Journal Name

ARTICLE

Detection of Cortisol in Serum Using Quantitative Resonance Raman Spectroscopy

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Measurement of cortisol in serum is used commonly as an indicator of stress and disease. Conventional analytical techniques have limited utility given that they remain largely laboratory based, they do not directly measure the deemed biologically active free cortisol, and there is no robust correlation between the free cortisol measurements within serum and saliva. It would therefore be desirable to measure both the free and total cortisol readily within the same matrix in a portable device in the field or at the bedside. This paper demonstrates the utility of a portable Raman approach to measure both the biological active free cortisol as well as total cortisol in human serum, compared to a laboratory-based chemiluminescence analysis technique. This alternative portable Raman method produced results that were consistent with results obtained from previous methods, which has the potential for further miniaturisation for point of test applications.

Introduction

Cortisol is a lipophilic steroidal hormone produced in the zona fasciculate within the adrenal cortex of the adrenal gland. It is released in response to physiological stress and implicated in a number of essential roles such as glycogenolysis, immune function, bone metabolism, and assists in maintaining blood pressure. It regulates the stress response along the hypothalamic–pituitary–adrenal axis. Cortisol is the key biomarker of interest in monitoring stress, and its robust measurement has been a subject of much study. The physiological range of human total cortisol in serum is up to 600 ng/mL with normal levels of 140.60 ± 9.62 ng/mL and 101.03 ± 8.61 ng/mL in males and females, respectively).[1, 2] Aberrant cortisol measurements may be indicative of disease; cortisol is elevated in Cushing's syndrome and reduced in Addison's disease[3]. Cortisol measurements are also used to assess athlete performance, as over-stressed athletes are prone to injury or infection.

Cortisol has been studied in a variety of matrices, primarily in serum, plasma and saliva, but also in urine and hair. In serum or plasma, approximately 90 % of circulating cortisol is bound to cortisol (or corticosteroid) binding globulin (CBG)[4] when a subject is at rest. A further 5 % or so is sequestered by albumin. The remaining 5 % or so of cortisol remains unbound, or free, and deemed biologically active, as the protein-bound cortisol cannot readily permeate through cell membranes[5-7].

Unbound serum cortisol enters the saliva *via* active intracellular mechanisms rather than passive filtration of the serum. There are many competitive immunoassay kits available for the detection of total cortisol in blood serum and free cortisol in saliva. However, there are a number of constraints with the use of saliva for cortisol measurements[8]. There is a need to ensure that passive drool is collected, and for the sample to be free from contaminating food, drink or oral bleeding resulting from gum disease or injury. Recovery of a useable sample cannot always be achieved, especially if the subject has a cold, which may only lead to the precipitation of mucin. Where stress measurements are made for athletes, both before and after their training regime, dehydration can prevent provision of a saliva sample for analysis. Although the cortisol in saliva is primarily the biologically active free cortisol, the salivary cortisol is subject to parotid metabolism to cortisone[9], thus affecting the accuracy in the measurement of cortisol. Thus, whilst there have been reports of non-linear correlation between total cortisol in serum and salivary cortisol[10], any apparent correlation could result simply from the high measurement variability existing within patient groups[11]. It would therefore be desirable to measure both the free and total cortisol readily within the same matrix,

Urinary cortisol exists primarily in the unbound form, and so may be used to gauge adrenal function by measuring the metabolically

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active form of the hormone. Free cortisol in urine is elevated with Cushing's syndrome as the CBG is unable to bind the high concentration of cortisol in the bloodstream. Given that free cortisol in plasma is filtered through the glomeruli with partial tubular reabsorption, the amount of free cortisol recovered in urine is theoretically dependent upon the glomerular filtration rate (GFR). Hence urinary free cortisol excretion is determined from a 24 h urine collection[12], although this approach would be impractical to implement for ascertaining athlete responsiveness to their training regime.

Kirschbaum *et al.*, 2009 postulated that hair may provide the retrospective analysis of cumulative cortisol secretion over extended periods of time, although laborious extraction methods are required prior to the cortisol assay[13]. More recently, the use of fingernails to provide the retrospective analysis of cortisol levels has also been explored[14]. However, this measure of chronic stress would not be useful in ascertaining physiological stress in response to a particular athlete's training regime.

Conventionally, free cortisol measurements in serum are determined by performing a total cortisol assay on serum sample fractionated to recover the free cortisol. Extensive dialysis, ultracentrifugation, or ultrafiltration is employed to fractionate the free cortisol. However, these methods are either labour intensive, time consuming or require the use of large dedicated equipment which is not amenable for a field-based or bedside study. A direct immunoassay to measure the biologically active free cortisol in serum has not previously been reported. To bypass this stumbling block, researchers have for many years investigated the prospect of measuring the Free Cortisol Index (FCI) as a substitute for measuring free cortisol in serum; this strategy requires the measure of the total cortisol concentration in serum and the concentration of CBG, the key serum binding protein[15, 16]. More recently, investigators have also attempted to take into account the concentration of albumin in refining the FCI, as albumin has a high capacity to bind cortisol[17]. Evidently, the measurement of two or even three analytes to derive the FCI is rather labour intensive, but highlights the importance of deriving a measurement that correlates to the biologically active free cortisol rather than total cortisol within serum.

Raman spectroscopy involves the inelastic scattering of light, where the scattered photons have the same energy as the incident light[18-20]. Raman is a relatively weak process (with only one in 10^6 photons being Raman scattered), however resonance Raman scattering (RRS) involves the use of a laser beam with a frequency that is coincident with the frequency of an electronic transition in a molecule. The main advantages of this method being that it is non-destructive and highly selective. In the case of the work presented here, the laser beam frequency of 638 nm coincides with the absorption maximum of the utilised chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) so giving rise to molecularly specific Raman spectra of the analyte under analysis.[21] Recent advances have led to the development of portable Raman instrumentation for rapid discrimination between boar meat samples[22], and direct detection of erythropoietin in blood plasma[23] and clinically relevant concentrations of uric acid in urine samples[24].

Here we report a preliminary study that demonstrates the portable detection of both free and total cortisol within serum, without requiring the fractionation of the pool of free hormone, through use of the portable Snowy Range resonance Raman Scattering (RRS) spectroscopy instrumentation, compared to data obtained by chemiluminescence.

Materials and Methods

Reagents and Samples

Measurements of total cortisol were performed using screened pooled normal human male serum certified reference material (CRM) from the National Institute of Standards and Technology (NIST), USA with certified concentrations of total cortisol, as designated by liquid-chromatography mass spectrometry (LC-MS). Samples were prepared by spiking this CRM with exogenous known quantities of NIST cortisol (also a CRM) gravimetrically prepared, according to the manufacturer's protocol.

Measurement of free cortisol was made within female-pooled screened human serum from NIST, as well as the male pooled NIST serum sample (SRM 971 Hormones in Human Serum). Charcoal stripped pooled normal female human serum (Sera Lab, UK) was used as a negative control.

Assay Procedure

Briefly, the total cortisol assay entailed the coating of a black 96-well microtitre plate with solid flat-bottomed wells with the capture antibody, a mouse anti-cortisol monoclonal antibody (CalBioreagents, USA) overnight at 4 ^{o}C. The antibody was removed by inverting the plate, and then washed twice with PBS, 0.05 % Tween20 wash buffer. Blocking buffer, comprising of 1 % BSA in PBS, was added to each reaction well to saturate the vacant binding sites and the plate was incubated at room temperature for 1 h without agitation. The plate was then emptied by inversion and rinsed with the wash buffer once. The reaction mixture, which comprises of each test serum supplemented with a fixed quantity of the tracer, cortisol-HRP, in a phosphate-based buffer with reagents that dissipate the binding of cortisol to serum proteins, is added to the plate. The test sera were NIST SRM 971 Hormones in Human Serum consisting of two serum pools, one from normal adult males, and one from normal, premenopausal adult females, as well as charcoal stripped serum (from Sera Lab). The reaction mixture was incubated for 15 min at room temperature with agitation of the plate at 450 rpm. The contents were decanted by inversion, before the plate was rinsed four times with wash buffer prior to the addition of the TMB substrate. The plate was subsequently incubated for 30 min at room temperature before the addition of the TMB BlueSTOP solution, which stabilises the chromogenic substrate as a blue reaction product.

Raman Analysis

Raman analysis was performed using the Snowy Range Sierra Series™ with an excitation wavelength of 638 nm. Raman measurements were obtained from each well by focussing the laser below the meniscus and measuring the signal intensity at a wavenumber of 1611 cm^{-1}. The calibrants comprise of known concentrations of NIST cortisol standards that are spiked into charcoal stripped serum, and the signals observed for the test samples were interpolated from the standard curve. The free cortisol assay was similar to the total cortisol assay, with the exclusion of the reagents used to displace the bound cortisol from serum binding proteins. The serum was sourced commercially; hence no blood samples were collected for this study. However, typically when blood is collected in a serum gel tube, it is inverted six times and allowed to clot for 30 min at RT before a 10 min spin at 1300-2000 *g* at RT to obtain the serum.

Chemiluminescence Analysis

For the assays using chemiluminescence for detection with the Aushon Cirascan plate reader, a black 96-well microtitre plate with clear flat-bottomed wells was used as the reaction vessel. The analyte/tracer incubation step was performed for 1 h instead of only 15 min that was required for the Raman based assay. For the Aushon assay, luminol is used as the substrate (*in lieu* of TMB), which is oxidised by the horseradish peroxidase (HRP) enzyme component of the tracer to omit light that is detected using a cooled charge-coupled camera. The read time for each plate is approximately 4 min. While the plate reading is more rapid by this method, overall the assay time including the plate reading is slightly longer for the Aushon assay than for the Raman based assay.

Results and Discussion

Competitive assays were devised using cortisol conjugated with HRP as the labelled analogue of the analyte, to compete with the analyte cortisol for capture by the anti-cortisol antibody coated on a 96-well plate. Certified reference materials from NIST[25] were used as the calibrants for the standard curve. For detection by chemiluminescence, the HRP enzyme conjugate of the tracer hydrolyses luminol to emit light, which is measured using the cooled charged-coupled camera of the Aushon Cirascan reader. For detection *via* resonance Raman scattering spectroscopy (Figure 1), the HRP enzyme hydrolyses the chromogenic TMB to give rise to a blue reaction product, which generates a specific Raman signal. TMB BlueSTOP was then added to stabilise the blue product for detection by focussing a laser below the meniscus of the well at a wavelength of 633 nm, and measuring the peak intensity at a wavenumber of 1611 cm^{-1}. This specific Raman peak corresponds to a combination of ring stretching and CH bending vibrations within the oxidised TMB molecule.[26, 27]

During the initial assay, both microtitre plates of high binding (Greiner Fluorotrac 600) and medium binding (Greiner Fluorotrac 200) capacity were used to illustrate the concentration dependent response for the Raman spectroscopy based cortisol assays. While the high capacity plate gave rise to signals almost three-fold higher, the medium binding capacity plate exhibited a wider working range and greater sensitivity than the assay using the high binding capacity plate (Figure 2). As the cortisol assay is in a competitive format rather than a sandwich assay, a lower concentration of capture antibody is desirable as this encourages competition between the analyte and the tracer (the labelled analogue of the analyte).

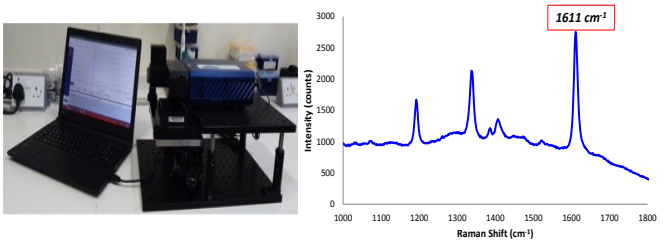

Figure 1 Photograph of the portable Raman instrument, Snowy Range Instruments (SnRI) Sierra Series with a 638 nm laser. The spectrum on the right is that of TMB (3,3′, 5,5′-Tetramethylbenzidine) highlighting the principal peak at 1611 cm^{-1} used for data analysis.

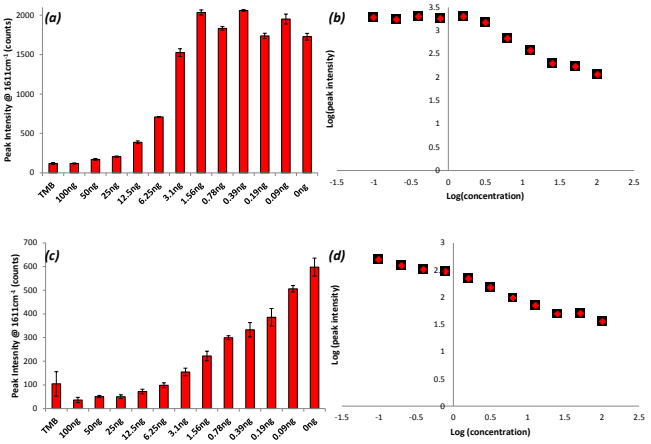

Figure 2 The concentration dependent response for the detection of cortisol using the high binding capacity Greiner Fluorotrac 600 (a) and the medium binding capacity Greiner Fluorotrac 200 plates (c). "TMB" represents the control where no cortisol was present. The peak intensity at 1611 cm^{-1} for the single determinations of each calibrant was measured three times, and the data represent the mean values of the triplicate readings and the error bars denote ±1 SD of the mean. The relationship between the logarithm of the mean signal, in terms of peak intensity at 1611 cm^{-1} and the logarithm of the concentration of the cortisol calibrants was also plotted to obtain the concentration-dependent curve for both the Fluorotrac 600 (b) and the Fluorotrac 200 (d).

The quantity of free cortisol within selected test samples were then determined using Fluorotrac 200 plates as the reaction vessels for the assays. As each of the individual wells required manual alignment under the fixed laser during signal detection, only a single determination for each calibrant (from separate rows on the microtitre plate) was read, followed immediately by a single determination of the test samples. The order of processing of the distinct rows of calibrants was row C, row D and then row E. The

single determination for each test sample was then interpolated from the linear portion of the calibration curve (derived from single determinations that were processed immediately prior to the sample readings), which encompassed the 0.78 -12.5 ng/mL range of each curve (Figure 3). The linear trendline for each row is shown in Figure 3, and the interpolated concentrations of the test samples are collated in Table 1.

Table 1 The interpolated concentrations of each test sample was derived from single determinations of the full range of calibrants for each distinct row, as each single determination of the samples was analysed immediately after obtaining a calibration curve from the peak intensity measurements of single determination calibrants. The mean, SD and CV of the interpolated data from the three distinct rows have also been determined.

	Free [Cortisol] (ng/mL)					
Serum test sample	Row C	Row D	Row E	Mean Free Cortisol (ng/mL)	SD	CV (%)
Male NIST (CM)	4.50	5.25	5.31	5.02	0.45	8.93
Female NIST (CF)	3.99	5.15	5.22	4.79	0.69	14.51
Heavy charcoal stripped (CH)	1.09	1.08	1.24	1.13	0.09	7.97

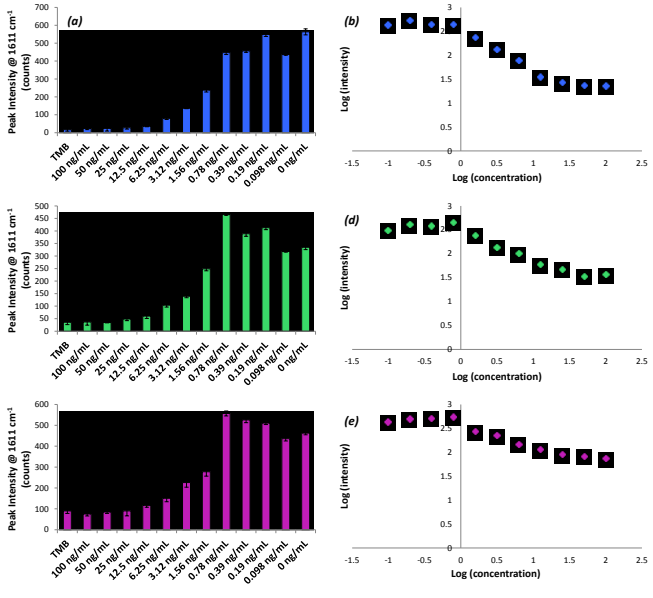

Figure 3 The calibration curves for three separate rows C to E of the microtiter plate. Figures a, c and e represent the peak intensity at 1611 cm^{-1} for the single determinations of each calibrant measured three times, and the data represent the mean values of the triplicate readings and the error bars denote ±1 SD of the mean for rows C, D and E respectively. Figures b, d and f show the relationship between the logarithm of the signal for Rows C, D and E respectively, in terms of peak intensity at 1611 cm^{-1} and the logarithm of the concentration of the cortisol calibrants was also plotted to obtain the concentration-dependent curve.

While it is apparent that the standard curves constructed from single determinations of each calibrant from separate rows do not overlay exactly, due to the increasing signal upon delayed reading of each reaction well in spite of the addition of the TMB BlueSTOP solution (Figure 4), there is good reproducibility between the interpolated cortisol concentrations among the test samples between rows. The measurements of free cortisol with the selected test samples were comparable to results obtained from triplicate determinations with the Aushon assay, performed in parallel (Table 2). This points to the concept of Raman based detection of cortisol being a valid approach, possibly through the use of single determinations.

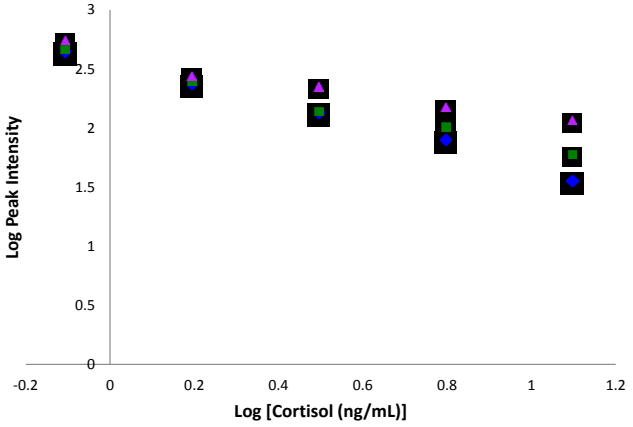

Figure 4 Portions of the linear correlations of the calibration curves for each row of calibrants on the microtitre plate, Row C (blue), Row D (green) and Row E (purple), encompassing the calibrants ranging from 0.78 – 12.5 ng/mL cortisol, inclusive. R^2 values were of 0.99453, 0.98731 and 0.95883 were obtained for Rows C, D and E respectively.

Table 2 A summary of the interpolated concentrations of free cortisol within the test samples, determined by resonance Raman scattering spectrometry and an Aushon chemiluminescence assay in parallel, and the mean % free cortisol. The latter calculation is based on the total cortisol measurements within the male and female NIST sera designated by LC-MS as 102.469 ng/mL and 86.417 ng/mL, respectively.

	RRSS		Aushon	
Serum test sample	Mean Free Cortisol (ng/mL)	Mean % Free Cortisol	Mean Free Cortisol (ng/mL)	Mean % Free Cortisol
Male NIST (CM)	5.02	4.90	4.64	4.53
Female NIST (CF)	4.79	5.54	5.99	6.93
Heavy charcoal stripped (CH)	1.13	-	1.25	-

These data are comparable with historical determinations of the free cortisol content within the test sera, using the Aushon Cirascan platform; male NIST serum: 4.11 ± 1.25 ng/mL (n=6), female NIST serum: 5.05 ± 2.04 ng/mL (n=5) and heavy charcoal stripped serum: 1.18 ± 0.44 ng/mL (n=6), which were obtained from the mean values from triplicate determinations for each sample for all the separate experiments. The mean percentage of free cortisol

determined in the pooled normal male and pooled normal female NIST sera are consistent with expected values.

A preliminary assessment of the total cortisol assay with serum using resonance Raman spectroscopy is shown in Figure 5. The test samples for the total cortisol assay comprised of male NIST serum (M4), with a known endogenous concentration of total cortisol as assigned by LC-MS, and the same serum spiked with known exogenous quantities of NIST cortisol to emulate the higher human cortisol concentrations (i.e. samples M1, M2 and M3). The recoveries of total cortisol within the test samples were assessed within triplicate determinations of serum test samples with four distinct concentrations (samples M1-4). The calibrants within row C were processed, followed by single determinations of samples M1-4, before the reading of the calibrants in row D, followed by the second loadings of samples M1-4.

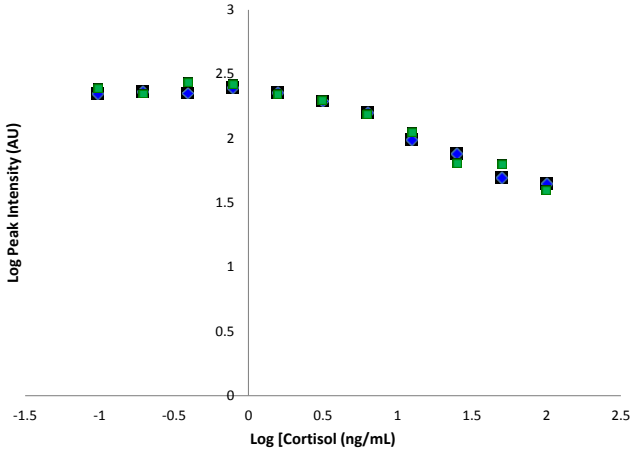

Figure 5 Scatter plots of the standard curve data for each separate row of calibrants of the Raman plate based assay, Row C (blue), and Row D (green), when determining the amount of total cortisol.

Alongside the Raman assay, a chemiluminescence based Aushon assay was performed in parallel, and the standard curve constructed from triplicate determinations of the calibrants is shown in Figures 6 and 7. Comparison of the data from the two platform assays is shown in Table 3.

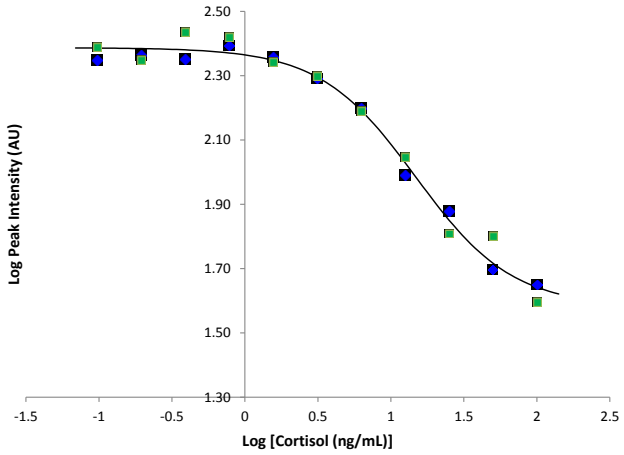

Figure 6 The line of best fit for the total cortisol Raman assay, based on calibrants in rows C (blue) and D (green) of the microtitre plate.

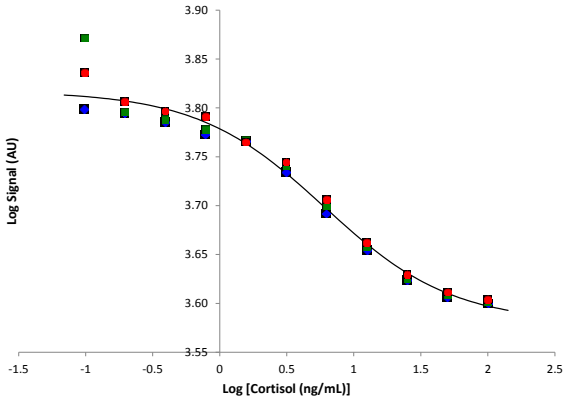

Figure 7 The 4 parameter logistics fit of the Aushon total cortisol assay calibration curve.

Table 3 The % recovery of total cortisol within male NIST serum (sample M4), and the same serum with additional spiked exogenous NIST cortisol CRM, as determined by resonance Raman spectroscopy and an Aushon chemiluminescence assay performed in parallel.

Sample	Predicted [Total Cortisol] (ng/mL)	Observed [Total Cortisol] by RRSS (ng/mL)	Observed [Total Cortisol] by Aushon assay (ng/mL)	% Recovery (RRSS)	% Recovery (Aushon)
M1	252.47	294.48	197.76	116.64	78.33
M2	177.68	202.92	144.06	114.20	81.07
M3	139.69	177.12	111.23	126.80	79.63
M4	102.47	116.16	75.99	113.36	74.16

There would appear to be a platform bias, as the recoveries of total cortisol were around 113-127% and 74-82% using the resonance Raman scattering spectroscopy and the Aushon Cirascan, respectively. However, there is no apparent concentration-

dependent recovery with either platform technology and the range in the recoveries for each platform is acceptable, as an acceptable range for analyte recovery for an ELISA based assay is between 80-120%. However, the recovery range when using the resonance Raman based method generally falls more within the desired range. Therefore, this allows Raman spectroscopy to be an alternative technique for the detection of free and total cortisol in serum samples.

Conclusions

We have demonstrated the ability to not only measure the free and total cortisol using resonance Raman scattering spectroscopy, but also shown the potential to obtain the measurements using single determinations in a reproducible manner. Given the as yet non-optimised analysis time of up to 45 minutes, this study highlights the applicability of Raman spectroscopy in a portable set up for the detection of biologically relevant targets that can rival current methods of detection. This has significant implications for on-field sample analysis, for example drug analysis or forensic testing, as this methodology can be readily adopted.

Acknowledgements

KG, KF and DG would like to thank the EPSRC for funding and the Royal Society of Chemistry for support from a Wolfson Research Merit award. Work undertaken by SP, GMJ and JB was funded by the UK government department for Business, Energy & Industrial Strategy (BEIS) and the English Institute of Sport (EIS). We thank Dr Scott Drawer (formerly at EIS), Dr Brian Cunniffe at EIS, and Dr Marco Cardinale (formerly at British Olympic Association) for addressing the need to devise point of care diagnostics for sport and health.

Notes and references

The research data associated with this paper will become available at the following link starting in August 2017. DOI: 10.15129/d70ec931-375a-452b-b9db-98d95c9f9fa5

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