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Chemical fingerprinting and quantitative monitoring of the doping drugs bambuterol and terbutaline in human urine samples using ATR-FTIR coupled with a PLSR chemometric tool†

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The use of performance-enhancing drugs is prohibited in sports competitions according to the World Anti-Doping Agency (WADA) regulations. Here, ATR-FTIR spectroscopy coupled with a partial least squares regression (PLSR) chemometric tool was used for the detection of the misuse of such substances. Bambuterol and its metabolite terbutaline have been included in the list of prohibited doping agents. Therefore, we used bambuterol and terbutaline as models for the accurate and simultaneous qualitative and quantitative analysis of bambuterol and terbutaline in human urine samples. The method was straightforward and once the urine samples were collected, they could be directly applied to the surface of the ZnSe prism (ATR unit) to get the results within one minute. A calibration set with a partial factorial design was used to develop the PLSR model that could be used to predict the concentration of unknown samples containing the two drugs. The developed method was carefully validated and successfully applied to the urine sample analysis of human volunteers. The drugs were quantified at nanogram level concentrations. A side-by-side comparison of the proposed method with the routine GC-MS method was performed to demonstrate the challenges and opportunities of each method.

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1. Introduction

Forensic analysis is expanding, as confirmed from the hundreds of advanced studies published annually.^{1,2} The World Anti-Doping Agency (WADA) establishes the regulations concerning doping drugs across all sports all over the world.³ The use or attempted use of prohibited substances by athletes is considered a violation of these rules. Since such issues correspond closely to criminal cases under forensic investigations, urine samples are collected in and out of competitions and exposed to a variety of analytical examinations developed to monitor the presence of a banned substance.^{4,5}

The WADA rules classify β_2 -agonists such as bambuterol HCl (BAM) and terbutaline (TER) as stimulants and anabolic agents

with a restricted specific regulation.³ Athletes often use BAM for therapeutic purposes as a treatment for exercise-induced asthma.⁶ However, there are some adverse effects of the use of β_2 -agonists, such as anabolic and stimulation effects with the increase in myocardial contraction and heart rate, increase in the accretion of muscle proteins and a reduced rate of fat deposition;⁷ thus, this drug and its metabolite TER⁸ have been included in the list of prohibited doping agents.³ The detection of BAM and TER as doping drugs for routine analysis in urine samples has been accomplished mainly by chromatography techniques such as GC/MS^{9,10} and LC-MS/MS.¹¹ Although these methods have high potential in the fight against doping drugs, they suffer from many disadvantages, such as the use of very expensive instruments, being non-portable, need for highly qualified operators, high operation cost, moderate throughput, limitations with respect to solvents and samples, long operation times, and too many sample preparation steps.

It has been proven that attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) has ability for the quantitative and/or qualitative determination of biological factors and samples such as lung and prostate cancer biomarkers,¹² protein analysis,¹³ and petroleum component analysis.¹⁴ Chemometrics is a well-established technique in forensic analysis,^{15,16} and partial least squares regression (PLSR) does not require the accurate knowledge of all the components

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present in urine samples. It can make concentration predictions even in the presence of interfering compounds.¹⁷ Also, the direct application of urine samples to the surface of the ATR prism as a sampling technique is sufficient for the quantitative determination of the early-stage malaria parasite,¹⁸ enzyme kinetics parameters,¹⁹ biomarkers of glomerulonephritis,²⁰ tumor cells,²¹ and malignant ovarian tissues.²² A recent study compared the performances of micro-ATR-FTIR and GC-MS as identifying tools for micro-plastics and fibers isolated from different water sources.²³

Therefore, this paper describes the application of ATR-FTIR in combination with a PLSR chemometric tool for the determination of the doping drugs BAM and TER. Low detection limits were obtained due to the high selectivity, sensitivity, portability, high throughput analysis, and straightforward nondestructive direct determination capabilities of PLSR/ATR-FTIR.^{24–26} Only a few microliters (μL) of urine samples were sufficient for the quantitative determination of the analytes. The developed anti-doping method may be considered as a preliminary step, opening the way for further investigations on other doping agents.

2. Experimental procedures

2.1. Materials

BAM was supplied from AstraZeneca, Egypt. TER was obtained from (Sigma-Aldrich, Germany). Bambec® tablets (AstraZeneca Ind Co., Egypt) labeled to contain 20 mg of BAM per tablet (Batch No. 120065) were purchased from the local community pharmacies, Cairo, Egypt.

2.2. Standard and working solutions

Powdered BAM and TER equivalent to 0.01 g were separately placed into two 10 ml volumetric flasks (VFs). The volumes were completed to the mark using human urine. Then, they were stirred until completely dissolved to get $1000 \mu\text{g ml}^{-1}$ solution. Only one milliliter was transferred into a 100 ml VF and the volume was completed to the mark using human urine to get a working solution of $10 \mu\text{g ml}^{-1}$.

2.3. Instrument

An IRaffinity-1 FTIR Spectrometer, (Shimadzu, Japan) fitted with a high energy ceramic light source (single beam), He-Ne laser for alignments, germanium-coated potassium bromide beam splitter, and DLATGS detector equipped with a temperature controlling unit was used in the analysis. A horizontal ATR accessory model 8200HA (PIKE Technology, USA) fitted with a zinc selenide (ZnSe) crystal, which is a rectangular-shaped yellow prism (dimensions: 80 mm long, 10 mm wide, 4 mm thick), was used.

2.4. Software

The FTIR instrument was controlled by IRSolution software, version 1.6 (Shimadzu, Japan). The ATR-FTIR absorption spectrum of each sample was obtained with software parameters of SqrTriangle apodization function and 45 scans using a high spectral resolution of 4 cm^{-1} in the mid-infrared region from 4000 to 400 cm^{-1} ; thus, each spectrum gave 2024 data points at

1.93 intervals. The Unscrambler software version X 10.4 (CAMO Software, Norway) was used for PLSR chemometric calculations.

2.5. Experimental design for the PLSR model

The experimental design is a significant factor in chemometric measurements. The calibration set concentrations have to be carefully designed to increase the chances of extracting useful information from the prediction samples. To get a representative calibration set, a multilevel partial factorial design²⁷ of two factors (BAM and TER) and five concentration levels was constructed. It required about 25 experiments to study the mixtures, as shown in Table 1. This orthogonal design was obtained after numbering the levels (*i.e.*, the concentrations) from +2 (highest) to -2 (lowest) to get a cyclic generator (-2, -1, 0, 1, 2) to ensure that there is no correlation between the under-investigation analyte concentrations so that the correlation coefficient will be zero.

A representative set of 25 mixtures was prepared by taking the proper volumes from the prepared working solutions into the volumetric flasks. Then, the volumes were completed to the mark with human urine. The prepared mixtures were separated into two sets: a calibration set of 15 synthetic mixtures and a validation set of 10 mixtures (Table 1).

2.6. Spectral data acquisition and calibration steps

2.6.1. Background scan to remove any interference. A background scan was conducted; only 10 μL of human urine

Table 1 The concentration levels of the calibration and validation sets

Coded levels				
BAM	TER	MIX no.	BAM (ng ml^{-1})	TER (ng ml^{-1})
0	0	1	5000	5000
0	-2	2	5000	50
-2	-2	3	50	50
-2	2	4	50	10 000
2	-1	5	10 000	2500
-1	2	6	2500	10 000
2	0	7	10 000	5000
0	-1	8	5000	2500
-1	-1	9	2500	2500
-1	1	10	2500	7500
1	2	11	7500	10 000
2	1	12	10 000	7500
1	0	13	7500	5000
0	2	14	5000	10 000
2	2	15	10 000	10 000
2	-2	16	10 000	50
-2	1	17	50	7500
1	-2	18	7500	50
-2	0	19	50	5000
0	1	20	5000	7500
1	1	21	7500	7500
1	-1	22	7500	2500
-1	-2	23	2500	50
-2	-1	24	50	2500
-1	0	25	2500	5000



was placed over the ATR prism and air-dried for two minutes and scanned as a background spectrum. The recorded background spectrum was stored in the computer to be subtracted from each real sample spectrum.

2.6.2. Spectral data acquisition of calibration and validation sets. For each real sample analysis, only 10 μ L from each mixture sample was dropped over the dry surface of the ZnSe prism and air-dried for two minutes. The ATR-FTIR scanning of real samples was started against the recorded background spectrum. Fig. 1A shows an overlay plot of the calibration set mixtures. Each sample was measured in triplicate to ensure method precision and reproducibility. A phosphate buffer and

ethanol were used for crystal surface cleaning between the measurements.

Complete sample dryness was ensured by the application of 10 μ L of human urine over the ATR prism; then, a spectrum was obtained each minute during the air-drying process. Complete sample dryness occurred after two minutes. The cleanliness of the ATR prism was tested regularly between the measurements, where an absorption spectrum was recorded without the sample to make sure that it had no peaks higher than the normal environmental noise level.

2.6.3. 1st derivative Savitzky–Golay algorithm. Savitzky–Golay smoothing and derivative algorithm are a common signal

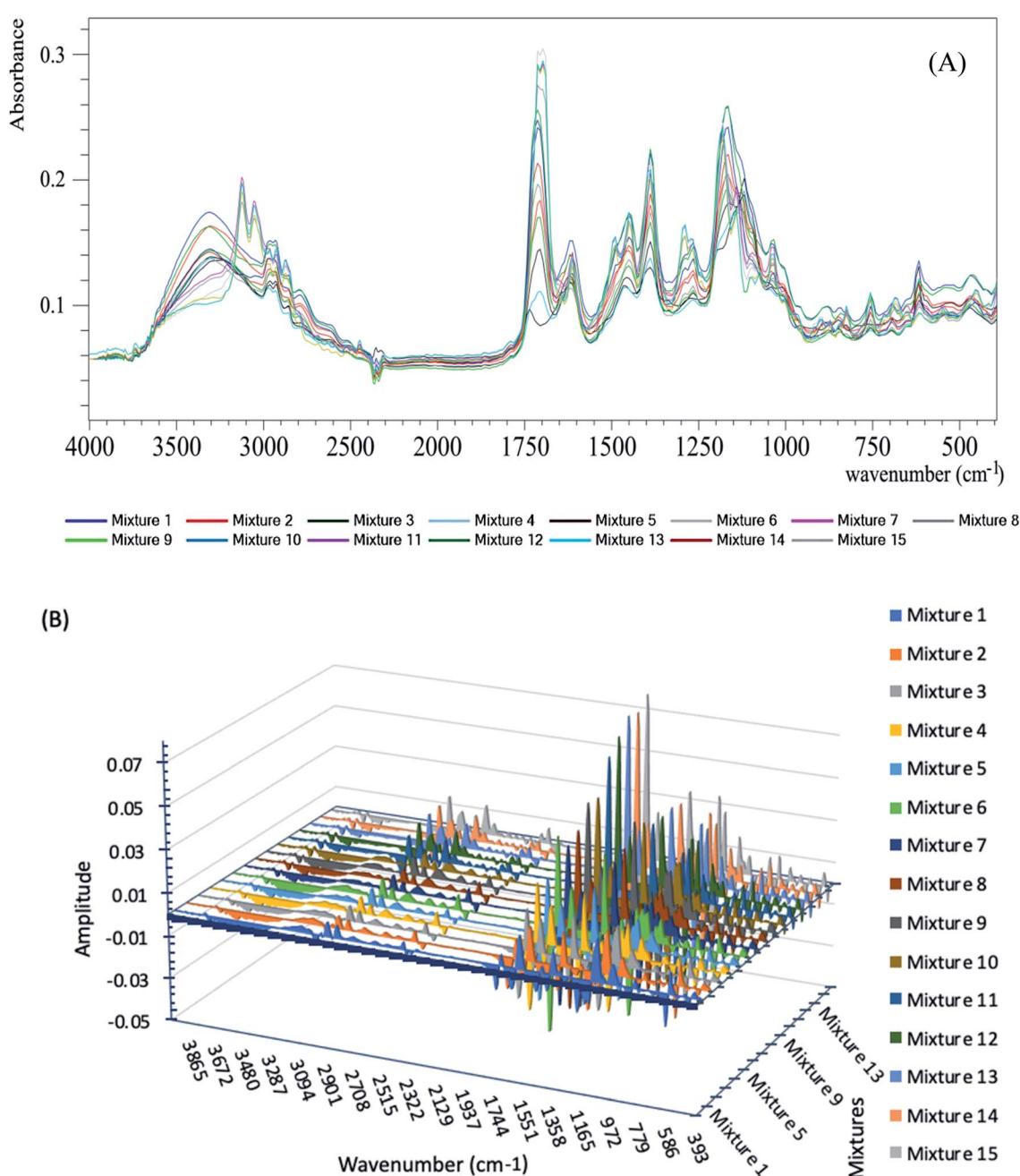


Fig. 1 (A) ATR-FTIR absorption spectra of the calibration set mixtures; (B) 3D plot of the 1st derivative of the calibration set mixtures.



smoothing methods in chemometrics. This algorithm is based on achieving a linear regression fit of a polynomial window around each point in the spectrum with the aim of data smoothing; then, the derivative calculation is applied.²⁸

The derivative calculations were applied to the recorded spectra from the earlier steps using the following parameters: derivative order: 1, polynomial order: 2 and smoothing points: 5, as shown in Fig. 1B.

These pre-processing steps could be registered on the Unscrambler X software to be performed automatically for the prediction of unknown sample concentrations.

Finally, the PLSR chemometric algorithm²⁹ was applied to the derivative spectra obtained from the last step (shown in Fig. 1B). The recorded absorbance spectra were related to the concentrations by Beer's law.³⁰ The Regression calibration curves were plotted between the known and predicted concentrations.

2.7. Analysis of drugs in urine samples

Healthy volunteers were fully informed verbally and in writing about the objectives and nature of this study. They received a single oral dose of 20 mg Bambec® tablets in the morning after breakfast and no extra food was allowed for at least two hours (h). Urine samples were collected in sterile screw-top containers in consecutive pools with at least 5 drops of each sample during random time intervals. The volunteers took a pre-dose sample and then at 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, and 12–24 hours after the dose. The collected urine volume was measured after every period. The containers were labeled with date and time and then stored at –80 °C. The concentrations of BAM and TER were determined following the described procedure. About 10 µL of the sample was transferred from each container to be placed over the ZnSe prism and rapidly air-dried. The samples were scanned using the same device parameters mentioned above and the collected data stored in a computer. PLSR chemometric calculations were performed on the stored spectra.

The study involving human participants was performed in strict accordance with the institutional ethical standards of the Helsinki Declaration of 1964 and its later amendments. It was approved by the local research ethical committee of faculty of pharmacy, Cairo University, Egypt. Healthy volunteers were fully informed verbally about the objectives, nature and possible risks of this study and a written informed consent was obtained from all the volunteers involved in the study.

3. Results and discussion

Based on the fact that an infrared spectrum is considered as a fingerprint of each compound, structurally related compounds can be differentiated using their spectra. To the best of our knowledge, this is the first method for the determination of BAM and TER as doping drugs in urine samples using the ATR-FTIR technique coupled with PLSR chemometrics. FTIR spectroscopy is a well-established technique with a wide application range. The direct application of biological samples over the ATR prism is a widely used sampling

technique with high sensitivity and selectivity, as reported in literature.^{18,20–22} The PLSR algorithm was chosen as it does not require prior accurate knowledge of all the components present in the samples under investigation.

The analysis of BAM in the presence of its metabolite TER is a challenge as they are structurally related compounds. Fig. 2 shows the structures of BAM and TER. BAM (*RS*-5-(2-*tert*-butylamino-1-hydroxyethyl)-*m*-phenylene bis dimethyl carbamate) is metabolized in the liver by the cleavage of the ester bond and replacement of the branches of *N,N*-dialkyl carbamate by OH groups to give TER.³¹ This hydrolysis causes the main differences in the structures.⁸

Fig. 2 shows the overlay plot of the two spectra of BAM and TER and their characteristic peaks can be distinguished. For more illustration, the spectra were analyzed by the assignment function of the ACD/SpecManager software to show the peak differences between BAM and TER, as summarized in Table 1S.† The FTIR spectra of BAM and TER can be considered as their own fingerprints. The BAM spectrum has a characteristic C=O group that shows a peak at 1720 cm^{–1}, which disappears in the TER spectrum. The proposed method was convenient for the simultaneous qualitative and quantitative determination of BAM and TER.

3.1. PLSR model calibration and validation

In order to get a representative calibration set, the calibration samples have to be distributed over the concentration space of both analytes. Thus, a representative wide range calibration set of uncorrelated mixtures was constructed. It was efficiently applied for the analysis of BAM with a linear range of 50–10 000 ng ml^{–1}, covering the reported excretion range of BAM in urine samples.¹¹ The method showed high accuracy over the calibration range with the mean recovery percent values of 98.92 and 99.97% for BAM and TER, respectively.

The performance of multicomponent analysis is based on the spectral model used and the wavelength selected. PLSR with a non-linear iterative partial least squares algorithm (NIPALS) was calculated using the Unscrambler software on the Savitzky–Golay first derivative algorithm for the selected spectrum range (400–4000 cm^{–1}), due to which all the available measured wavelengths are often used without neglecting any region that may contain important data.

The key step in building the PLSR model is the selection of optimum number of factors to achieve correct concentration prediction in PLSR calibrations to prevent data over-fitting. PLSR models with 1 to 7 factors were calculated for each wavelength region and validated using “leave-one-out cross-validation”.^{32,33} Briefly, the model is repeatedly recalculated leaving out a single sample individually at a time and then used to predict the concentration of the left out observation. PLSR calibration using 14-mixture spectra was performed and the regression equation was calculated. The concentration of the left-out sample was calculated using the obtained regression equation. The same procedure was employed for the 15-mixture analysis; each sample was left out once and the predicted concentration was calculated for each sample. The predicted concentrations were plotted against the known concentrations



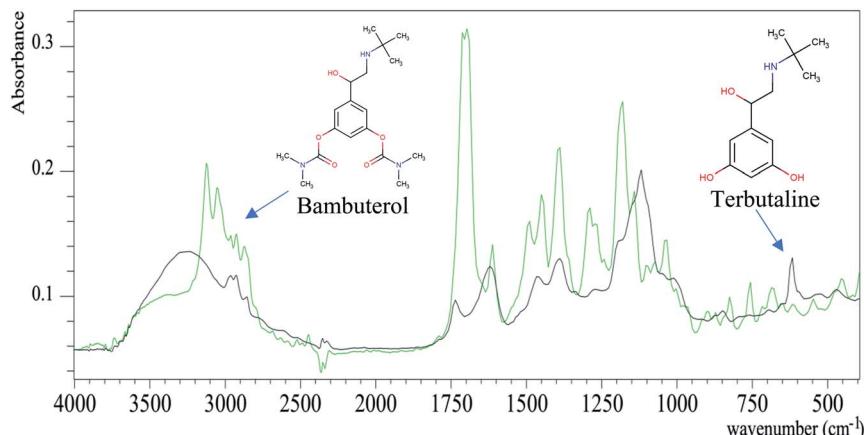


Fig. 2 ATR-FTIR absorption spectra of bambuterol (green) and terbutaline (black).

of each mixture. The obtained recoveries (between 95 and 105%) indicate the validity of the method for its application to unknown samples. The latent factors that explain the spectral matrix are sorted in decreasing order according to their contributions to the spectral features. Using the optimal pre-processing method, latent factors were selected with a cross-validation method, as mentioned above. This method depended on the selection of the model including the smallest number of factors that give the minimal root mean square error

of calibration (RMSEC) and the minimum root mean square error of validation (RMSEV), by plotting the difference of RMSEV against the number of factors for the analysis of each mixture. The PLSR method proved that (2) factors were found to be optimum for BAM and TER determination. The PLSR calibration model was constructed to achieve correct quantification and prediction with minimum errors.^{34,35}

The PLSR model's predictive ability was evaluated using two procedures: the 1st procedure was achieved by plotting the

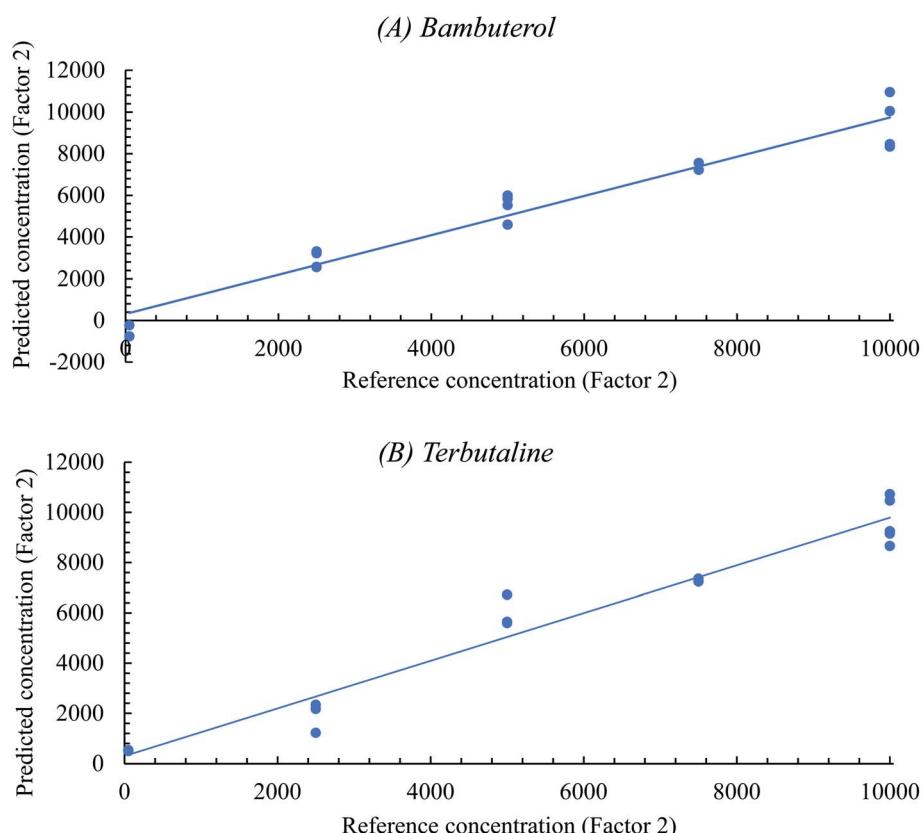


Fig. 3 Plots of reference concentrations against the predicted concentrations of the calibration sets of (A) BAM and (B) TER.



Table 2 The characteristic parameters of the PLSR chemometric method

Parameter	Compound	
	BAM	TER
Elements	15	15
Range (ng ml ⁻¹)	50–10 000	50–10 000
Slope	0.9842	0.9892
Offset	76.98	53.97
Correlation	0.9921	0.9945
<i>R</i> ²	0.9842	0.9892
RMSEC	456.01	365.67
SEP	432.87	510.50
Bias	13×10^{-4}	13.3×10^{-4}

reference concentrations against the corresponding predicted concentrations, as shown in Fig. 3; then, the internal validation method was performed (estimation of BAM concentrations in its own calibration set). A satisfactory correlation coefficient (r^2) (0.9988) and good recovery (101.09%) were obtained, indicating high predictive abilities of the model. The 2nd procedure for the

determination of the predictive ability of the method was conducted by calculating the standard error of prediction (SEP).³⁶

Table 2 summarizes the characteristic parameters of PLSR chemometric urine analysis. The obtained very low BIAS (0.00048) means a very small acceptable error of prediction. Correlation is the linear relationship between the predicted and reference values in the plot, which is very close to 1, indicating that the predicted and reference concentrations are highly correlated. RMSEC measures the dispersion of the calibration samples around the regression line. BIAS is the mean of all the numbers that lie above or below the regression line and the values close to zero show a random distribution of points around the regression line. The obtained RMSEC and standard error of calibration (SEC) are very close, indicating insignificant BIAS.

External validation was achieved by a validation set of 10 mixtures and it was not implemented in model building. The concentrations of BAM and TER in the validation set solutions were selected randomly but were in the range of the calibration set. The method has high resolving power with good mean recoveries (97.3 and 99.1%) for BAM and TER, respectively.

Regression coefficient plots were obtained to summarize the relationship between all predictors and responses by analyzing the 15-mixture 1st derivative spectra to find the areas

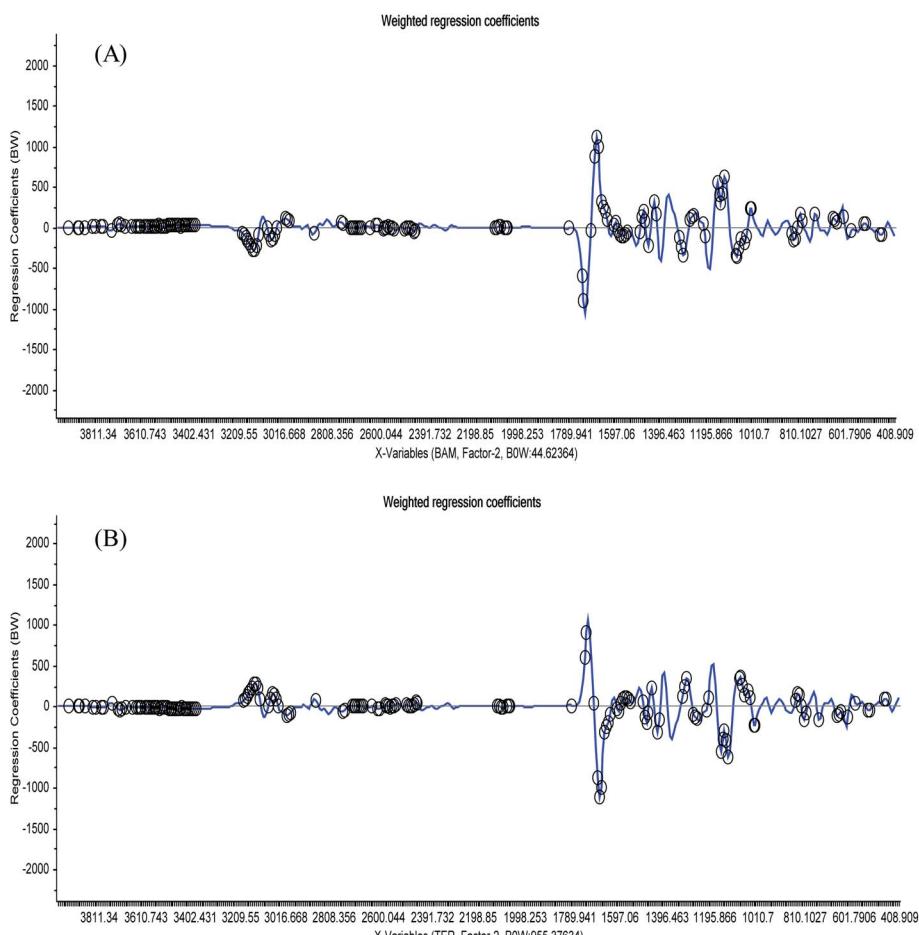


Fig. 4 Plots of the regression coefficient weights against the wavelength variables of the 1st derivative of (A) BAM and (B) TER. The important variables used by the software during building the PLSR model are highlighted by black circles except the spectral areas above 3250 cm⁻¹.



(highlighted with black marks) that the software depended on during the building of the model for the detection of either BAM or TER, as shown in Fig. 4. This plot proves and validates the ability of the developed method to differentiate between BAM and TER within the matrix of urine components.

3.2. Quantification of BAM and TER in human urine samples

The proposed PLSR chemometric algorithm was applied for the quantification of BAM and TER in the collected urine samples. They can be detected and quantified in the urine of healthy volunteers up to about 24 h after receiving a single oral dose of Bambec® tablets.

Urine samples collected from the same volunteer before and after the oral administration of the Bambec® tablets were scanned. The urine sample that did not contain any drug was used as a blank and scanned as a background spectrum. Then, the samples collected after the oral administration of Bambec® tablets were scanned against this background to subtract any substances that may be found in urine and may interfere with the analysis results. Thus, the obtained peaks were due to the presence of BAM and TER in urine with different concentrations, as confirmed from Fig. 1S.†

Fig. 5 shows the obtained spectra of human volunteers' urine at different times (1, 2 and 6 h). It can be easily noticed that the peak intensity moderately increases with time, which may be due to the increased excretion of the drugs in urine with time.

The obtained spectra were highly related to the spectra of original BAM and TER shown in Fig. 2.

The mean cumulative excretion profiles of BAM and TER in healthy volunteers are shown in Fig. 6. It shows that the highest drug concentration in urine is detected within the first six hours; then, the concentration decreases gradually with the analysis time. Most of the drugs (BAM and TER) were eliminated within 10 hours of oral administration. The maximum elimination rates of BAM and TER were 180 and 530 $\text{ng ml}^{-1} \text{h}^{-1}$, respectively, and this was observed within 0–5 h after their oral administration.

3.3. Challenges and opportunities of the proposed method in comparison with GC-MS

In order to compare the proposed ATR-FTIR multivariate method with the reported GC-MS method³⁷ for the quantification of β_2 -agonists in human urine, a statistical comparison was applied. The developed ATR-FTIR-PLSR algorithm was tested for the quantification of BAM and TER. Then, the obtained results were compared with the reported data, as summarized in Table 3. The computed t and f values were compared to t and f tabulated values. The computed values were smaller than the tabulated values, indicating that there was no remarkable difference between the results obtained through different methodologies and the proposed method was suitable for the quantification of BAM in biological samples.

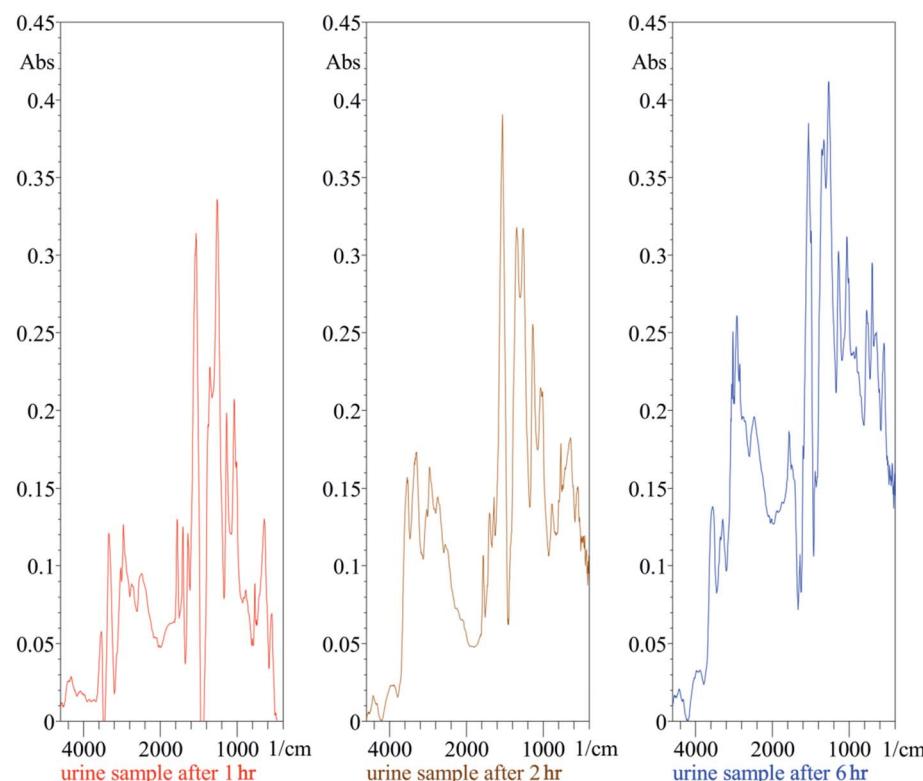


Fig. 5 Typical ATR-FTIR spectra of the urine samples obtained from volunteers at various time intervals (1, 2 and 6 h) after a single oral dose of 20 mg bambuterol tablet.



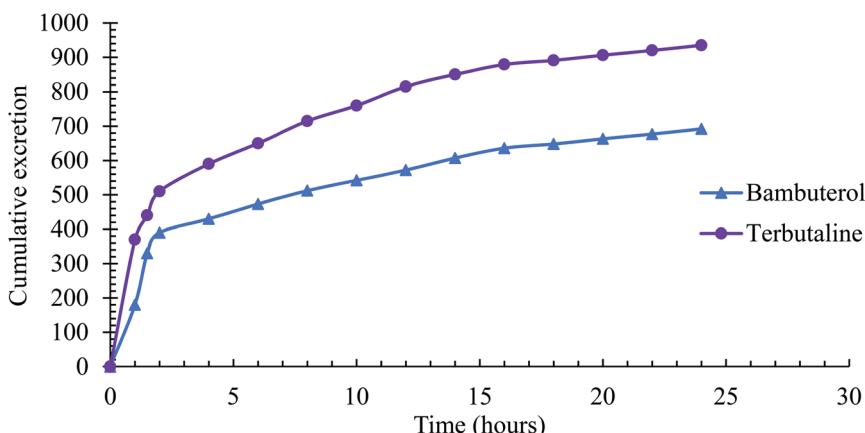


Fig. 6 Cumulative excretion of BAM and TER in urine samples of healthy volunteers after a single oral dose of BAMBI[®] tablet.

Table 3 Statistical comparison of the proposed method and the reported GC-MS method

Parameter	PLSR chemometrics urine analysis		GC-MS reported method ref. 9	
	BAM	TER	BAM	TER
M.R.%	98.92	99.97	98	100.2
S.D.	0.982	1.67	0.4	0.3
N	5	5	5	5
Standard error	0.439	0.747		
Student's <i>t</i> test ^a	1.94 (2.31)	0.303 (2.31)		
<i>F</i> test ^a	0.166 (6.39)	0.032 (6.39)		

^a The values in parenthesis are the corresponding theoretical values of *t* and *f* at the 95% confidence level.

Obviously, the ATR-FTIR method was straightforward; once the sample was collected (10 μ L of urine), it could be directly placed over the ZnSe prism and scanned using the FTIR device to detect if the doping agent is present or not. Also, it has many advantages, which are as follows: nondestructive techniques, a wide range of samples with different masses can be analyzed, provides a spectrum of the analyte, which is considered as a fingerprint of each compound, only one minute is needed to get the results, and no chemical extraction or sample pre-treatment is required.

The disadvantages of the GC-MS method are as follows: it is a destructive technique, *i.e.*, measurements cannot be repeated using the same sample, inorganic compounds cannot be detected, nonvolatile substances have to be chemically modified to be volatile, several sample pre-treatments have to be performed before measurements, not portable, chemical extraction steps of the analytes from the urine samples are required, analysis time is nearly 15 minutes for each sample, needs highly qualified operators, and the instruments are very expensive.

However, it gives complete data about the chemical composition of the sample and contamination that may be present in the sample.

The application of ATR-FTIR as a universal technique for the determination of doping drugs still needs much more investigation and application to all the doping drugs under all possible

conditions. Our study can be considered as a preliminary study that opens the way for much more ATR-FTIR measurements for doping control.

4. Conclusion

The experiments illustrate that ATR-FTIR coupled with a chemometric tool is qualitatively and quantitatively useful for the determination of the doping drug BAM and its metabolite TER in human urine samples. The drugs can be detected in the matrix of human urine without any sample pre-treatment or several preparation steps. Only 10 μ L sample was sufficient for the analysis, which is less than one drop of volume and nearly the same as the volume used for regular HPLC injections. It was also a viable approach for the quantification of very small concentrations that reach nanogram levels with high selectivity and accuracy.

It has many advantages in comparison with the traditional techniques by eliminating the errors during sample pre-treatment that can consume time and may lead to bad recoveries.

Conflicts of interest

The authors declare that there is no conflict of interest.



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