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Short communication

Quantitative analysis of liquid formulations using FT-Raman spectroscopy and HPLC The case of diphenhydramine hydrochloride in Benadryl®

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Abstract

The capability of FT-Raman spectroscopy for the fast and non-destructive quantitative analysis of liquid formulations was tested and the results were compared to those obtained by HPLC. Diphenhydramine hydrochloride (DPH), the active ingredient of Benadryl®, was determined in the presence of the numerous excipients of the elixir. A Raman calibration model was developed by measuring the peak intensities of different standard solutions of DPH vibration at 1003 cm^{-1}. Application of the calibration model on the peak intensity recorded from the as-received commercially available sample with 2.5 mg ml^{-1} DPH nominal value yielded a value of 2.49 ± 0.05 mg ml^{-1} DPH. The reliability of this method was verified by testing it against the conventionally used HPLC. The results from both methods were in excellent agreement. The main advantage of Raman over HPLC method during routine analysis is that is considerably faster and less solvent consuming. Furthermore, Raman spectroscopy is non-destructive for the sample. On the other hand, the detection limit for Raman spectroscopy is much higher than the corresponding for the HPLC methodology.

Keywords: Raman spectroscopy; Benadryl®; Quantitative analysis; Liquid formulations; Diphenhydramine hydrochloride; HPLC

1. Introduction

There is an ever-growing need for development of reliable, simple and non-destructive methodologies for quantitative analysis of pharmaceutical formulations. In the recent past, FT-Raman spectroscopy, a technique that requires minimal sample preparation, has been used for the quantitative analysis of numerous solid formulations as can be seen in the recent review article by Pelletier [1]. Quantitative Raman analysis of solid powder mixtures is usually complicated because the intensity depends on the reproducibility of some factors such as particle size, packing density of the sample and homogeneity of the mixture. On the other hand, for isotropic liquid formulations the analytical approach is expected to be less complicated and the concentration of a species can be determined easier. There are several recent publications where Raman spectroscopy has been used for quantitative analysis of liquid mixtures. These publications fall into two main categories: (a) those dealing with hydrocarbon mixtures, e.g. Refs. [2–4] and (b) and those referring to glucose in water and biological fluids, e.g. Refs. [5–7]. There are also some scatter studies dealing with other materials, such as ethanol and water mixtures [8,9], rhodamine-6G and methanol mixtures [9]. Quantitative Raman spectroscopy has also been used for studying acid dissociation in solvents [10]. There is also a report on the quantitative application of Raman spectroscopy on the aqueous suspension of active ingredient (medroxyprogesterone acetate) in commercial formulation [11].

In the present study, the feasibility of using FT-Raman spectroscopy for non-destructive quantitative determination of the active ingredients in liquid formulations was tested. Therefore, Benadryl®, consisting of the active ingredient

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diphenhydramine hydrochloride (DPH), an antihistaminic, and numerous excipients (sucrose, orange oil, cinnamon oil, coriander oil, clove oil and anethole) in 14% ethanolic solution was chosen.

DPH has been assayed in environmental sediments formed from wastewater treatment plants [12], in pharmaceutical sleep aid products [13], and in cough–cold syrup [14] using HPLC. Usage of chromatographic techniques for quantitative purposes are not only destructive for the sample but also time consuming and cumbersome since extensive sample preparation is needed. First order UV spectroscopy was also used to determine DPH in binary mixtures with naphazoline hydrochloride in nasal jelly [15]. Application of UV spectroscopy, although successful in some binary mixtures was hindered by the presence of the numerous elixir excipients that absorb at the same wavelength (i.e. 216 nm) as DPH [16]. Furthermore, a 100-fold dilution of the elixir is needed.

In this work, DPH was assayed using Raman spectroscopy and the results were compared to those obtained from the application of an HPLC based methodology that was developed for Benadryl®.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of analytical USP-NF grade and were used without further purification. Diphenhydramine hydrochoride reference standard was obtained from Sigma–Aldrich Company. DPH was characterized prior to its use by FT-IR Spectroscopy (EQUINOX 55, Bruker, Karlsruhe, Germany) and by FT-Raman spectroscopy (FRA-106/S FT-Raman, Bruker), and the spectra found to match those reported in literature [17].

Benadryl® is an elixir with 14% (v/v) ethanol and 2.50 mg ml^{-1} DPH as the active ingredient. Two formulations Benadryl® (B1 and B2) from Pfizer were bought from a Patras pharmacy store and one (B3) from Thessaloniki pharmacy store. The lot number in all three was the same. The excipients, according to the manufacturer, were sucrose, orange oil, cinnamon oil, coriander oil, clove oil and anethole.

All chemicals and reagents used for HPLC were USP-NF grade. The solvents, methanol and water were "gradient grade for liquid chromatography" supplied from Merck company. All solution preparations were made using deionised water, filtered by a "Millipore-Q plus 185" equipment.

2.2. Raman spectroscopy

The Raman spectra were recorded using a FRA-106/S FT-Raman (Bruker) with the following characteristics: the laser excitation line used, was the 1064 nm of a Nd:YAG laser. A secondary filter was used to remove the Rayleigh line. The scattered light was collected at an angle of 180°. A Hellma QS quartz cell with a coated backside and a PTEF lid was used for the liquid samples. The system was equipped with a liquid N_2 cooled Ge detector (D 418). The power of the incident laser beam was about 370 mW on the sample's surface. Typical spectral line width was 0.5 cm^{-1} while the recorded spectra were the average of 300 scans.

2.3. HPLC instrumentation and chromatographic conditions

A Shimadzu series LC-6A pump and an SIL-10ADVP programmable auto sampler with the volume injection set to 100 μl were used for HPLC analysis. Detection was via a LC-75 UV-detector operated at 210 nm. The chromatographic peaks were recorded by a HP DeskJet 940c series printer and elaborated automatically by employing a computerized Shimadzu program "CLASS-VP".

Separation was achieved on a Hypersil BDS C-18 column (150 mm × 4.6 mm) with particle size 3 μm. The isocratic elution system consisting of an aqueous 0.015 M 1-pentanesulfonic acid sodium salt adjusted with sulfuric acid to pH 3.1 and acetonotrile (60:40, v/v). The flow rate was 1 ml/min whereas the mobile phase was degassed by filtering through a Millipore HV 0.45 μm pore membrane filter.

2.4. Sample and standard preparation

2.4.1. For the Raman method

Raman spectroscopy method was based on a simple linear regression model obtained from a series of 14% (v/v) ethanolic standard solutions containing 1.21, 1.81, 2.40, 3.01 and 3.60 mg ml^{-1} DPH, respectively. Each standard was measured three times.

The spectra were obtained on different days and, as it was anticipated, there were changes in the recorded intensities. In order to be able to compare Raman spectra from different days, five spectra from the same formulation were recorded every day. The average absolute intensity of the strong vibration at 2932 cm^{-1}, after the subtraction of the background, was compared with the respective intensity that was recorded on the first day and the ratio between these intensities was calculated. All subsequent spectra recorded in the same day were multiplied by this ratio.

Quality control (QC) concentrations (low, medium and high) were prepared separately by spiking to Benadryl® (B1 formulation, 2.50 mg ml^{-1}) elixir, quantities of pure standard DPH to produce three QC samples containing 1.25, 1.51 and 2.53 mg ml^{-1} DPH, respectively.

2.4.2. For the HPLC method

For the development of the HPLC calibration model, five different concentrations, after appropriate dilutions of DPH (10.68, 16.02, 21.36, 32.04, 42.72 and 53.40 μg ml^{-1}) were prepared and assayed ($n = 3$ concentration). Responses were measured as peak areas and plotted against concentration.

Each elixir was diluted (1/100) with bidistilled water to give a final concentration of 25.0 μg ml^{-1}. Thus, the concentration of each sample solution approached the concentration of that in the middle of the standard solution range. The sample solutions

were homogenized by shaking and filtered with acrodisc GHP 0.45 μm.

3. Results and discussion

3.1. Development of the Raman analytical methodology

The intensity of a Raman line depends on a number of factors including the incident laser power, the frequency of the scattered radiation, the absorptivity of the materials involved in the scattering and the response of the detection system. Thus, the area under a Raman peak, $A(\nu)$, can be represented as [18]:

$$A(\nu) = I_0 K(\nu) C \quad (1)$$

where I_0 is the intensity of the excitation laser line, ν the Raman shift and $K(\nu)$ is a factor which includes the frequency dependent terms: the overall spectrophotometer response, the self-absorption of the medium and the molecular scattering properties. C is the concentration of the Raman active species.

The Raman spectra of an aquatic solution of DPH can be seen in Fig. 1 along with the spectrum of the as-received Benadryl® and the spectrum of powder DPH. It is apparent that the most intense vibration at 1003 cm^{-1} of DPH should be used for the quantitative analysis.

Among the sample-related factors affecting quantification changes in refractive index are included. In order to minimize such problems, the calibration curve was constructed by dissolving DPH in 14% (v/v) ethanolic solution while the concentration range chosen was in the area of the 2.5 mg ml^{-1}, i.e. the concentration of DPH in the commercially available elixir.

Three Raman spectra were recorded from each solution (Fig. 2) and for reasons of simplicity the peak intensities I, after background subtraction were used, instead of the areas A of the Raman vibration (Eq. (1)) used. A plot of I versus C_{DPH}, as expected, yielded a straight line, Eq. (2).

$$I_{DPH}^{1003} = 0.13 \times 10^{-5} + 3.96 \times 10^{-5} C_{DPH} \quad (2)$$

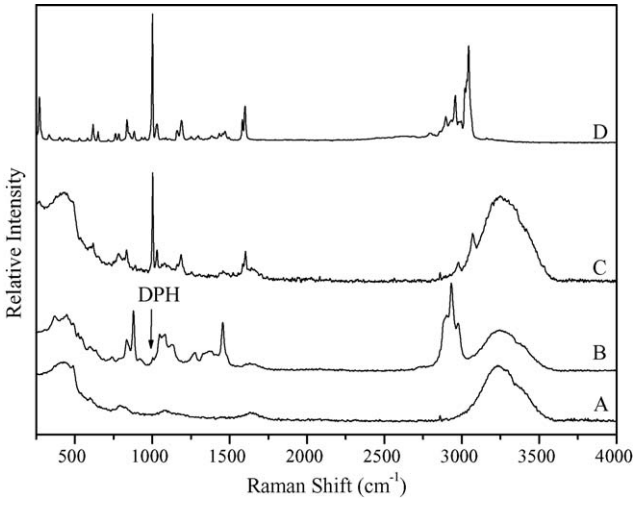

Fig. 1. FT-Raman spectra of (A) water (in quartz cell), (B) the as-received Benadryl® (in quartz cell), (C) DPH (50 mg ml^{-1}) dissolved in water (in quartz cell) and (D) DPH in powder.

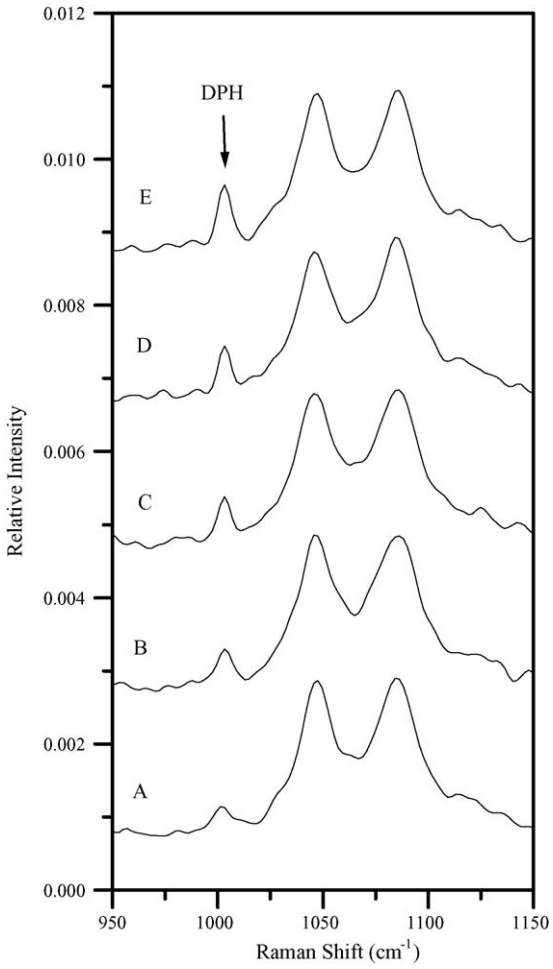

Fig. 2. FT-Raman spectra of DPH solutions (14% ethanol) in various concentrations: (A) 1.2 mg ml^{-1}, (B) 1.8 mg ml^{-1}, (C) 2 mg ml^{-1}, (D) 3.0 mg ml^{-1} and (E) 3.6 mg ml^{-1}.

The correlation coefficient, r, was 0.9989 while the standard deviation (S.D.) for the slope was found to be 5.26×10^{-7} and for the intercept 1.34×10^{-6}. The S.D. of the intercept is slightly higher than the value of the intercept, indicating, as expected, that the intercept is practically zero.

3.2. HPLC chromatography

USP suggests an HPLC method for determination of DPH in an elixir [19]. The Pharmacopeia methodology does not allow differentiation of excipients and is rather slow. In order to make the HPLC more competitive to the generally faster Raman spectroscopy, an effort was made to improve the USP methodology which resulted in the DPH retention time and in the ability to monitor each excipient individually.

Peak interference and broadening in reversed phase liquid chromatography of DPH were observed and this phenomenon prompted investigation of the influence of operating conditions (pH, columns residual silanols and ion pairing agent) on the analyte chromatographic behavior.

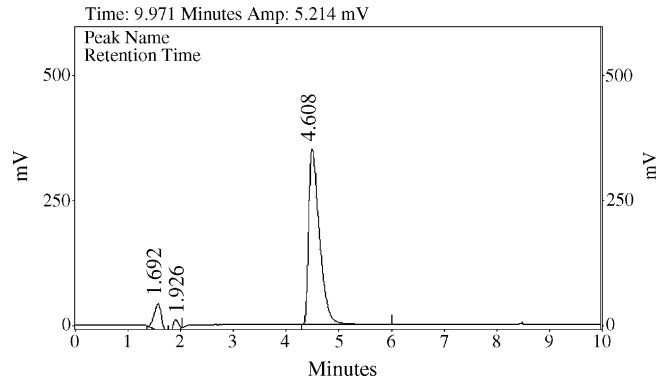

Fig. 3. Typical chromatogram of diphenhydramine-HCl (21.36 μg ml^{-1}) reference standard at 210 nm. Retention time 4.6 min.

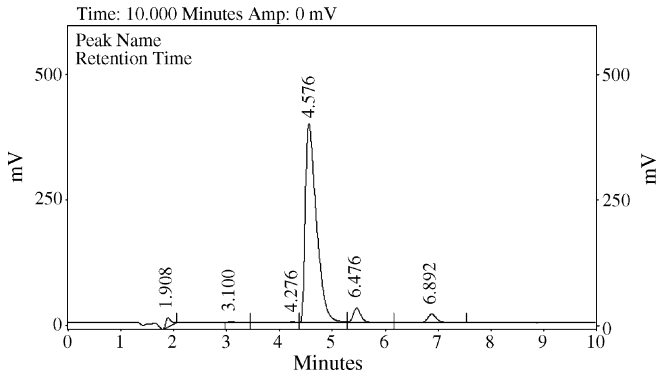

Fig. 4. Determination of diphenhydramine-HCl (25.0 μg ml^{-1}) in Benadryl® formulation.

pH has a determining influence on diphenhydramine and excipients separation, since differences in the degree of ionization change elution order and modify resolution.

Moreover, at pH as low as 3.1, pentanesulfonic acid sodium salt proved to be a useful ion pairing agent able to improve the analyte retention and resolution, providing a single symmetric peak for DPH (Fig. 3). The resolution R_s ($R_s = 2(t_{R2} - t_{R1})/W_2 + W_1$) between the chromatographic peak of DPH and each separate peak of the excipients was no less than 2.4, signifying complete separation (Fig. 4). The tailing factor ($T = b_{0.05}/2A$) for diphenhydramine was 1.3, the capacity factor ($k' = (t_R - t_0)/t_0$) 1.8 and the number of theoretical plates ($N = 16(t_R/W_R)^2$) was 1913.

From the chromatogram presented in Fig. 4, it can be seen that the DPH retention time was reduced to approximately 6 min. Despite this improvement, the total time needed for DPH determination in Benadryl®, including verification of the calibration model, cleaning the column, etc. is approximately 8–9 h.

The choice to use a common wavelength set at 210 nm was considered satisfactory, permitting the detection of the drug with adequate sensitivity. Acetonitrile was also selected in mobile phase, instead of methanol, because it yielded a more stable base line at this wavelength.

3.3. Validation of the methods

3.3.1. Linearity

The linearity of the above methods for determination of DPH was evaluated by analysis of different concentrations of the drug. In this study, according to the International Conference on Harmonization (ICH) [20], five concentrations were chosen, ranging between 1.21–3.60 mg ml^{-1} for FT-Raman method and 10.68–53.40 μg ml^{-1} for HPLC. The analysis for each concentration was repeated three times (Table 1). On the results the unweighted linear least squares method was used and the relevant tests verifying the validity of this choice were applied:

Table 1
The measured signals (three per standard), the residual and the *t*-test results ($a = 0.02$), for checking the normal distribution of the residuals around zero

FT-Raman				HPLC			
Concentration (mg ml^{-1})	Peak intensities $\times 10^{-5}$	Residuals $\times 10^{-6}$	$t_{experimental}$ value[a]	Concentration (μg ml^{-1})	Peak areas	Residuals	$t_{experimental}$ value[a]
1.212	5.0500	1.19708	1.40	10.68	2468496	−31991.2	6.70
1.212	4.9196	−0.10692		10.68	2469532	−30955.2	
1.212	4.9800	0.49708		10.68	2481178	−19309.2	
1.811	7.2357	−0.66243	2.79	21.36	4882121	−41117.2	0.12
1.811	7.2775	−0.24483		21.36	4919878	−3360.2	
1.811	7.2768	−0.25143		21.36	4977614	54375.8	
2.403	9.6140	−0.31878	0.33	32.04	7409265	63275.8	6.53
2.403	9.6129	−0.32998		32.04	7385211	39221.8	
2.403	9.6868	0.40975		32.04	7389238	43248.8	
3.012	11.6028	−4.54321	0.39	42.72	9783399	14658.8	0.17
3.012	12.3204	2.63237		42.72	9741352	−27388.2	
3.012	12.0059	−0.51199		42.72	9789304	20563.8	
3.603	14.3645	−0.32693	0.71	53.4	12120860	−70631.2	0.44
3.603	14.3702	−0.26930		53.4	12284640	93148.8	
3.603	14.6800	2.82883		53.4	12087750	−103741.2	

[a] $t_{critical} = 6.96$.

Table 2
The critical and experimental *f*-values at significant level 0.05 for both methods

Statistical *F*-test	FT-Raman		HPLC	
	f-critical	*f*-experimental	*f*-critical	*f*-experimental
Goodness-of-fit	4.67	1.66	4.67	3.74
Lack-of-fit	3.71	0.49	3.71	1.35

(a) The residuals at every standard concentration were normally distributed with mean zero at every *x*-value (concentration) as proven mathematically by using a *t*-test. The calculated values for this test were smaller than the critical (6.96) at $n = 3$ and $a = 0.02$ (Table 1).

(b) The absence of curvature on the calibration curve, as proven mathematically by applying an analysis of variance (ANOVA) lack-of-fit and goodness-of-fit test [21], indicated homoscedasticity of the normal distributed residuals.

As a measure of the quality of fit, the *f*-value for the goodness-of-fit was calculated for the linear model from:

$$f(p-1, n-p) = \frac{\mathrm{MSS_{fact}}}{\mathrm{MSS_R}} \quad (3)$$

where $\mathrm{MSS_{fact}}$ is the mean sum of squares due to the factors, $\mathrm{MSS_R}$ is the mean sum squares of the residuals, $p-1=1$ and $n-p=13$.

The lack-of-fit test was based on a comparison of the mean sum of squares due to the model and the experimental error with $(f-p, n-f) = (3.10)$:

$$f(f-p, n-f) = \frac{\mathrm{MSS_{lof}}}{\mathrm{MSS_{pe}}} \quad (4)$$

Since the calculated *f*-values for both tests, at the significant level 0.05, were lower than the critical (Table 2), the variance due the random measurement errors was higher than the variance due to the error of the model, so the chosen linear models appeared to be adequate.

(c) The linearity (r) of the two calibration plots, were also validated using a two-tailed *t*-test with $(n-2)$ degrees of freedom and 95% confidence interval ($t_{\mathrm{exp\,erimental}} = |r|\sqrt{n-2}/\sqrt{(1-r)^2}$) [22]. Since the calculated values of $t_{\mathrm{experimental}}$ for FT-Raman and HPLC methods were greater than the critical ($t = 2.16$) the correlation between *x* and *y* values is significant. The results for both methods were presented in the following regression equations:

$$y = 3.959(\pm 1.14) \times 10^{-5} x + 1.316(\pm 2.9) \times 10^{-6} \; (r = 0.9989) \quad \text{FT-Raman} \quad (5)$$

$$y = 226849(\pm 2054)x + 77736(\pm 2782) \; (r = 0.9999) \quad \text{HPLC} \quad (6)$$

3.3.2. Accuracy

Due to the large number of excipients and since neither the placebo nor its recipe were available, the exclusion of the possibility of DPH Raman peak at 1003 $\mathrm{cm^{-1}}$ overlapping with vibrations of excipients, was difficult. The influence of the excipients on DPH intensity was tested, according to ICH (4.1.2. b), by applying the standard addition method. Three solutions were prepared from B2 formulation, having 1.25, 1.51 and 2.53 $\mathrm{mg\,ml^{-1}}$ DPH in addition to the nominal (2.5 $\mathrm{mg\,ml^{-1}}$) concentration. Five Raman measurements from each solution were obtained and the plot of the average *I* versus C_{ADPH} was found to yield a straight line, Eq. (7).

$$I_{\mathrm{DPH}}^{1003} = 10.16 \times 10^{-5} + 3.99 \times 10^{-5} C_{\mathrm{ADPH}} \quad (7)$$

where C_{ADPH} is the concentration of the DPH added in the B2 formulation. The S.D. for the slope was 8.34×10^{-7} and for the intercept 4.51×10^{-7}. The intercept *A* of Eq. (7) should be equal to $A = C_{\mathrm{DPH}} \times B$, where *B* is the slope of Eq. (7) and C_{DPH} is the total concentration DPH in formulation B2. Therefore, $C_{\mathrm{DPH}} = A/B$. C_{DPH} was found to be 2.55 ± 0.04 $\mathrm{mg\,ml^{-1}}$ indicating that the influence of the excipients on DPH Raman peak, if any, was minimal.

Additionally, the drug content calculations for the commercial elixir evaluated by assaying the three as-received Benadryl® five times each formulation. The excellent recoveries of the samples (Table 3) suggested the high accuracy of the proposed FT-Raman method.

3.3.3. Precision

For the evaluation of the precision six determinations at 100% of the test concentration were performed using FT-Raman and HPLC methods on B1 elixir. The developed HPLC system was applied for the determination of DPH in the same Benadryl® elixir used in Raman spectroscopy, injecting the

Table 3
% Recovery results obtained from three commercial formulations (Benadryl®)

Elixir	FT-Raman		HPLC	
	Mean ± confidence limits[a] ($\mathrm{mg\,ml^{-1}}$)	% Recovery	Mean ± confidence limits[a] ($\mathrm{mg\,ml^{-1}}$)	% Recovery
B1	2.48 ± 0.06	99.2	2.49 ± 0.04	99.6
B2	2.53 ± 0.04	101.2	2.52 ± 0.05	100.8
B3	2.46 ± 0.05	98.4	2.45 ± 0.04	98.0
Average	2.49 ± 0.05	99.6	2.49 ± 0.04	99.5

[a] 95% confidence limits ($n = 5$).

formulation six times. A mean recovery value 2.496 ± 0.038 with %R.S.D. = 1.11 mg ml^{-1} was obtained. The FT-Raman results yielded an average value of 2.477 ± 0.038 mg ml^{-1} with %R.S.D. = 1.13. The nominal value, according to manufacturer was 2.50 mg ml^{-1}.

Statistical comparison of the results was performed using *F*-test. Since the experimental *f*-value (1.03) is lower than critical (7.15), there was no statistically significant difference at the 95% confidence level [22].

3.3.4. Detection and quantitation limits

Limit of detection (LOD) = 0.14 mg ml^{-1} for FT-Raman and 0.81 μg ml^{-1} for HPLC and limit of quantitation (LOQ) = 0.44 mg ml^{-1} for FT-Raman and 2.45 μg ml^{-1} for HPLC were calculated according to the following ICH recommendations:

$$\mathrm{LOD} = 3.3S_{y/x}/m \qquad \mathrm{LOQ} = 10S_{y/x}/m$$

where $S_{y/x}$ is the residual standard deviation and m is the calculated slope of the corresponding calibration [20,22].

4. Comparison of Raman and HPLC

From Table 3, it is apparent that both techniques yield equally reliable results. The detection limit of the HPLC methodology was found to be much lower than the respective detection limit yielded by the FT-Raman method. Furthermore, the HPLC methodology has the potential of differentiating the numerous excipients present in the elixir (Fig. 4) as opposed to the Raman spectroscopy where the presence of several of them cannot be detected. Despite these shortcomings, application of the non-destructive and much less time consuming Raman spectroscopy for monitoring the presence of the active ingredient is still an attractive possibility.

5. Conclusions

The main advantage of FT-Raman spectroscopy in routine quantitative analysis over the well-established HPLC methodology in the determination of active ingredients in liquid formulations is the time and solvents consumption. Application of both techniques for the determination of DPH in Benadryl® elixir yielded practically the same value for the ingredient. The differences between these two techniques were mainly the lower detection limit for HPLC, as opposed to the non-destructive no-pretreatment advantage of FT-Raman.

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