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Research Article

A Non-invasive Method for the Determination of Liquid Injectables by Raman Spectroscopy

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Abstract. Drug safety has become a very important subject, and more countries have joined in the fight against counterfeit drugs. This study demonstrated a non-invasive Raman spectroscopy method that could be utilized for screening liquid injectable drugs for spurious/falsely-labeled/falsified/counterfeit medical products (SFFCs). Two problems were solved to remove the blocks in identification and quantitation: one problem was the weak API signal extraction from the non-invasive Raman spectra and the other was the problem of Raman absolute measurement. Principal component analysis (PCA) and classical least square (CLS) algorithms were performed to establish the models. Water was chosen as the "internal standard" to normalize the spectra to solve the problem of Raman absolute measurement. The results showed that the 11 positive samples and 66 negative samples were all well identified with a threshold of 0.95. One of the positive samples contained the excipient propylene glycol, which was identified successfully at the same time. The accuracy of quantitative results was approximately 5% for doxofylline liquid injectables and about 10% for the low-concentration and big glass bottle-containers of Levofloxacin Lactate and Sodium Chloride Injections as compared to the results using an HPLC method, this is satisfactory for fast screening of SFFCs. In conclusion, with the development of a database of identification and quantitation models, this method may determine liquid injectable drugs in a fast and non-invasive way and become one of the most powerful weapons against SFFCs.

KEY WORDS: CLS; liquid injectables; non-invasive Raman fast screening method; PCA; SFFCs (Spurious/falsely-labeled/falsified/counterfeit medical products).

INTRODUCTION

Drug safety has always been a focus in the health care system. Spurious/falsely-labeled/falsified/counterfeit medical products (SFFCs) is a newly defined term by the World Health Organization (WHO) to replace the previous term of "Counterfeit drugs" and is defined as medicines containing incorrect amount of active pharmaceutical ingredients (APIs) or toxic or contaminated materials that result in the patient being untreated or poisoned. In these cases, public health and safety are threatened and public confidence in the health systems may be eroded following use and/or detection of suspected SFFCs. Drug alerts on SFFC use from WHO involve almost every region of the world (1,2).

Traditional wet chemical methods and chromatography methods for screening of SFFCs are effective, but are time consuming and invasive. Instead, spectroscopic methods have become the most highly efficient and non-invasive methods in battling SFFCs over the past 20 years (3,4). However, most of the work has been done with drugs in solid dosage forms, such as tablets and capsules (5,6), while there is an increasing demand for rapid, non-invasive analysis of liquid injectables in ampoules, vials, or bottles. Near infrared (NIR) has proven to be an effective tool to measure tablets and capsules either in diffuse reflectance or diffuse transmittance. Furthermore, tablets can even be measured with NIR through plastic blister packaging material. However, when applying NIR to liquid injectables, especially those in the aqueous phase, samples are measured in transmittance without breaking the containers, so the pathlength of measurement will depend on the diameter of the injectable ampoules, vials, or bottles. These diameters vary enormously and in general the pathlengths of the containers are too big to acquire a reliable NIR spectrum. Therefore, NIR is inappropriate as the universal solution for liquid injectables, especially in thick bottles. On the other hand, water has extremely strong absorbance in the NIR spectra, which makes it difficult to determine the API in the solution.

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Table I. Information about Liquid Injectables Samples

Samples No.	Products	API	Excipient	Package	Labeled amount
1	Manufacturer A	Doxofylline	NONE	Ampoule	10 mL:0.1 g
2	Manufacturer B	Doxofylline	Propylene glycol	Ampoule	10 mL:0.1 g
3	Manufacturer C	Doxofylline	NONE	Ampoule	10 mL:0.1 g
4	Manufacturer D	Doxofylline	NONE	Ampoule	10 mL:0.1 g
5	Manufacturer E	Doxofylline	NONE	Ampoule	10 mL:0.1 g
6	Manufacturer E	Doxofylline	NONE	Ampoule	20 mL:0.3 g
7	Manufacturer D	Doxofylline	NONE	Ampoule	20 mL:0.3 g
8	Manufacturer A	Doxofylline	NONE	Ampoule	10 mL:0.2 g
9	Manufacturer F	Levofloxacin	NONE	Bottle	100 mL:0.2 g
10	Manufacturer G	Levofloxacin	NONE	Bottle	100 mL:0.2 g
11	Manufacturer H	Levofloxacin	NONE	Bottle	100 mL:0.2 g

Raman spectroscopy is a scattering-based technology, and the measurement point is at the focus point of the laser beam. Various focus lengths in the range of a few millimeters or longer can be achieved by using different optics. Raman spectroscopy contains rich chemical and structural information for pharmaceutical materials, while water and glass have weaker signals in Raman spectroscopy than that in infrared spectroscopy. For these reasons, the Raman spectrum of a liquid sample inside an ampoule, vial, or bottle can be acquired non-invasively by choosing fiber optic probes with suitable focus lengths. Thus, in principle, Raman technology may be the best, and possibly the only choice, for the non-invasive measurement of liquid injectables. However, limitations in manipulation and evaluation mean there are difficulties in eliminating the interference of different glass containers. Therefore, the existing studies in this field all measure Raman spectrum of samples removed from ampoules, vials, or bottles (7–9). The extraction of weak signals and Raman absolute measurement has been the key problems blocking the development of non-invasive Raman spectroscopy fast screening for liquid injectables.

In this paper, chemometric methods were used to overcome three problems: first, eliminating the interference of different glass containers; second, extracting the weak signals; and third, solving the Raman absolute measurement problems. At last, a non-invasive Raman fast screening method was developed to determine the APIs in liquid injectables. Weak, but useful, API signals could be extracted from the measured Raman spectra of liquid drugs in their glass containers for identification, and the problem of Raman absolute measurement could also be solved for quantitation. In our studies, the method was proven to be efficient by hundreds of different injectable drugs for both Identification and Quantitation. In this article, only the doxofylline injectable and Levofloxacin Lactate and Sodium Chloride injections were used to demonstrate the results of this method.

MATERIAL AND METHODS

Apparatus and Software

Portable Raman spectrometer, Metage OPAL-3000 (Metage Scientific, Banbury, UK), was used for this study. All spectrometers are equipped with a fiber optic probe, a 785-nm diode laser excitation source with a maximum output power of 400 mW and a TE-cooled −50°C CCD detector with a maximum spectra range of 200–3000 cm^{-1}. A specially designed sample compartment was used to assure measurement under dark environment, and samples in ampoule, vials, or bottle were at the focus point of the probe.

EssentialFTIR software (version 3.00.047, Operant LLC, USA) was used for data collection, and the CLS-based advanced-ID (10) module and software were used for qualitative analysis. Spectra were calculated in the range of 500–2500 cm^{-1} and first derivative pre-processing was used during method development.

Samples

Doxofylline and levofloxacin standards were purchased from the National Institutes of Food and Drug Control and were used as the API reference standards. The standards were dissolved in Millipore Milli-Q (18 MΩ cm^{-1}) water. Here, solutions rather than solids to more closely approximate the targeted samples and eliminate the influence of polymorphs on the Raman spectra.

Doxofylline liquid injectables from six manufacturers with three specifications were used as validation samples. Table I lists the doxofylline samples used in this study. All doxofylline samples were in 10 or 20 mL ampoules with labeled concentrations ranging from 10 to 20 mg/mL.

Levofloxacin Lactate and Sodium Chloride Injections from three manufactures contained in glass bottles with labeled concentrations of 2 mg/mL were used as low-concentration and big-package examples to show whether the method developed worked well in those conditions. Sixty-six different other injectable drugs from various manufacturers were used as negative control samples to challenge the validity and applicability of the method.

Another 60 doxofylline injectables and 20 Levofloxacin with known concentration information were used as a training set for quantitative evaluation.

Each sample used in our study was proved to be qualified by a qualification report from manufacture itself or drug regulatory department.

Raman Spectra

Offset correction, *x*-axis correction and *y*-axis correction, was done on each Raman spectrometer before collecting the spectra (11). Raman spectra were collected with a resolution of 4.5 cm^{-1} and scan time of 200 s in the spectral range of 200–

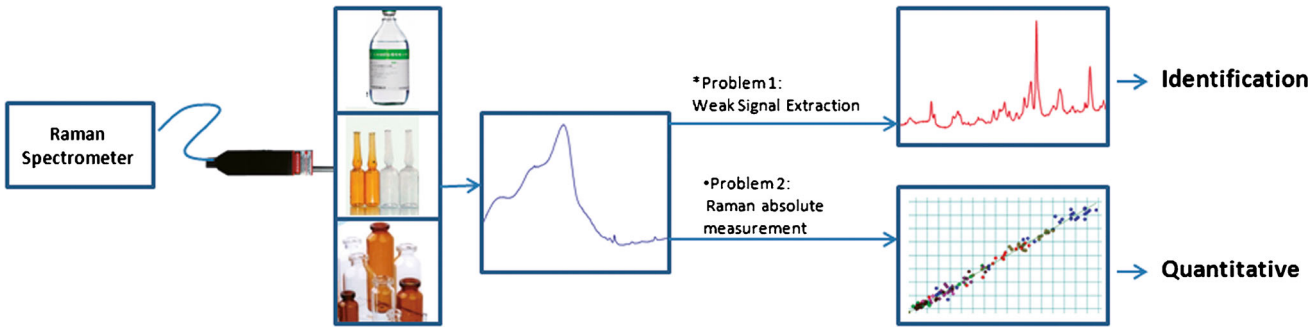

Fig. 1. Schematic of the implementation process of the methods in the study

3000 cm^{-1}. Drug samples in their original ampoules, vials, or bottles were placed in the specially designed sample compartment, and Raman spectra were collected directly through the ampoules, vials, or bottles. The API reference standard water solutions and excipients (propylene glycol) were measured in 1 cm quartz cuvettes. Water spectra were collected in the same cuvettes.

Reference Methods

Reference data for the doxofylline injectable mentioned in this paper were measured by HPLC following the method of SFDA Standard WS1-(X-130)-2003Z. The concentration values of levofloxacin in levofloxacin lactate and sodium chloride injections were also measured by HPLC following the method in China Pharmacopeia 2005 (12).

THEORY AND CALCULATION (13)

Set-Up

In this study, measurements were simplified to a one-key operation to fit the demand for fast screening of SFFCs. The schematic of the implementation process is illustrated in Fig. 1, and it depicts that both identification and quantitation came from the same information of the non-invasive spectrum by solving the two problems separately. The main problem was how to extract weak signals from API for identification. A further problem of Raman absolute measurement for quantitation also needed to be resolved.

Identification

The total non-invasive Raman spectrum of injectables included the signals of container, water, excipients, and API; in which the container contributed over 90% in most cases tested. APIs concentrations varied from 0.1 to 10% (*w/w*), but more than 90% of them lower than 1%. Therefore, the contribution from API was at the level of 0.1%, which is no doubt a weak signal.

The first step to extract the weak signal of the API should be to separate the signal of the container from the total non-invasive Raman spectrum. In this article, glass containers were mainly examined and discussed. Containers made of other materials have similar behaviors as glass. For injectables, the glass used for the ampoules, vials, and bottles varies from manufacturer to manufacturer resulting in Raman spectrum variations. In addition, glass exhibits photoluminescence that is not reproducible between different bottles.

If a large number of target spectra or spectra of one or more known components are available, then it may be advantages to use the principle component analysis (PCA) method. Factoring the spectra casts them into a new space, in which it is often possible to choose the subset of the factors such that noise is reduced with loss of only a small amount of other information. The factor loadings for the subset of these factors, rather than the original spectra, are then used in the calculations. After the calculations, we compared the extracted spectrum to the spectrum collected by pouring the injectable sample into the cuvette, where the signal from cuvette could be ignored. Increased similarity in spectra resulted in increased accuracy in the calculated results.

In this study, Raman spectra were gathered for over 160 kinds of ampoules, vials, and glass bottles used by different manufacturers including transparent and brown colored ones. PCA was performed and we found that the first ten loading spectra could represent 99.8% of the variations in the glass containers collected as showed in Table II. Instead of the original Raman spectra from empty glass containers, the first ten loading spectra were used in the calculation to separate the glass signals from the total non-invasive Raman spectrum.

This pretreatment with PCA factors improved the correlation coefficient of the extracted spectra and the liquid contents spectra from an average of 0.9513 up to an average of 0.9843, compared with using the original empty glass containers' spectra for calculation, as shown in Fig. 2.

The second step of extracting the API signal was easier than the first step because water had a relative weak Raman response, mainly in the range of 1500–1800 cm^{-1}. Let S be the spectrum of a sample that consists of n components that have

Table II. PCA Analysis of Glass Packages

Factor	Cumulative variance explained (percent)
1	74.1805
2	91.6525
3	95.4495
4	97.9948
5	98.6185
6	99.1566
7	99.5000
8	99.6460
9	99.7331
10	99.8003

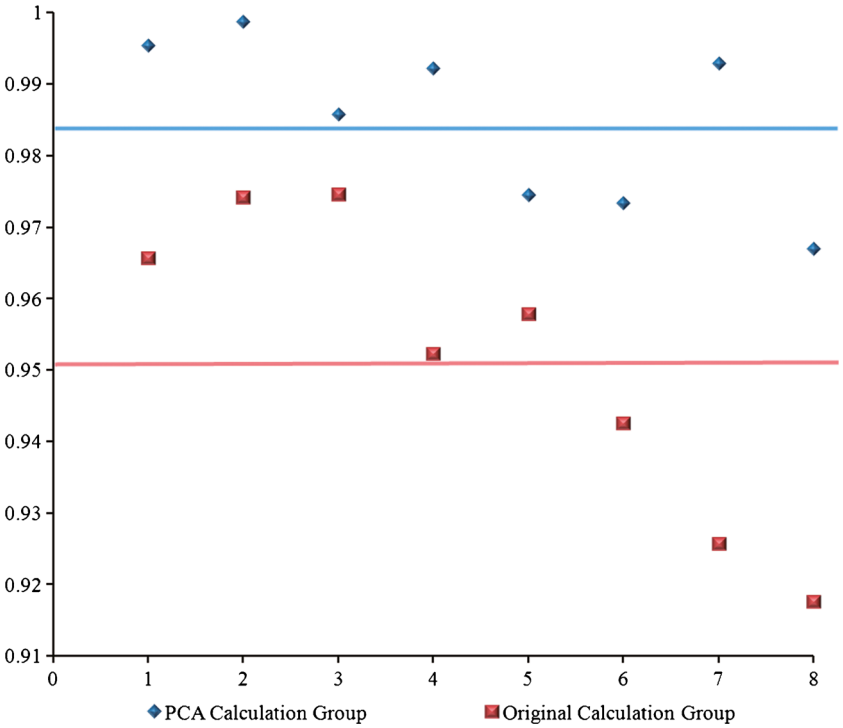

Fig. 2. Calculation improvement with PCA pretreatment. The PCA calculation group was on an average level of 0.9513, while the original calculation group was on an average level of 0.9843

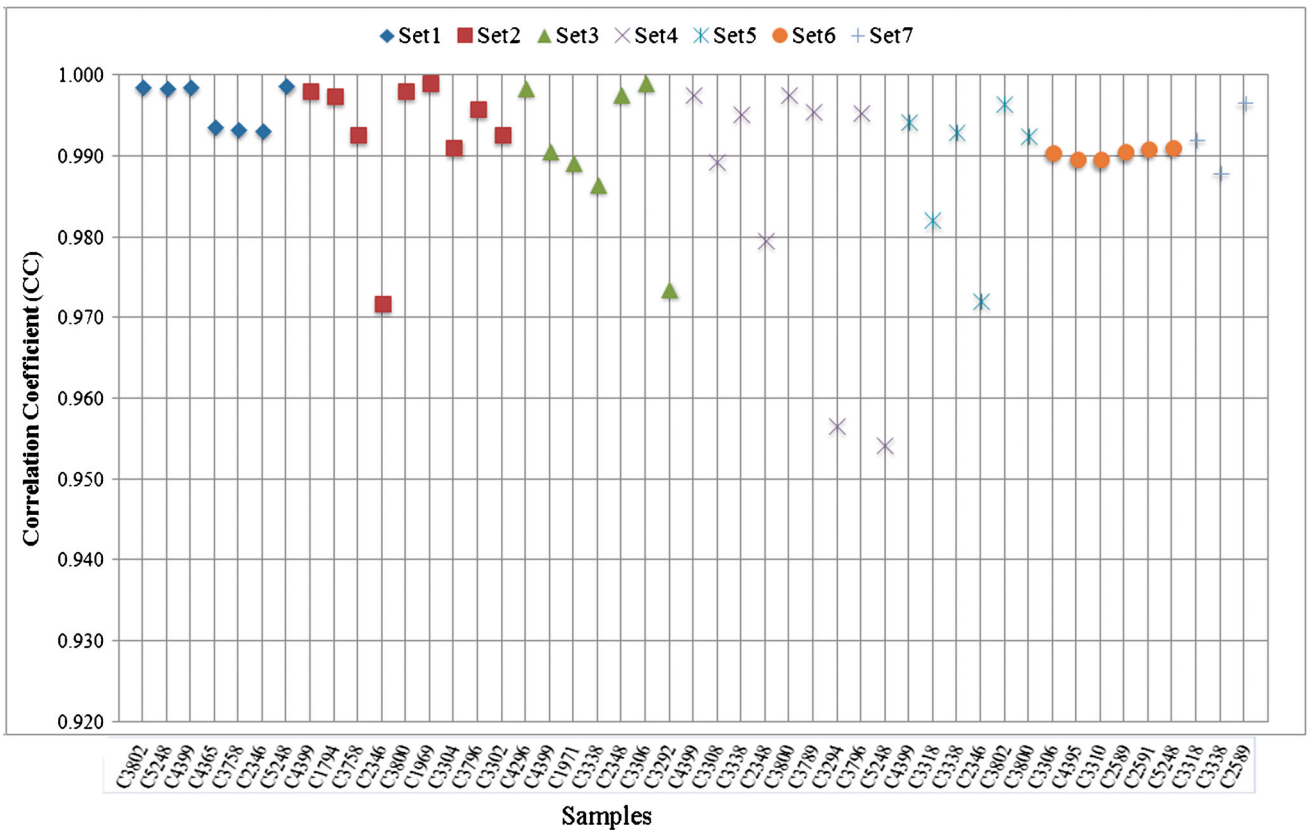

Fig. 3. Positive control results. Seven sets of instruments and operators were involved to show the variation of correlation coefficients, and all results were not lower than 0.96

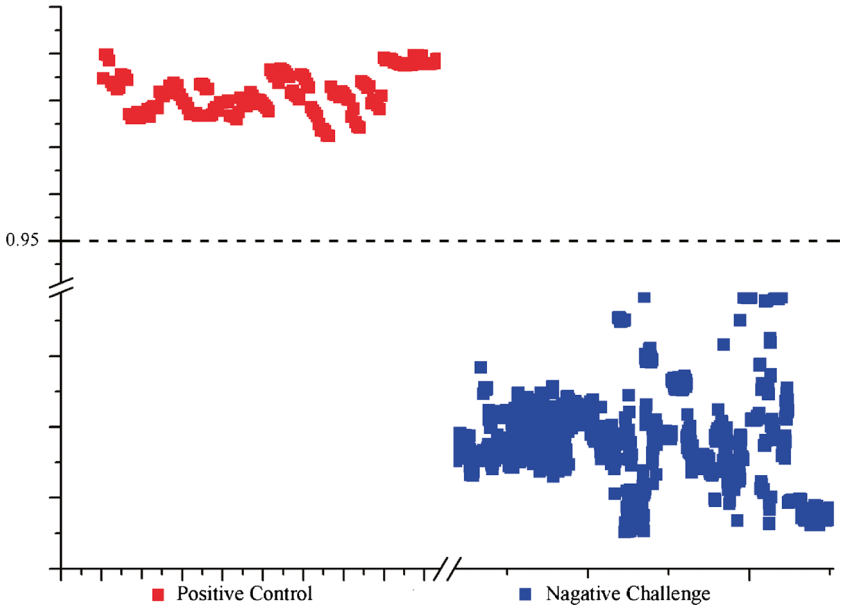

Fig. 4. Threshold settings. Positive control and negative challenge were considered and the threshold was set to 0.95

spectra $A_1 \ldots A_n$. The spectrum from the first step can be modeled as Eq. (1).

$$S = \sum_{i=1}^{n} (c_i \cdot A_i) + e \quad (1)$$

where A is the matrix of reference spectra of the sample components, $c_1 \ldots c_n$ are unknown coefficients, and e is the residual error. The least squares solution to this equation for the coefficients $c_1 \ldots c_n$ could be found by standard matrix algebra. In standard chemometric terminology, this is referred to as classical least squares (CLS). The CLS method was used to determine the coefficients $c_1 \ldots c_n$. Constraints, such as non-negativity, were applied in the regression. With known values of coefficients $c_1 \ldots c_n$, the API spectrum A_{API} could finally be extracted successfully. For the reference spectrum of API, the standard was dissolved in a solvent to eliminate the influence of polymorphs on the Raman spectrum. Then, the pure API reference spectrum could be extracted following the step 2.

Then, we could obtain the correlation coefficient between the API spectrum extracted A_{API} and the spectrum collected from the API reference $A_{reference}$. The nearer the correlation coefficient was to 1, the more the API signals extracted from the injectable sample were similar to the API reference. A "positive or negative" identification result could be achieved by setting the appropriate threshold.

Meanwhile, excipients in the injectable samples could also be identified by seeing how close its coefficient was to zero. A "positive or negative" identification result of excipient also could be achieved by setting the threshold of 0.1.

Quantitation

The quantitation was compiled based on the results of the identification. If the identification results of the injectables were positive, further work would be performed on the calculation of the concentration of the API.

As described previously, when compared to IR, one of the drawbacks of Raman spectroscopy in the context of

Table III. Identification Results of Doxofylline Injectables and Levofloxacin Lactate and Sodium Chloride Injections

Samples No.	Correlation coefficient	Threshold	API identification results	$c_{excipient}$	Threshold	Excipients identification results
1	0.9990	0.95	Positive	0.03	0.1	Negative
2	0.9931		Positive	0.66		Positive
3	0.9944		Positive	0.06		Negative
4	0.9922		Positive	0.06		Negative
5	0.9988		Positive	0.00		Negative
6	0.9983		Positive	0.01		Negative
7	0.9970		Positive	0.04		Negative
8	0.9943		Positive	0.03		Negative
9	0.9831	0.95	Positive	0.07	0.1	Negative
10	0.9827		Positive	0.08		Negative
11	0.9871		Positive	0.08		Negative

Table IV. Quantitative Results

Samples No.	Predicted API concentration (mg/mL)	HPLC results (mg/mL)	Relative error (%)
1	10.21	10.17	0.39
2	9.72	9.90	1.82
3	9.90	9.58	3.34
4	10.42	10.37	0.96
5	9.88	9.93	0.50
6	15.45	15.12	2.18
7	14.28	14.99	4.74
8	19.62	20.16	2.68
9	1.93	2.05	5.85
10	1.84	1.97	6.60
11	2.16	2.02	6.93

quantitative analysis is that Raman is an absolute measurement. When the laser power changes or if the samples are measured in a different geometry, the Raman signal from the drug will change correspondingly, even if the identical drug sample is measured under apparently identical conditions.

This problem can be resolved if the Raman signal is scaled using the signal from a common component. In this study, nearly all products examined contained water and the signal from the water meets the criteria for an "internal reference signal" very well. Thus, the concentration of the drug could be estimated from the relative contributions of water and the drug, which had been quantified in Eq. (1).

The Raman signal of component i (I_i) is proportional to laser strength (L), concentration of component i (C_i), collection geometry (m), and Raman scatter coefficient (r_i) as described in Eq. (1):

$$I_i = m^*L^*r_i^*C_i \quad (2)$$

Then, the Raman signal of API and water can be described in the formulas below:

$$I_{API} = m^*L^*r_{API}^*C_{API} \quad (3)$$

$$I_{water} = m^*L^*r_{water}^*C_{water} \quad (4)$$

Using water as the internal reference signal, the signal from API was normalized to that from water. The impact of laser intensity (L) and collection geometry (m) could be eliminated. This is the key to solve the problem of Raman absolute measurement, so quantitation might be possible.

Therefore, if a series of concentrations of the API were known, the concentration of the API in the unknown drug could be estimated from the CLS calculation (14,15).

RESULTS AND DISCUSSION

Threshold Settings

The setting of the threshold takes into consideration both the positive control and negative challenge.

For the positive control, more Raman spectra were collected by our partners using their own qualified doxofylline liquid injectable samples and Raman spectrometers. In total, seven more sets with the same equipment were used and each followed the series of corrections before data collection. Then, these spectra followed the aforementioned data analysis progression and finally got the results of correlation coefficients (CC). As shown in Fig. 3, the results were consistency not lower than 0.96, which indicated that with the introducing of many influences such as different instruments,

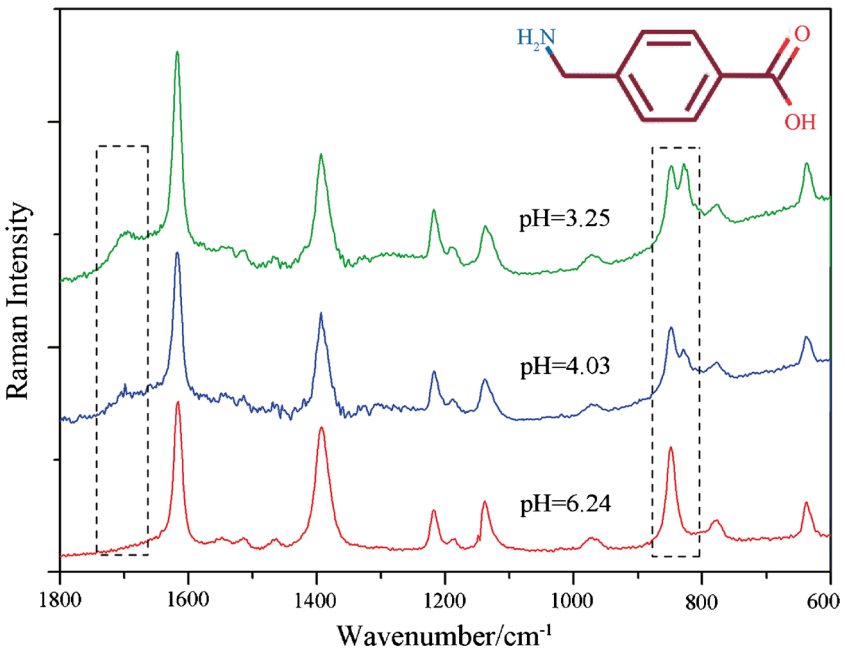

Fig. 5. The impacts of pH on the Raman spectra. Aminomethylbenzoic acid was taken as an example to show the variations on Raman spectra under the pH of 3.75, 4.03, and 6.24

operators, and samples, the results could be kept in the threshold of 0.96.

On the other hand, the CC values of all 66 negative challenge samples are all smaller than 0.80. Considering both the positive control and negative challenge, as shown in Fig. 4, based on a higher tolerance and our experience, the threshold was configured to 0.95.

Identification

Correlation coefficients between A_{API} and $A_{Reference}$ were calculated, and the results of eight doxofylline samples and three Levofloxacin Lactate and Sodium Chloride Injections are shown in Table III.

The correlation coefficient values of the doxofylline liquid injectable samples from six manufacturers (sample No. 1–8 in Table III) and Levofloxacin Lactate and Sodium Chloride Injections from three manufactures (sample No. 9–11) were all larger than the threshold of 0.95. Therefore, their identification results were all "positive". As what could be seen from Table III, sample No. 2 had a $c_{excipient}$ value larger than the threshold 0.1, which means sample No. 2 contained excipient, while the rest of the samples had no excipients ($c_{excipient}$ values of less than 0.1).

Quantitation

After normalization with water, 60 qualified doxofylline liquid injectables and 20 Levofloxacin in Levofloxacin Lactate and Sodium Chloride Injections samples of known API concentrations were used as training set to set up the CLS models for quantitation. With the calculations from CLS models, the quantitative analysis results of the 11 samples previously described in Table I are shown in Table IV. Compared to the Raman predicted values with HPLC reference results, the relative error of all eight doxofylline liquid injectable samples were within 5% and of the three low-concentration Levofloxacin in Levofloxacin Lactate and Sodium Chloride Injection samples were within 10%. These results were enough for fast screening to determine which drugs were suspected to be SFFCs.

Potential Influence of pH

In other studies, we found that pH of the injectables was another important factor. For example, the Raman spectra of aminomethylbenzoic acid are different in region 780–840 cm^{-1} and 1690–1790 cm^{-1} at different pH conditions as shown in Fig. 5. Under conditions like this, the pH value of the injectable has to be measured and then use it to adjust the pH value of water solution of standard material.

CONCLUSIONS

In this article, PCA combined with CLS chemometrics method was used to overcome the problems of the interference signals of glass containers and solutions, and weak signals from API finally were extracted for qualification. Using water as an internal standard for normalization, the Raman absolute measurement problem was solved and the CLS quantitation models were established.

In conclusion, the method we developed is a fast, reliable, and non-invasive approach to identify and quantify liquid injectables in the aqueous phase. Therefore, with the development of a database with enough models, this method could be an efficient tool for fast screening of SFFCs in liquid dosage forms.

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