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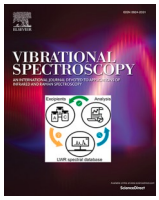

Label-free assaying of testosterone and growth hormones in blood using surface-enhanced Raman spectroscopy

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ABSTRACT

This work reports the potential use of surface-enhanced Raman spectroscopy (SERS) in rapid, label-free assaying of testosterone (TE) and growth hormone (GH) in whole blood. Biomarker SERS spectral bands from the two hormones (TE and GH) in intentionally spiked water for injection and in male Sprague-Dawley (SD) rat's blood are reported. These concentration-sensitive Raman bands as deduced through Principal Component Analysis (PCA) and Analysis of Variance (ANOVA), were centered around 1490 and 1510 cm^{-1} for GH, 1614 and 1636 cm^{-1} for TE; and 684 cm^{-1} for the hormone mixture (GH+TE) in blood. These bands exhibited significant intensity changes with the concentration of GH and TE hormones in blood. They were tentatively assigned to C-C stretching (684, 786, 856, 1614 and 1636 cm^{-1}), CH_2 bending (1490 cm^{-1}) and CH_2 stretching (1510 cm^{-1}). These bands may be used in SERS assaying of the respective hormones in blood using a customized and calibrated Raman system thus utilizing the strengths of the SERS method that include, being label-free, rapid (<1 min), chemically specific, minimal sample preparation among others. Besides, the method may potentially be used in detecting abuse of TE and GH in sports where they are often abused concurrently.

1. Introduction

Testosterone (TE) and growth hormone (GH) are potent anabolic hormones that play crucial roles in the regulation of many physiological processes in humans, including muscle growth, development and power, bone density, and sexual function [1,2]. In addition, these hormones are of interest in the sports world, where they are often abused singly or simultaneously as performance-enhancing drugs [3,4], often without knowledge of their prolonged side effects such as hypertension and liver failure [5]. This is due to the drive to win competitions and improve athletes' visual appearance [6]. These dopants harm users and their use is against clean competition [7]. Therefore, detecting these hormones in blood is of paramount importance in both medical and sports-related fields.

The most common methods for determining the levels of these hormones in blood are currently based on immunoassays [2,7] and mass spectrometry [8]. For instance, GH has been detected in athletes' blood using the radioimmunoassay (RIA) method, which has been reported to be highly sensitive but is limited by safety concerns due to the radioactive materials used [9]. The LC–MS/MS technique has been reported to detect traces of TE with high sensitivity and is capable of screening many tiny molecules in a single encounter; however, it suffers from low resolution when measuring proteins with high molecular weight [8]. Other methods include serum and enzyme-linked immunoassay (ELISA) [2,7], liquid chromatography-mass spectrometry (LC/MS) [8], and ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC/HRMS) [10]. These methods provide accurate results but are often limited in cost, sensitivity, and selectivity, require complex sample preparation, and cannot simultaneously assay GH and TE [11]. Therefore, there is a great need to develop novel methods for the detection of these hormones.

Spectroscopic techniques have recently shown potential for application in hormone detection. For instance, Raman Spectroscopy has successfully detected female reproductive hormones in mouse's blood

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[12,13], Fourier Transform Infrared Spectroscopy has identified reproductive hormones in urine [14], and surface-enhanced Raman spectroscopy (SERS) has identified estrogen [15] and detected TE [16,17]. SERS is the most powerful analytical spectroscopic technique for the detection and characterization of molecules among these techniques. It enhances the weak Raman scattering signals by utilizing, in addition to the incident, induced local fields produced by the driven plasmonic oscillations in metallic nanostructures that are in proximity to the probed molecules [18]. The enhancement factor of SERS can be as high as 10^{14}, which makes it possible to detect single molecules [19]. In addition, SERS provides molecule-specific information that can be used to identify, characterize, and quantify complex molecular mixtures. Consequently, SERS biomedical applications have recently increased in popularity, with distinct types of bacteria being detected and identified based on their unique SERS spectra [20]. This may enable rapid and reliable identification of bacterial diseases [20]. Biomarkers such as type 2 diabetes [21] and cancer [22] have been detected using the SERS technique.

The purpose of this work was to obtain the characteristic SERS spectra of both the TE and GH and investigate the existence of concentration-sensitive SERS bands for these hormones (TE and GH) in blood. Our findings revealed that unique SERS bands that are sensitive to the levels of the two hormones in blood exist, as well as some common hormone bands.

2. Materials and methods

SERS substrates (AgNPs) (150 μL) (synthesized as described in [23]) were mixed with 30 μL of the sample (pure reference solution and blood) and stirred thoroughly to obtain a homogenous mixture. 2 μL of the resulting mixture were then dropped onto aluminum-wrapped microscope glass slides (25.4 mm × 76.2 mm × 1.2 mm dimensions) and left to dry in air at room temperature for sixty (60) minutes. The sample sets included pure GH (3.7 mg/ml) (GeneScience Pharmaceuticals Co., Ltd., China), pure TE (250 mg/ml) (HEPIUS Pharmaceuticals, Hong Kong), and blood supplied from the Department of Zoology, University of Nairobi. The blood was drawn from male SD rats (weighing about 300 g), fed with standard rat chow, kept on a 12-hour light-dark cycle at room temperature of 23 ± 2 °C, and kept in metallic cages with dimensions of 109 cm by 69 cm by 77.5 cm as described in [21]. Different concentrations of each of these hormones were prepared in the range of 0.01–60 ng/ml via serial dilution of each of the pure hormones using water for injection as a diluent. Thirty microliters of each concentration were then thoroughly mixed with 30 μL of blood and 150 μL of AgNPs. The major goal of adding these varied amounts to blood was to separately identify the GH and TE concentration-sensitive SERS bands in blood. Concentrations were then grouped as follows: low (0.01–2) ng/ml, medium (3–22) ng/ml, and high (25–60) ng/ml for TE; and low (0.01–2) ng/ml, medium (3–25) ng/ml, and high (30–60) ng/ml for GH. Blood was collected from SD rats and stored at the Department of Medical Physiology of the University of Nairobi under the conditions outlined by Ondieki et al. [23]. For SERS (Raman) measurements, spectral data were collected and recorded from 12 random spots on each sample using a portable Raman spectrometer (EZRaman-N Portable Analyzer System, Enwave Optronics, USA). The experimental parameters were: ~150 mW excitation power, 5 s exposure time, 5 s; spectral averaging, and boxcar, 1. Data pre-processing included background correction (Auto Baseline 2) and smoothing (box car of 1) using built-in EZRaman Analyzer software. The spectrometer spectral range was 100–4200 cm^{-1} with 7 cm^{-1} spectral resolution and was equipped with a 1.2 m HRP-8 high throughput high Rayleigh rejection fiber optics probe. The probe (785 nm excitation wavelength) delivered the excitation beams and collected Raman-scattered radiation to and from the sample. Raman spectral data analysis together with further preprocessing that included normalization to maximum intensity, principal component analysis (PCA), and analysis of variance (ANOVA) was done within the 'fingerprint' spectral region 600–1850 cm^{-1} using a script in MATLAB R2021a (version 9.10.0.1602886, The MathWorks Inc., Natick, USA).

3. Results and discussions

3.1. Characteristic SERS spectra of pure testosterone and growth hormones

The chemical structures of GH and TE hormones (see Fig. 1) contain functional groups such as carbonyl (C═O), carboxyl (COOH), and hydrocarbon (CH) groups; therefore, it was expected that some vibrations would be isoenergetic.

Indeed, as shown in Fig. 2, common Raman bands (TE and GH) were observed at approximately 770, 948, and 1000–1400 cm^{-1} which were attributable to vibrations in the hydrocarbon [24], carbonyl, and carboxylic [25] functional groups, respectively. These bands can be tentatively ascribed to C–C bending (770 cm^{-1}) [12], C–O and C–C stretching (800–1200 cm^{-1}) [21,23,26], and Amide III (1220–1340 cm^{-1}) [27,28] vibrations. Unique bands were observed at approximately 668, 914, 1488, and 1652 cm^{-1} for TE and 1440, 1518, and 1700 cm^{-1} for GH. These bands can be tentatively assigned to C–O–C stretching (668 cm^{-1}) [12], CH_2/CH_3 scissoring (1440 cm^{-1}) [12], Amide II (1518 cm^{-1}) [29], and C═O stretching of Amide I (1650–1700 cm^{-1}) [28,30]. These latter TE and GH characteristic Raman bands may not necessarily be observed in blood because the different solvent environments influence their bond vibrational energies. Therefore, it was necessary to spike the blood drawn from SD rats with the two hormones and measure their Raman spectra.

3.2. The SERS spectra of testosterone and growth hormones when mixed with blood

Blood from male SD rats was spiked separately at various concentrations with TE and GH, and the Raman spectra were measured. Male rat blood was chosen because the levels of the two hormones (GH and TE) are known to fluctuate less [31]. Fig. 3 shows the average SERS spectra for various hormone concentrations (GH and TE). As expected, the Raman spectral profiles from the hormone mixture in water (Fig. 2) and blood (Fig. 3) were dissimilar because of changes in the local environment around the molecules of the two hormones. The only similar bands (Figs. 2 and 3) with slight shifts were those at approximately 1064 cm^{-1} for GH; 910, 1190, 1490, and 1338 cm^{-1} for TE; and 1658 cm^{-1} for both hormones (TE and GH). Other bands associated with various compounds in blood, such as proteins, carbohydrates, and lipids, were observed at approximately 658, 798, and 1410 cm^{-1}.

These bands can be tentatively attributed to C–O–C stretching (658 cm^{-1}) [12], C-C bending (798 cm^{-1}) [12], C–O/C–C stretching (910 cm^{-1}) [21,28,32], C–C stretching (1064 cm^{-1}) [23], C–C stretching of β-carotene/tyrosine (1190 cm^{-1}), CH_2 wagging of proteins (1338 and 1410 cm^{-1}) [21,27], CH_2 bending (1490 cm^{-1}) [33], and carbonyl stretching of proteins (1658 cm^{-1}) [23,32]. These spectral profiles and bands were largely identical to those reported elsewhere [34–36] from blood thus indicating the suitability of the SERS substrates employed in this study. The distinction between the two Raman spectral data sets, however, was observed in the amount of band intensity variation with concentration. As displayed in Fig. 3, band intensity variations with concentration were evidently more in blood mixed with GH (Fig. 3(a)) compared to that in TE (Fig. 3(b)).

PCA was used to further reveal the subtle Raman spectral profile differences between the two hormone samples in blood. Segregation based on the spectral profile patterns is shown in the score plot in Fig. 4 (a). The two spectral datasets (from GH and TE) were distinctly different. The bands responsible for segregation are shown in the loading plots in Fig. 4(b). From the loading plots, one can observe a unique band centered around 1510 cm^{-1} in GH, which is also overt in

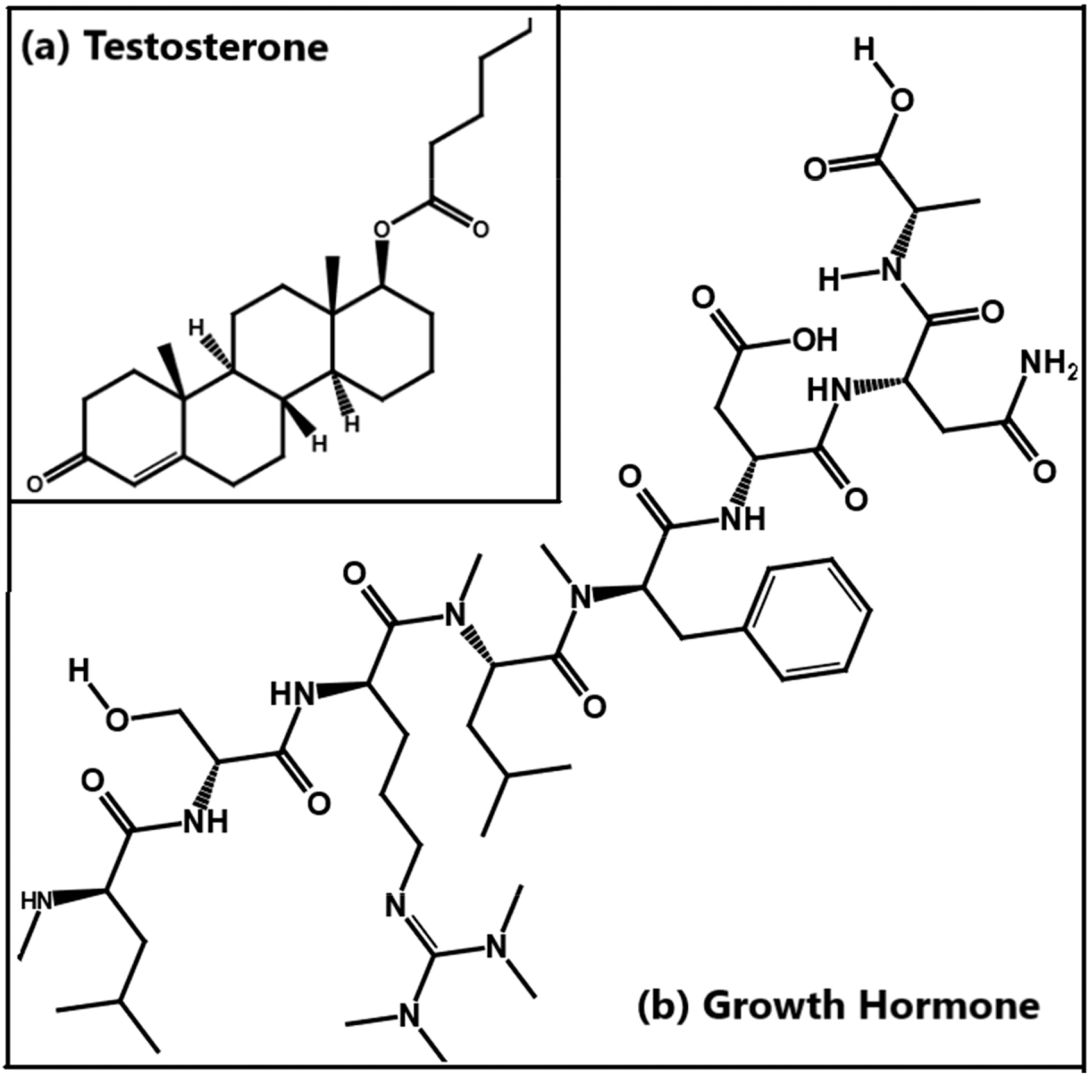

Fig. 1. Testosterone and Growth Hormone structures.

the spectra displayed in Fig. 3.

As also revealed by the PCA loading plot (Fig. 4(b)), the spectral segregation was caused by several other bands that displayed a large intensity variance. The bands that significantly contributed to segregation, as seen from the loading plot scores, were around 1378 cm^{-1} (for GH and TE), 684, 786, 856, 1042, 1240, and 1510 cm^{-1} (for GH), and 1166 and 1614 cm^{-1} (for TE). The two PCs had a total explained variance of 84.1 %, with PC1 and PC2 accounting for 74.7 % and 9.4 % of the variance, respectively. The remaining 15.9 % of data not explained by the two PCs could be due to the components present in blood such as haemoglobin (1378 cm^{-1}). These bands, apart from the unique band at 1510 cm^{-1}, with large loadings distinguish between GH and TE in terms of the amount of sensitivity to concentration level changes in blood, with GH Raman spectral bands being more sensitive. Intensity changes at around the 1510 cm^{-1} band can be used to reveal elevated levels of GH in blood.

For the SERS method to be applied to detect or assay GH and TE separately, the Raman band intensities should vary with the concentration of each hormone. In this study, the Raman spectral data sets from various concentrations of GH and TE in blood were grouped as Low (0.01–2 ng/ml), Medium (3–22 ng/ml), and high (25–60 ng/ml) before performing PCA. PCA was performed to identify the bands (including subtle bands) that were sensitive to concentrations. Fig. 5 shows the PCA scores and loading plots for the GH and TE datasets.

In the Raman dataset from GH samples, it was evident from the score plot in Fig. 5(a) that the spectra from non-spiked blood were segregated from the spiked ones. At the same time, low concentrations were distinctly segregated from the medium and high concentrations. The latter two (medium and high) were not significantly segregated. The bands responsible for the segregation were those centered at 1378 cm^{-1} for both blood and blood with low concentrations of GH; 1166 and 1614 cm^{-1} for low concentrations; 798, 1240, and 1510 cm^{-1} for both medium and high concentrations; and 684, 896, and 1042 cm^{-1} for high concentrations. For the Raman dataset of the TE samples, the groups (blood, low, medium, and high) were slightly segregated (Fig. 5(c)). The bands that brought about the segregation were those centered around 786 cm^{-1} for blood, low and medium concentrations of TE; 1378 cm^{-1} for both blood and low concentrations of TE; 856 and 1042 cm^{-1} for low and medium concentrations of TE; 1614 and 1658 cm^{-1} for medium concentrations of TE; and 658, 896, 1166, 1240 and 1480 cm^{-1} for high TE concentrations. As shown in Fig. 5(b) and (d), some of the bands identified to bring about the differences in the spectral profiles of different concentrations of the two hormones matched exactly, and some were slightly different from those in pure hormones (see Fig. 2). These bands include those around 798, 1042, and 1490 cm^{-1} for GH and 658, 786, 896, 1042 1166, 1378, and 1658 cm^{-1} for TE. A spectral band at 1378 cm^{-1} was present for both hormones at low concentrations because of their components in blood. The concentration-sensitive

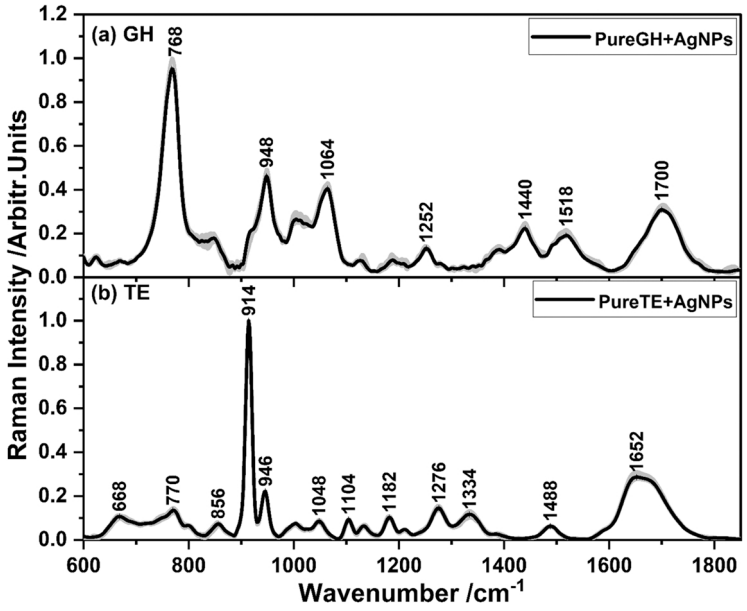

Fig. 2. Average SERS Spectra (black lines) derived from 12 spectra in each sample (gray lines) of (a) pure Growth Hormone and (b) pure testosterone hormones dissolved in water for injection.

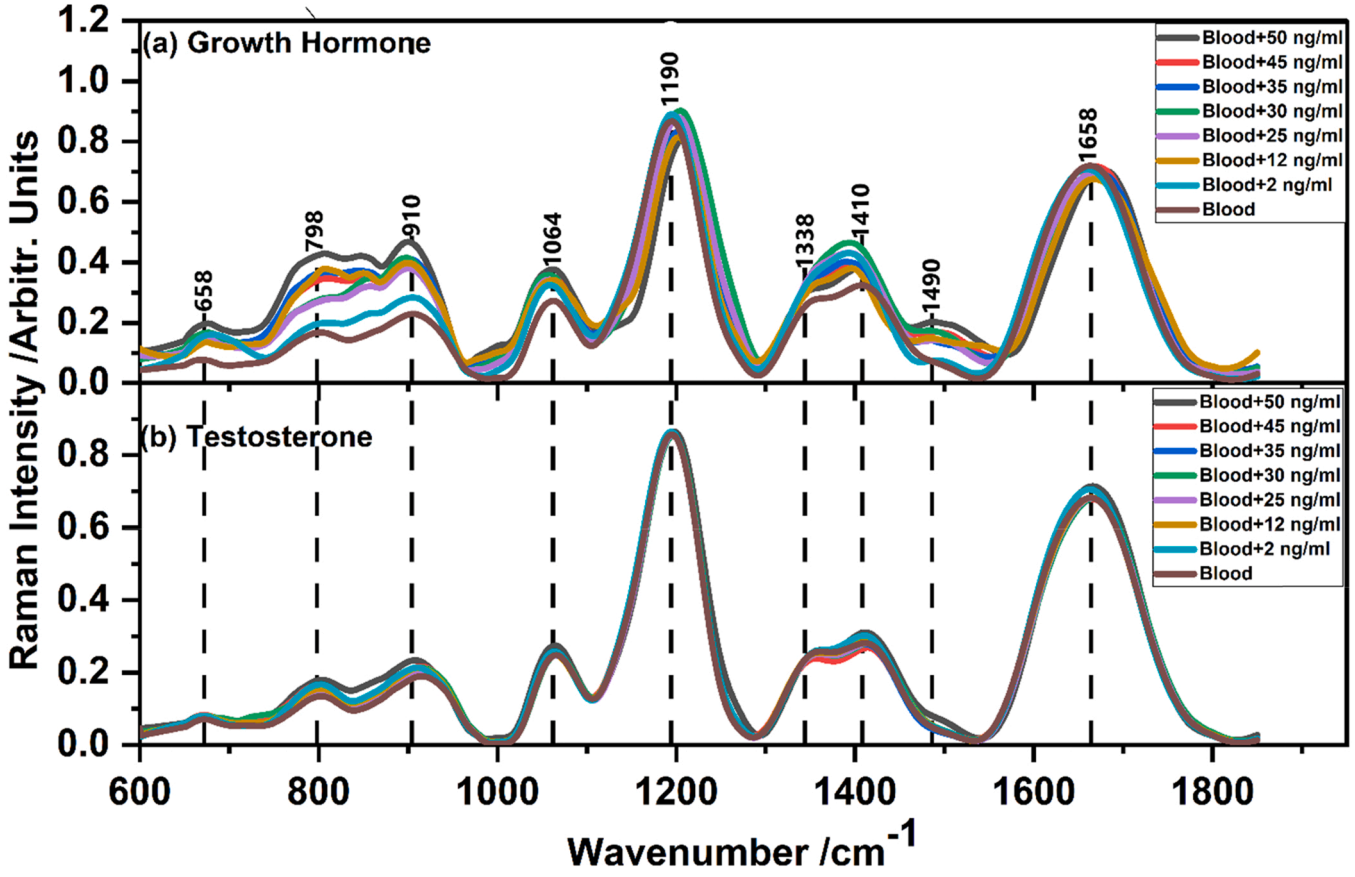

Fig. 3. Mean SERS spectra derived from 12 spectra of each sample for different concentrations of (a) growth hormone, and (b) testosterone hormones in blood dried on a clean aluminum-wrapped glass slide.

bands observed in the PCA results for the two hormones were centered at 896 and 1240 cm^{-1} for all hormones; 684,798,1042 and 1510 cm^{-1} for GH, and 658, 1166, and 1480 cm^{-1} for TE.

Because both hormones (TE and GH) are generally present in blood at normal concentrations, it was important to investigate whether the concentration-sensitive bands in the GH and TE spectra also exist in a sample spiked with both at identical concentrations. As seen in the score plot displayed in Fig. 5(a), there was some degree of segregation of the Raman spectral data sets from low, medium, and high concentrations, and non-spiked blood. The bands responsible for the segregation (with

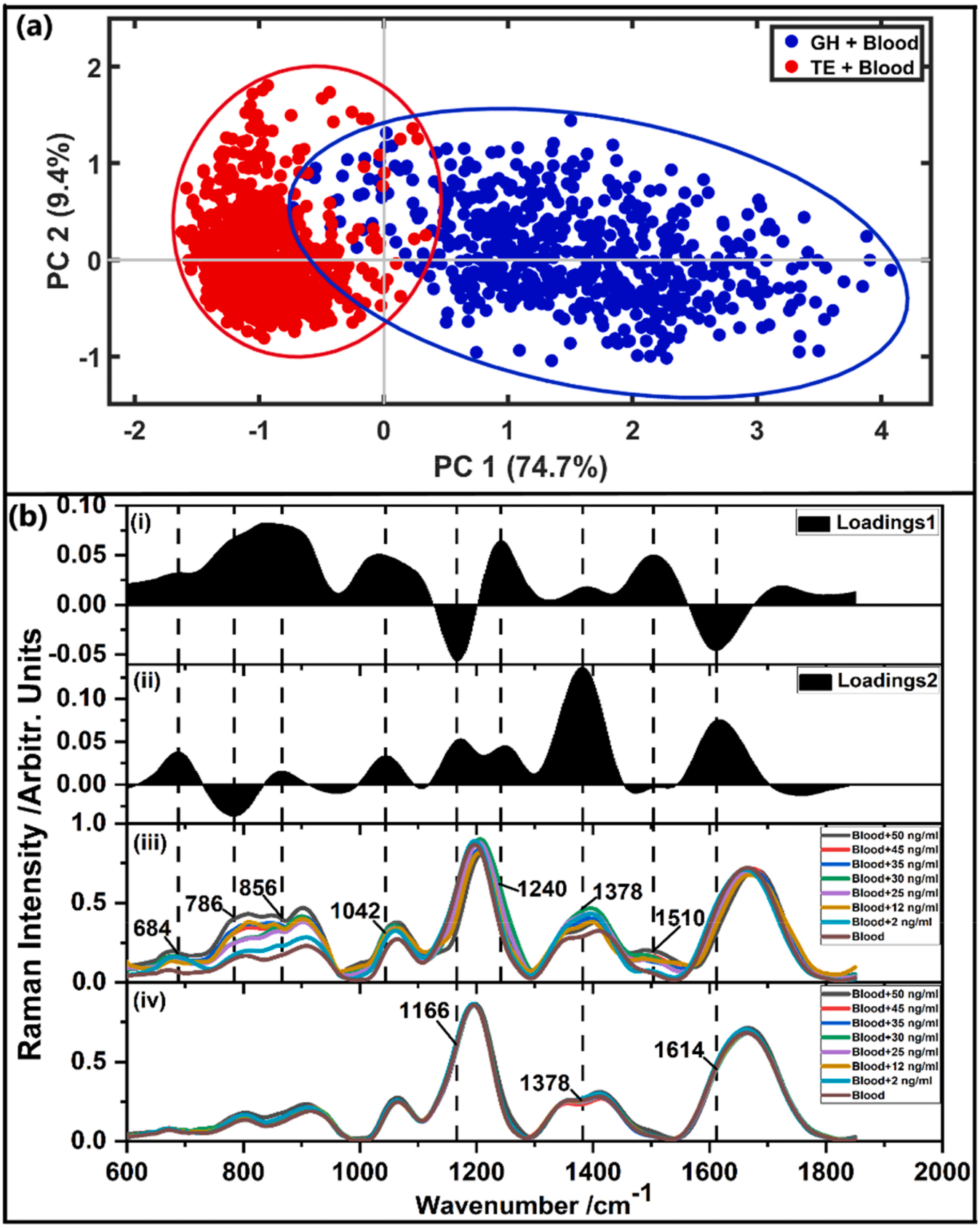

Fig. 4. (a) Two-dimensional PCA score plot for different hormone (GH and TE) concentrations in blood and (b) PCA loading plots ((i) and (ii)), Raman spectra of Growth hormone in blood (iii), and testosterone in blood (iv). The explained variances are indicated in percentages, and were 74.7 % and 9.4 % for PC1 and PC2, respectively.

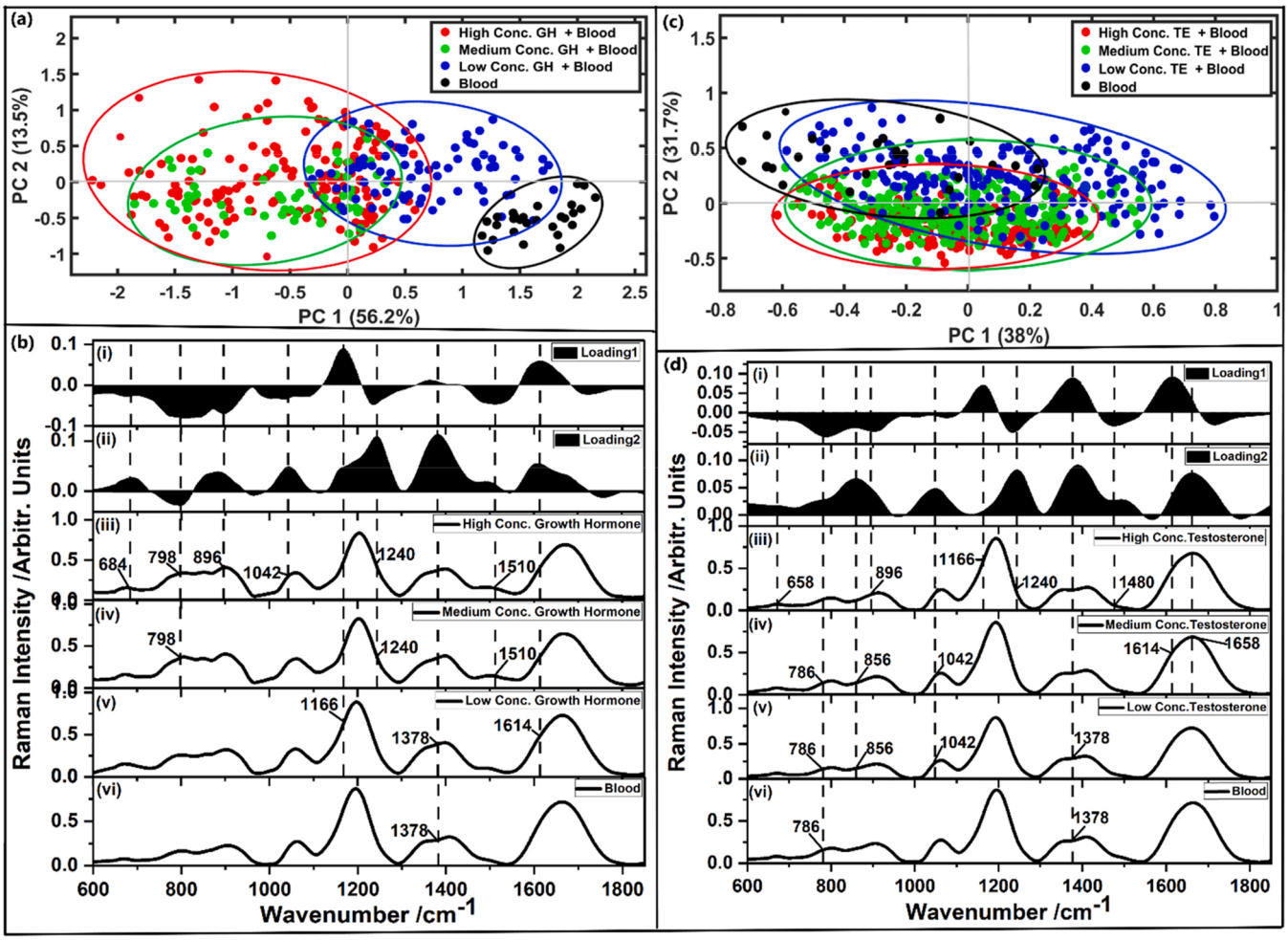

Fig. 5. The 2-dimensional PCA score plots and loading plots for GH and TE mixed with blood at different concentrations. The explained variances are indicated in percentages and were 56.2 % and 13.5 % for GH, and 38 % and 31.7 % for TE, for PC1 and PC2, respectively.

large and unique PC loadings) were those around 684 and 1614 cm^{-1} in GH and 786, 856, and 1490 cm^{-1} in TE. These bands qualify as biomarker bands for the respective hormones in blood, as they are also noticeable in the TE and GH spectra displayed in Fig. 5.

ANOVA was used to further confirm the concentration-sensitive bands obtained by PCA (Fig. 7). It was expected that ANOVA would reveal the specific spectral bands that showed significant differences across the groups (un-spiked blood, spiked blood with low, medium, and high concentrations). Thus, bands centered at wavenumbers with significantly different means could serve as potential biomarkers or concentration-sensitive bands.

As shown in Fig. 3, the spectral profiles of different concentrations of GH and those of TE in blood are fairly identical, with variations in the intensity of some bands (see Fig. 6(a) and (b)). The profiles were identical since all the hormones investigated here are naturally present in blood at various concentration levels. ANOVA results for each hormone (Fig. 7(a) and (b)) and those from the combined (both GH and TE) hormones (Fig. 7(c)) showed prominent bands centered at wavenumbers around 684 cm^{-1} (assigned to C–C Stretching) [37] and 1614 cm^{-1} (assigned to C–C stretching) [37] in GH, and 786 cm^{-1} (C–C stretching) [38,39], 856 cm^{-1} (assigned to C-C stretching) [40], and 1490 cm^{-1} (assigned to CH_2 bending) [33] in TE. Other concentration-sensitive bands noted in each of the two hormones were around 896 cm^{-1} (C–C Stretching) [32], 1042 (assigned to C–C stretching vibrations) [12,20], 1166 (assigned to C-O stretching and COH bending) [41], 1240 (assigned to Amide III) [32,41], and 1378 (C–C stretching) [21,27] (see Table 1). In addition, some variance was noted in bands centered at 1510 cm^{-1} (attributable to CH_2 bending) [12,33] for GH and 1636 cm^{-1} (assigned to Amide I) [28,42] for TE, which could be potential biomarker bands for GH and TE in blood, respectively. These bands were largely identical to those revealed by PCA (see Table 1) thus confirming them as of interest in SERS assaying of level changes of the two hormones (TE and GH) in blood.

To further reveal the most concentration-sensitive Raman bands based on the size of variance, a histogram was plotted for the significantly variant bands as shown in Fig. 8. As shown, GH level changes (blue bars) were significant in most of the bands with its change being proportional to that of the mixture i.e., GH+TE (red bars). Minimal changes in TE (green bars) were in 1490 and 1510 cm^{-1} which can serve as GH level change marker Raman bands in blood. The band around 684 cm^{-1} could serve as a GH+TE marker band as GH and TE individually varied minimally. The highest intensity variation with TE concentration was in bands centered at 1614 and 1636 cm^{-1}.

3.3. Discussion

The primary goal of this study was to obtain SERS marker bands for assaying GH and TE in blood. Since these hormones are normally present in blood, the SERS spectral profiles from blood with various concentrations of GH and those with TE (Fig. 3) displayed comparable spectral profiles. The prominent bands noted were centered around 658 (C-O-C stretching), 798 (C–C bending), 910 (C-O/C–C), 1064 (C–C stretching), 1190 (C–C stretching), 1338 and 1410 (CH_2 wagging of proteins), 1490 cm^{-1} (CH_2 bending), and 1658 cm^{-1} (carbonyl stretching of

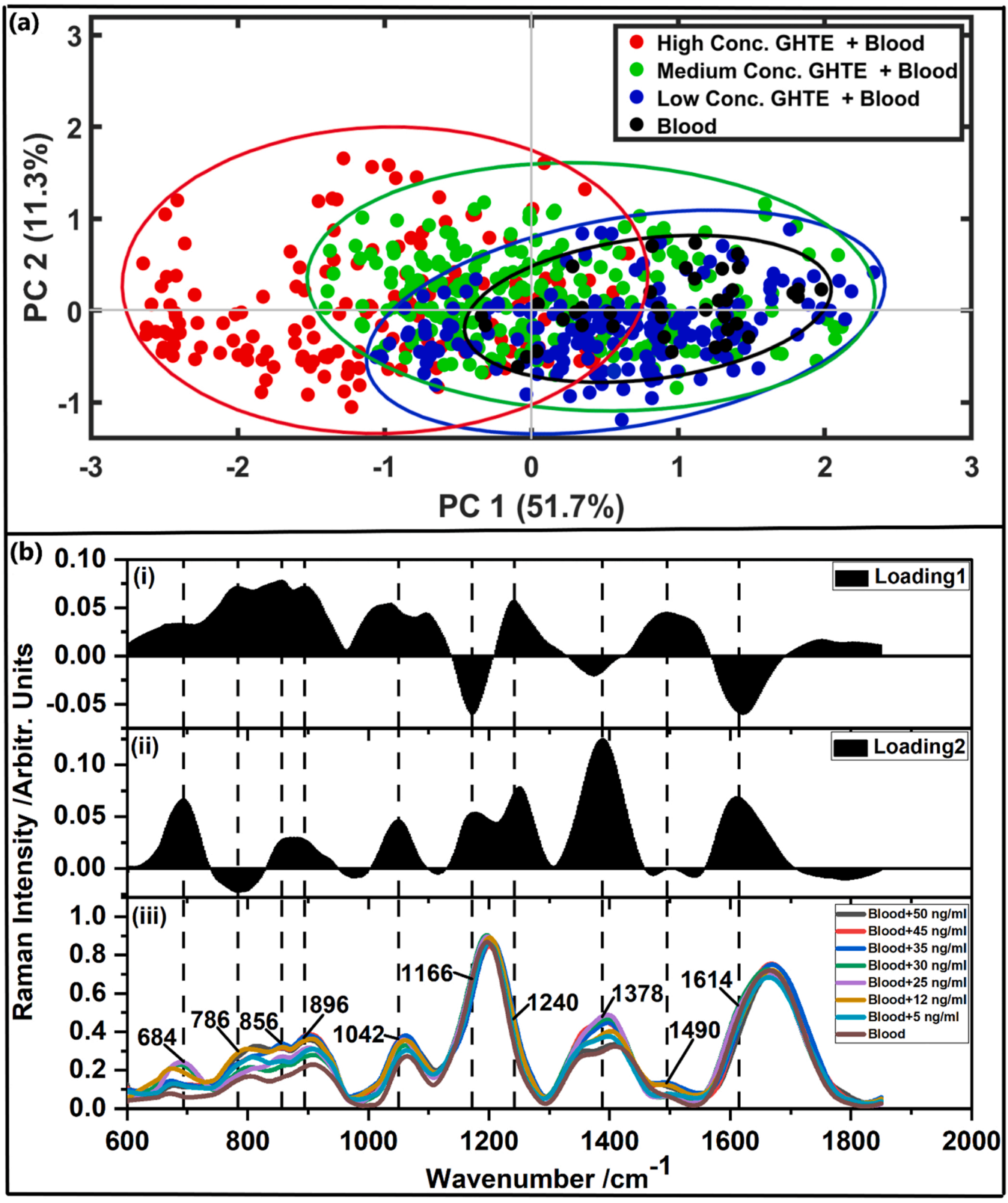

Fig. 6. (a) Two-dimensional PCA score plot, (b) PCA loading plots ((i) and (ii)), and Raman Spectra of blood (iii), with both GH and TE mixed at different concentrations. The explained variances are indicated in percentages and were 51.7 % and 11.3 % for PC1 and PC2, respectively.

proteins) [12,21,23,27,28,32,33]. When these profiles were compared to those obtained by [34,35,43], it was clear that the SERS substrates (AgNPs generated by laser ablation in liquids) used in this work were viable. In addition, the vibrational assignment of these significant bands corresponded closely with those in [12,21,23], implying that SERS can provide a sample's molecular-specific information. A noticeable difference was detected between the two spectral patterns where the intensity of most bands from blood with various concentrations of GH were more

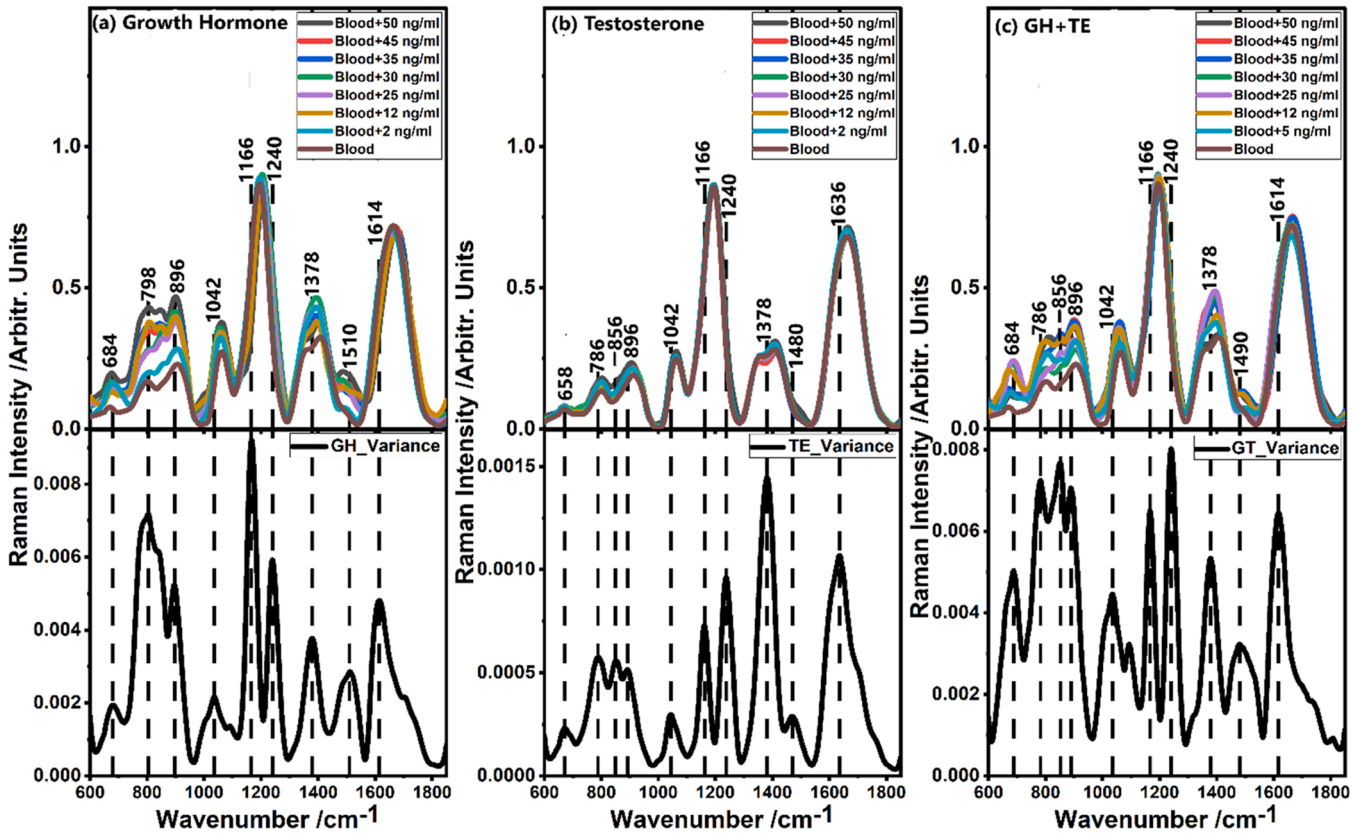

Fig. 7. Analysis of Variance results for SERS spectra for different concentrations of a) GH, b) TE, and c) both GH and TE mixed with male SD rat blood.

Table 1
Raman bands obtained from PCA and ANOVA for hormones (GH and TE) in blood.

Wavenumber	Growth hormone		Testosterone		Growth hormone + Testosterone		Vibrational Assignment
	PCA	ANOVA	PCA	ANOVA	PCA	ANOVA	
658			√	√			C–O–C stretch[12]
684	√	√			√	√	C–C stretch[37]
786			√	√	√	√	C–C stretching[38,39]
798	√	√					C–C bending[12]
856			√	√	√	√	C–C Stretch[40]
896	√	√	√	√	√	√	C–C Stretch[32]
1042	√	√	√	√	√	√	C–C Stretch[12,20]
1166	√	√	√	√	√	√	C–O Stretch and COH bending[41]
1240	√	√	√	√	√	√	Amide III[32,41]
1378	√	√	√	√	√	√	Stretch (COO-) and C–H bend[20]
1490			√	√	√	√	CH_2 bending[33]
1510	√	√					Amide II[29]
1614	√	√	√		√	√	C═C stretch[28]
1636				√			Amide 1[28,42]
1658			√				C-O/C═O Stretching[12,27]

responsive to changes in concentration compared to those of TE. The PCA and ANOVA results for various concentration of GH, TE and those from the combined (both GH and TE) hormones (Figs. 5–7) showed prominent bands centered at wavenumbers; 684 (assigned to C-C Stretching) [37]; 786 (C-C stretching) [38,39]; 856 cm^{-1} (assigned to C–C stretching) [40]; 1490 (assigned to CH_2 bending) [33]; 1510 (attributable to CH_2 bending) [12,33]; 1614 (assigned to C-C stretching) [37]; and 1636 cm^{-1} (assigned to Amide I) [28,42]. In addition, when these prominent bands for each hormone were examined and compared (Fig. 8), it was evident that changes in the GH levels had a significant impact on most bands. These changes were proportional to those seen in the mixture of GH and TE, indicating that variations in band intensity for the mixture concentration were closely related to GH concentration. Conversely, minimal band variations (more especially those centered around 1490 and 1510 cm^{-1}) were noted in response to TE level changes, suggesting that TE had a less pronounced effect on the spectral bands compared to GH. This implied that SERS is highly sensitive to detect even subtle differences between the samples. The concentrative-sensitive for the respective hormones deduced included 1490 and 1510 cm^{-1} (for GH), 1614 and 1636 cm^{-1} (for TE), and 684 cm^{-1} (for GH+TE).

The marker SERS band acquired in this study could be used to create

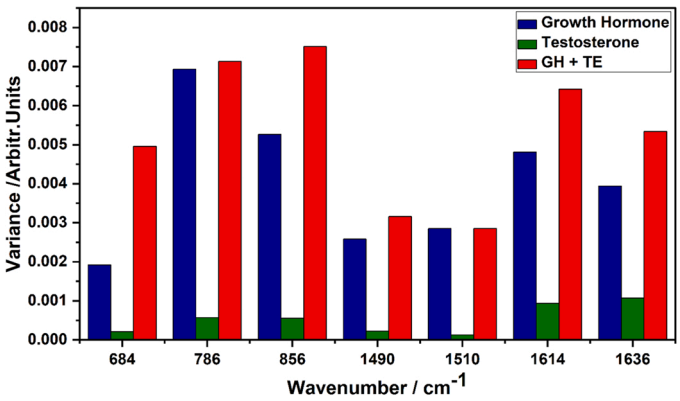

Fig. 8. Concentration-sensitive SERS band variation for each hormone.

and configure a portable Raman spectrometer specifically intended for GH and TE hormone detection. This could be accomplished by developing a SERS spectra library based on concentration-sensitive SERS bands for the two hormones that could be used to calibrate a SERS portable device. This device would be small, easy to use, and capable of providing real-time assay results for GH and TE levels in blood samples in less than a minute. This innovative approach could have significant implications in sports medicine for monitoring hormone levels in athletes for doping control and performance enhancement assessment. It should be made clear that no SERS system has been specialized to perform such tests, as many people are unaware of its existence and potential.

4. Conclusion

This research demonstrated the potential use of the SERS technique for assaying GH and TE hormones in blood, marking a novel application in hormone analysis, in which the following are important:

- The distinct spectral profiles observed for pure hormones in this study highlight the possibility of using SERS as a tool for quality checks in commercial hormone samples, thereby introducing a novel quality control application.
- The identification of concentration-sensitive SERS bands (1490 and 1510 cm^{-1} in GH; 1614 and 1636 cm^{-1} in TE; and 684 cm^{-1} in the mixture (GH+TE)) that showed significant intensity variations with GH and TE concentrations provides a unique and sensitive method for hormone level determination.
- Future research directions include developing a customized SERS system adapted for GH and TE assays in blood, employing SERS metallic nanoparticles of constant size and shape to ensure repeatability, and emphasizing innovation and precision in the methodology.
- These findings pave the way for a larger application of SERS in a variety of domains, including sports science, clinical diagnostics, and biological research, demonstrating its adaptability and potential effects.

CRediT authorship contribution statement

All of the experiments were carried out by Annah Ondieki, a doctorate student in physics, with the assistance of Moses Juma and Boniface Chege, and the data were analyzed and interpreted under the supervision of Dr. Birech, Prof. Kaduki, and Dr. Waweru. All supervisors directed Annah Ondieki's paper writing.

Declaration of Competing Interest

There are no financial interests or personal relationships that could have appeared to influence the work reported in this paper and potential competing interests don't exist.

Data Availability

Data will be made available on request.

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