88) (10%)	13.1 ± 3.0	ND	1.05 ± 0.45 (0.82) (8%)	19.1 ± 6.3	0.76 ± 0.38 (0.42) (4%)	1.53 ± 0.59 (0.85) (8.0%)	29.5 ± 9.2	2.79 ± 1.13 (0.76) (9.5%)	3.69 ± 1.31 (0.91) (12.5%)
10 min Exercise	119.3 ± 106.7	33.6 ± 6.5 (0.55) (3%)	110.8 ± 12.7 (0.84) (10%)	10.6 ± 4.9	ND	0.90 ± 0.28 (0.84) (8.5%)	18.3 ± 7.1	0.75 ± 0.51 (0.44) (4%)	1.47 ± 0.94 (0.88) (8.0%)	70.8 ± 11.5	8.01 ± 2.86 (0.84) (11%)	8.57 ± 1.94 (0.89) (12%)
20 min Exercise	1205.8 ± 114.3	34.3 ± 7.1 (0.54) (3%)	118.1 ± 17.4 (0.86) (10%)	6.3 ± 3.8	ND	0.51 ± 0.23 (0.83) (8%)	21.5 ± 7.4	0.83 ± 0.39 (0.55) (4%)	1.74 ± 0.54 (0.82) (8%)	91.3 ± 19.7	8.75 ± 4.31 (0.71) (9.5%)	11.5 ± 2.8 (0.91) (12.5%)
30 min Exercise	1509.7 ± 201.4	35.1 ± 8.3 (0.61) (2%)	143.4 ± 19.6 (0.85) (10%)	5.4 ± 4.1	ND	0.46 ± 0.21 (0.83) (8.5%)	26.7 ± 9.2	0.85 ± 0.22 (0.42) (3%)	2.14 ± 0.86 (0.84) (8.0%)	115.7 ± 15.8	10.04 ± 3.11 (0.79) (9.0%)	14.4 ± 3.7 (0.92) (12.5%)
40 min Exercise	1519.3 ± 186.5	36.0 ± 7.5 (0.69) (2%)	147.5 ± 21.3 (0.88) (10%)	6.9 ± 3.8	ND	0.57 ± 0.33 (0.80) (8%)	19.4 ± 4.0	0.68 ± 0.26 (0.68) (3.5%)	1.53 ± 0.51 (0.84) (8%)	109.4 ± 13.1	9.19 ± 3.76 (0.85) (8.5%)	13.7 ± 2.4 (0.90) (12.5%)
Postexercise	1005.4 ± 103.7	32.1 ± 8.3 (0.65) (3%)	96.5 ± 19.2 (0.89) (10%)	14.1 ± 5.5	ND	1.15 ± 0.48 (0.83) (8%)	13.9 ± 5.1	0.45 ± 0. (0.53) (3%)	1.11 ± 0.55 (0.83) (8.0%)	49.6 ± 11.3	4.02 ± 1.95 (0.81) (8%)	6.07 ± 1.14 (0.88) (12%)

Means ± S.E. are displayed.
( ) Indicates r^2 value for that period.
(%) Relative recovery rounded to the nearest 0.5% based on mean values.
Preexercise, exercise and postexercise values for transdermal and blood samples represent pooled measurements for the periods indicated.
For saliva samples, preexercise includes saliva samples at 10, 20 and 30 min into exercise, exercise values for saliva are 40, 50, 60 and 70 min from start of exercise.
This allows a 30-min lag between values in the blood and their reflection in saliva.

ization, but little response to saliva collection, and no obvious response to the electrosonophoresis. In the case of jugular catheterization, data (not shown) suggested that with 50 min of rest following the catheterization, any hormone level change associated with this procedure had subsided to baseline.

Cortisol, 17-β estradiol, testosterone and glucose were all measurable in saliva. Insulin was not detectable. Prior to the exercise stress, all values showed a high correlation (all $r^2 \geq .67$) with blood serum values (both free and total for hormones). This was best achieved when the saliva sample was compared to the blood sample obtained

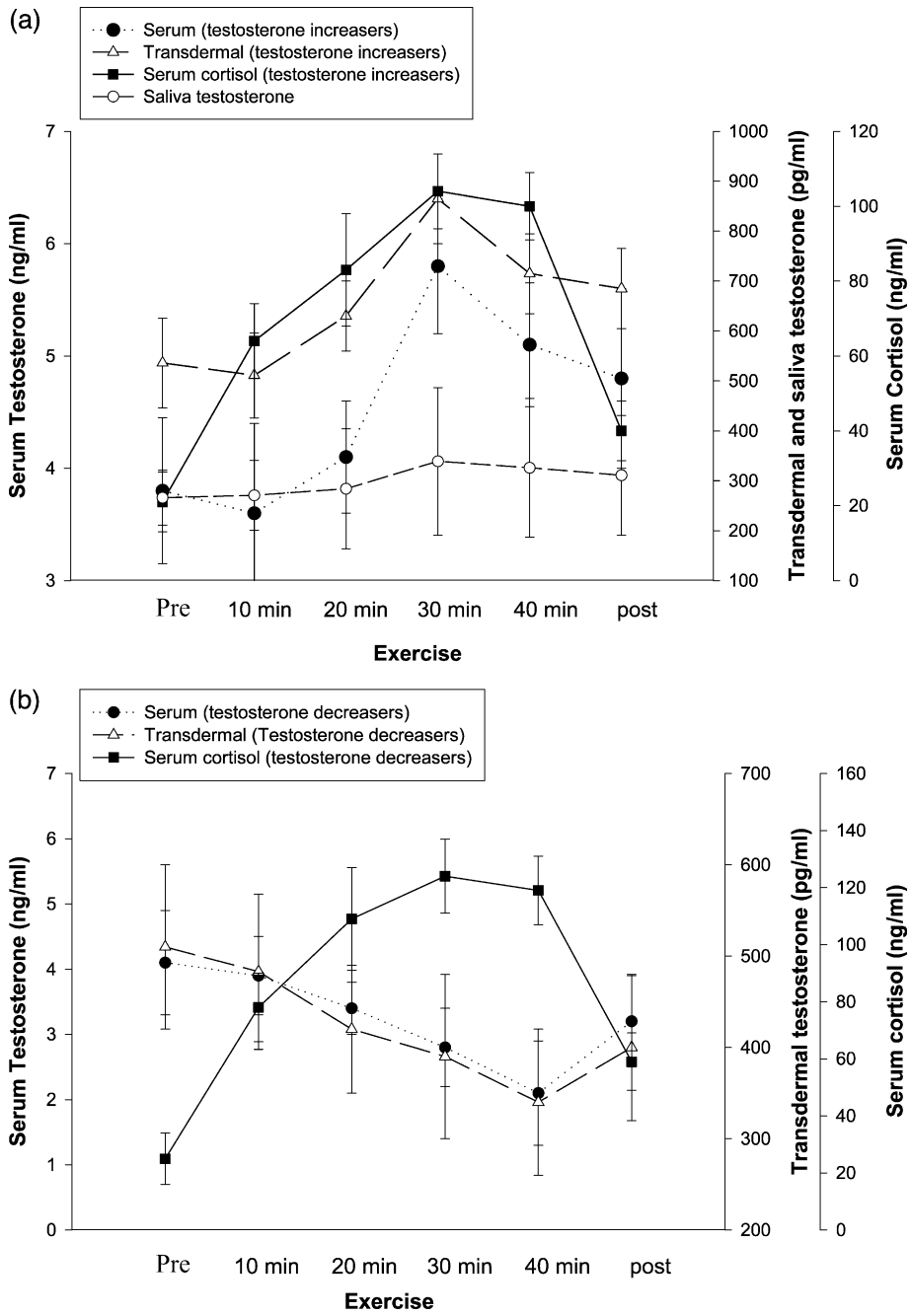

Fig. 3. (a) Changes in concentration of testosterone as measured in serum, saliva and transdermal samples and cortisol as measured in serum samples obtained from human subjects, before, during and after exercise, who showed consistent increases in the level of testosterone (increasers) during the exercise sessions. Means ± S.E. are presented for serum (ng/ml), transdermal (pg/ml) and saliva (pg/ml) values. Saliva values represent those obtained at or up to 30 min after both serum and transdermal samples both for pooled values (pre and post) and exercise time points. (b) Changes in concentration of testosterone as measured in serum and transdermal samples and cortisol as measured in serum samples obtained from human subjects, before, during and after exercise, who showed consistent decreases in the level of testosterone (decreasers) during the exercise sessions. Means ± S.E. are presented for serum (ng/ml) and transdermal (pg/ml) values.

20–30 min previously (i.e. there was a time lag between blood values and associated changes in saliva values). At rest, the levels of the hormones and glucose detected in saliva were typically between 4% and 9% of those seen in the serum. During and subsequent to exercise stress, cortisol, testosterone and glucose increased, in all animals, in both saliva and blood (Fig. 2 and Table 2). Insulin decreased in blood samples over the stress. However, the values in saliva and blood did not correlate as well as when the animal was at rest, irrespective of allowing any time lag between blood values and reflective saliva values. This reduction in correlation included 17-β estradiol even though it showed little change in either blood or saliva across the stress. Fig. 2 and Table 2 displays this data.

Salivary values measured directly by off-line assay or in real time via the immunosensors were highly correlated ($r^2 \geq .92$) for all values across the experiment.

In transdermal exudates, cortisol, 17-β estradiol, testosterone, insulin and glucose were all measurable. Exudate values were typically between 9% and 15% of blood values in sheep, varying with both the measured hormone and the individual. Despite considerable interindividual variation, within an individual, the relationship between exudate and blood was consistent, for each hormone, before, across and after the exercise stress. Within individual animals, a strong correlation ($r^2 \geq .79$) was seen between the exudate values and blood values obtained at the same time (i.e. there was no lag between blood levels and reflective transdermal levels, provided 10 min was allowed after the initial application of ultrasound). To maintain this transdermal flux, ultrasound is needed to be applied at least every 30 min. Cortisol, testosterone and glucose rose significantly ($P \leq .05$) with the exercise, while insulin decreased ($P \leq .05$) and 17-β estradiol showed little change. Table 2 and Fig. 2 exhibit this data.

Measurements did not change significantly across the three repeat exercise stressors for transdermal exudates and blood values. Baseline saliva values also showed stability, but saliva values across exercise and recovery were also variable ($P \leq .05$) across the repeats.

3.3.2. Humans

Cortisol, testosterone, 17-β estradiol and glucose were detected in saliva while insulin was not. Correlation to blood values preexercise was high ($r^2 \geq .69$) when the salivary values were compared to blood values 30 min prior to the saliva collection (i.e. a time lag of 30 min). During exercise and recovery, correlation between saliva and blood for all values fell ($r^2 \geq .42$) considerably irrespective of any time lag considerations. Salivary values, at rest, were typically 3–10% of blood values, depending on the hormone. Salivary values from the same samples, measured directly by off-line assay or in real time via the immunosensors, were highly correlated ($r^2 \geq .91$) for all values across the experiment.

Transdermal exudates contained measurable quantities of cortisol, testosterone, 17-β estradiol, insulin and glucose. After the initial application of ultrasound (allowing 10 min), these correlated ($r^2 \geq .82$) with blood values obtained at a similar time (i.e. no time lag). This was not changed across exercise and recovery.

Exudate values were between 8% and 12%, depending on the hormone, of blood values. This value for each hormone was consistent within subjects and across repeat measures. Intersubject variation was also considerably less ($P \leq .05$) than seen in sheep.

In all subjects, cortisol increased ($P \leq .05$) with exercise, but insulin decreased ($P \leq .05$). Testosterone and 17-β estradiol were more variable, showing individual subject-related increases or decreases across exercise and recovery. Glucose increased ($P \leq .05$) across exercise and recovery. Table 3 and Fig. 3a and b display this data. Over the six exercise sessions, the type and magnitude of the stressor response did not significantly differ for individuals.

In all subjects, cortisol increased significantly ($P \leq .05$) with exercise. In some subjects, during exercise, testosterone levels fell, while in other subjects, testosterone increased. This was a consistent individual pattern over the six exercise repeats. In subjects where testosterone increased over exercise, an increase in distance rowed in the exercise time was seen ($P \leq .05$) progressively over the exercise repeats. When the subjects were questioned, they did not report any information that suggested differences in terms of other fitness work, sleep or diet.

Placement of sampling device at different sites of the volar forearm did not significantly affect recovery or measurement within subjects. Increasing the room temperature by 8–10 °C tended to increase recovery slightly by 1–3%, but this was not significant.

4. Discussion

Saliva equilibrates reasonably well with many blood components, but the partitioning between the two body fluids is complex and still poorly understood [3,8,13]. Saliva can provide diagnostic values, particularly for compounds that are relatively stable in the blood over a reasonable period, or while the animals are at rest and concentrations are within a narrow range. Repeat measures, herein, while the animals were at rest did show good consistency to blood values. This supports a wealth of literature, suggesting that saliva measures may be useful in basal situations [3,8,12–14].

Saliva collection is relatively easy in format for midsized animals and humans. It is low invasive in nature and has easy compliance, and collection involves only low risk to the subject. As shown here, it can be measured quickly at the site of experiment, in this case, using the collection head and sensors described, with values being produced by such rapid assessment virtually identical to off-line measures.

However, from my results, it appears that there are difficulties in using saliva in dynamic (nonrest) longitudinal endocrine studies. The lag time in partitioning between blood and saliva is not linear, peaks appear to be 'averaged' out in saliva as seen in falling recovery percentages, and some hormones detectable in the blood were not measurable in saliva. Other studies [12,14] have also pointed out the problems of saliva measures under stress conditions.

Transdermally collected exudate, in contrast, appeared to mirror blood changes with high consistency. Collection was made from subjects with relative ease and in human subjects without any report of pain or discomfort. Animals showed no obvious behavioral aversion to the procedure, and had been familiarized to the overall experiment procedures. The collection did take 2 min (1 min ultrasound followed by a further 1 min collection), necessitating an increased contact of experimenter to subject compared to the saliva format, however, in the case of animals, this collection required less manipulation than did saliva collection. An initial application of ultrasound was also needed to start the transdermal flux. In repeated measures, without imposed stress, neither the salivary nor transdermal method elevated cortisol, suggesting that they were both low stress in nature. The transdermal method also allowed measurement of insulin, which salivary methods did not. I cannot offer any experimentally proven explanation as to why insulin was not seen in saliva other than the fact that partitioning between blood and saliva is not entirely passive and can be complex. It has been suggested that saliva only reflects unbound hormones, although numerous studies have now suggested that the relationship is more complex than this and that saliva can, under certain circumstances, reflect total blood hormonal levels better than free hormonal levels [4,8].

As noted by others [16–18], it was found that a short initial application of ultrasound was needed to start transdermal flux. Once this flux reached a point of equilibration with blood levels, in these experiments, this took 10 min as compared to 30 min in previous studies [16], measurement closely reflected changes in blood levels. However, herein, this was only the case if a further application of ultrasound and electric field was applied at least every 30 min or as part of each measurement procedure. Unlike previous work where at least 4 h of transdermal glucose sampling was permitted following a single ultrasound application [16], it was found for hormones that a much more frequent reapplication of the ultrasound and electric field was needed. This may reflect transdermal flux differences between hydrophilic molecules such as glucose and more complex structured molecules such as hormones.

It was also found that a 1-min application of the ultrasound, when combined with the electric field, was sufficient compared to 2 min used in the previous studies [16] with similar ultrasound power. Apart from these two differences, other parameters of application were very similar. Relative recovery in transdermal samples with ultrasound alone, compared to serum levels, was of the order of 4%, which was very similar to that reported in a previous work [16]. Herein, this was increased slightly with application of an electric field and more so by the use of the collecting head device. In previous studies, r^2 values consistently greater than .90, between transdermal flux and serum concentrations, were reported [16]. In this study, those consistent levels of correlation were not achieved, however, the correlation achieved for glucose and hormones, particularly testosterone and cortisol, were high and would provide valid prediction of blood levels.

The collecting head appeared to substantially improve concentration and recovery of the hormones and glucose, and it certainly allowed adequate concentration for measurement by immunosensors in the exit line. The immunosensors themselves can, by design, further this concentrating of measured substance. To a small extent, this was shown by the ability of the immunosensors to adequately measure key analytes in smaller collected volumes than for off-line analysis (150 μl versus 300 μl). This property has been demonstrated in previous studies [4,5,7]. The main advantage of this comes by allowing measurement of samples collected in very short time intervals.

One of the problems of measuring peptides by the immunosensor method is that these analytes can stick to the immunosensor membranes making repeated measurement unreliable. In vitro studies suggested some sticking of certain hormones to the membranes, but, interestingly, this was constant, thus not affecting the relativity of measurement. Sticking to membranes was not seen when the ultrasound and electric field were active, perhaps suggesting that these agitated the collected analytes freeing them for antibody binding within the immunosensor. Measurement correlated well to both free and total hormones, suggesting that bound hormones were being recovered for measurement. In vitro work, data not shown, supported this. Earlier work with immunosensors has suggested this possibility [4].

Transdermal collection had a very low intrasubject variability in both sheep and humans, less than that seen in saliva. The intervariation was much greater in the sheep than in humans. In the latter, this low intervariation allowed good predictive measurement not only within but also across subjects. Position of the device, in humans, showed little contribution to variability, with most places on the volar forearm giving similar results. A high degree of intrasubject consistency but considerable intersubject variability has been reported for ultrasound collection of glucose [16]. In this previous study [16], variation in sampling site also contributed to variability within a subject. These were not such issues, for human subjects, in this present study, perhaps indicating a more robust sampling system. Room temperature was also controlled for all human subjects in my experiments, and this could conceivably influence sampling properties. An increase in room temperature was associated with a trend toward higher recoveries, but this possibility was not tested any further.

Transdermal recovery was slightly less from sheep than humans, and with much greater intersubject variability. This may reflect the sampling sites. In humans, the volar surface of the forearm offered a highly vascular area, while the ventral surface of the sheep's back did not, and skin layers differ in thickness. Preliminary data, not shown, suggest that heating the skin in sheep allows an improved recovery and reduced intersubject variability. Other more vascular sites such as the ear also appear to further improve this in sheep. Intersubject variability did not influence the usefulness of the device in this study, but it needs consideration in studies where absolute across-subjects calibration is needed.

The hormone levels, other than cortisol and insulin, in the sheep were low but typical of ewes outside of breeding season [9,15]. Human subject levels were consistent with that expected for young fit men [2,10,20,21]. An interesting point of observation was that despite the sheep being ruminant; saliva from sheep was relatively similar in recovery values and changes over exercise to the monogastric human subjects.

In human subjects, exercise-related increases were predictive of the subject's ability to increase work performance, as measured in distance rowed, on a subsequent training session. Increases in cortisol relative to testosterone, at rest, have been suggested as indicative of overtraining in human subjects [2,20], with a fall in testosterone being partly contributory. It was interesting, then, in this study to note that increases in testosterone relative to exercise were associated with improving training performance, but this remains highly speculative as insufficient data were collected.

Sonophoresis, the use of ultrasound, as a transdermal facilitator of certain analytes, such as glucose, has been demonstrated as a useful concept for sampling previously [16,18,19]. In the study presented herein, the addition of an electric field (electrosonophoresis), and a specialized collection head, enabled a good, in terms of relativity to blood levels, collection of hormones using similar short bursts of ultrasound. Hormones are complex molecules, often with accompanying charge and water insoluble. The addition of an electric field appeared facilitative in their movement, while the arrangement of the collecting head seemed to improve further the collection for measurement.

The technique, thus, appears very useful as a noninvasive sampling tool. It is portable and handy, more successful in prediction of blood levels than saliva over varied conditions and perhaps easier to use in animal studies. Certainly, with short sampling time and intensive longitudinal studies, it has advantages over attempts at repeated saliva collection. Combining immunosensors offers further extension from just collection of the sample to actual rapid measurement on-line, an added advantage to experimental design. No evidence of skin damage or pain, or of aversion, was seen with repeat measurement. Preliminary work, data not yet presented, suggests that this method is applicable to a broad spread of animal species including rats, cattle, horses and sharks. There are numerous benefits that such a method would also offer human and other animal endocrine studies, including addressing welfare and stress concerns surrounding invasive sampling.

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