Simultaneous determination of tapentadol HCl and paracetamol by ratio-spectra derivative spectrophotometry

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ABSTRACT

A new simple and sensitive ratio-spectra first derivative spectrophotometric method was developed for the simultaneous determination of tapentadol hydrochloride (TPH) and paracetamol (PCM) in binary mixtures. The method depends on the use of the first derivative of the ratio-spectra obtained by dividing the absorption spectrum of binary mixture by a standard spectrum of one of the compounds. TPH was determined at 280.4 nm while PCM was determined at 300 nm. Calibration graphs are linear over the range of 20–100 µg.ml⁻¹ and 2–14 µg.ml⁻¹ for TPH and PCM, respectively. The developed method was validated and successfully applied for the determination of TPH and PCM in laboratory prepared mixture containing all possible excipients present in tablet dosage form. The mean percentage recovery ± S.D. were found to be 99.855 ± 0.203, and 101.250 ± 0.190 for TPH and PCM, respectively.

Key Words: Povidone, zero-crossing, binary mixture, laboratory prepared mixture, validation.

INTRODUCTION

Paracetamol PCM; N-(4-hydroxyphenyl) acetamide (Mol.Wt. 151.2), is an analgesic and antipyretic agent. It is the active metabolite of phenacetin, and exerts its analgesic effect via inhibiting COX-2 enzyme. Paracetamol is sparingly soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride [1, 2]. Paracetamol is official in both BP 2013 [1] and USP 34 [3]. Tapentadol hydrochloride TPH; 3-[(1R,2R)-3-(3-dimethyl-amino)-1-ethyl-2-methyl propyl]phenol HCl (Mol.Wt. 257.8) is a recently FDA approved centrally acting analgesic drug. Tapentadol is effective for the treatment of moderate to severe acute or chronic pain in adults 18 years of age or older. It has a unique dual mode of action as an agonist at the µ-opioid receptor and as a nor-epinephrine reuptake inhibitor [4]. TPH is a white to off-white powder, freely soluble in water, 0.1 N HCl, soluble in ethanol, sparingly soluble in methanol and slightly soluble in 2-propanol. The melting point ranges from 204 to 210 °C. The pKa values are 9.36 and 10.37 [5]. TPH is not official in USP 34 [3] or BP 2013 [1]. The structures of TPH and PCM are shown in Fig. 1. Tapentadol was recently co-formulated with paracetamol in tablets which labeled to contain 50 mg TPH and 325 mg PCM. This fixed dose combination is effective in the treatment of pain associated with osteoarthritis, postsurgical dental pain, advanced cancer, and postoperative pain [6]. Dosage form contains povidone, magnesium stearate, and micro-crystalline cellulose (avicel) as excipients. Povidone is 1-ethyl-2-pyrrolidinone homopolymer (Fig. 1.). It is used as dissolution enhancer and tablet binder [7]. Being a weak UV absorber, povidone may interfere in the spectrophotometric determination of TPH and PCM.

Literature review revealed different analytical methods for determination of TPH. The reported methods include UPLC [8], HPLC [9] and LC/MS/MS [10] for the determination of the drug in various biological fluids and RP- HPLC [11, 12] and spectrophotometric [13] methods for determination of the drug in its pharmaceutical dosage form. Several methods have been reported for the simultaneous determination of TPH and PCM in pharmaceutical samples and biological fluids, including; RP-HPLC [14,15], derivative spectrophotometry [16,17], Q-absorbance ratio method [18], simultaneous equation and dual wavelength method [19]. Reviewing literature revealed that nothing has been published concerning simultaneous determination of TPH and PCM.
PCM in presence of povidone by ratio-derivative spectrophotometry. The aim of this study is to develop ratio-first derivative spectrophotometric method for the simultaneous determination of TPH and PCM in the presence of povidone in their laboratory prepared mixture. Elimination of interference produced by povidone can be done by measuring amplitudes of the first derivative of the ratio spectra of mixtures at zero-crossing points of povidone. The method was validated by using ICH guidelines [20].

MATERIALS AND METHOD

Apparatus and Software: Spectrophotometric measurements were carried out using Schimadzu (UV-1800) UV-VIS double beam spectrophotometer equipped with 1 cm quartz cells and connected to a personal computer loaded with UV-Probe 2.33 software. Absorption spectra were recorded on wavelength range 220-340 nm at scan rate 400 nm.min⁻¹, spectral band width 0.2 nm and slit width 1 nm.

MATERIALS:

Pure drugs: TPH (99.90%) was purchased from Beijing Huikang Boyuan Chemical Co., Ltd, (China). PCM (99.85%) was kindly donated by Sigma for Pharmaceutical Industries Co. (Quesna; Menofia; Egypt).

Chemicals and reagents: Methanol was analytical grade (Sigma Aldrich 99.8%), Excipients of tablet; povidone, magnesium stearate, and micro-crystalline cellulose (avicel) were kindly donated by Sigma Company for Pharmaceutical Industries.

Standard solutions: TPH stock standard solution (1mg.ml⁻¹) was prepared in methanol. Working standard solution (200µg.ml⁻¹) was prepared by diluting 20 ml of TPH stock solution to 100 ml with distilled water.

PCM stock standard solution (1mg.ml⁻¹) was prepared in methanol. Working standard solution (20µg. ml⁻¹) was prepared by diluting 2 ml of PCM stock solution to 100 ml with distilled water.

Povidone stock standard solution (1mg.ml⁻¹) was prepared in distilled water. Working standard solution (10µg.ml⁻¹) was prepared by diluting 1 ml of povidone stock solution to 100 ml with distilled water.

Binary mixtures for method validation: A set of binary mixtures containing different ratios of TPH: PCM (40:8, 60:6, and 80:4 µg. ml⁻¹) were prepared by transferring different aliquots of each of TPH and PCM working standard solutions into 10 ml volumetric flask and diluting to volume with distilled water. These mixtures were used for accuracy and precision calculations.

Construction of calibration curve: In a series of 10-ml volumetric flasks, different aliquots of the working solutions of TPH (200 µg.ml⁻¹) and PCM (20 µg.ml⁻¹) were transferred separately; several appropriate dilutions were carried out with distilled water to obtain solutions of TPH ranging from 20-100 µg.ml⁻¹ and solutions of PCM ranging from 2-10 µg.ml⁻¹. A standard solution of povidone (0.4µg.ml⁻¹) in distilled water was also prepared. Spectra of prepared standard solutions were scanned from 220 - 340 nm against distilled water as a blank and stored in the computer.

Calibration curve of TPH: The computer stored UV absorption spectra of solutions of TPH prepared at different concentrations (20-100 µg.ml⁻¹) were divided by the previously stored spectrum of 10 µg.ml⁻¹ PCM solution smoothed at δλ= 2 nm. First derivative spectra were calculated for the obtained ratio spectra with δλ= 4 nm and scaling factor of one. The same procedures were followed for the computer stored UV absorption spectrum of standard solution of povidone (0.4µg.ml⁻¹).

The calibration curve was obtained by plotting the amplitudes of the first derivative of the ratio spectra (D₆) at 280.4 nm, which is the zero-crossing point for D₆ spectrum of povidone, versus the corresponding concentration of TPH, and then regression equation was computed.

Calibration curve of PCM: The computer stored UV absorption spectra of solutions of PCM prepared at different concentrations (2-14 µg.ml⁻¹) were divided by the previously stored spectrum of 20 µg.ml⁻¹ TPH solution that is smoothed at δλ= 2 nm. First derivative spectra were calculated for the obtained ratio spectra with δλ= 4 nm and scaling factor of one. Similar procedures were conducted for povidone (0.4µg.ml⁻¹) spectrum.

The calibration curve for PCM was obtained by plotting the amplitudes of the first derivative of the ratio spectra (D₆) at 300 nm, which is the zero-crossing point for D₆ spectrum of povidone, versus the corresponding concentration of PCM, and then regression equation was computed.

Preparation of laboratory prepared mixture: Because fixed dose combination of TPH and PCM is not available in local market; laboratory prepared
mixture equivalent to one tablet was prepared. It contains 50mg TPH, 325mg PCM, 10mg povidone, 10mg magnesium stearate and 105mg avicel. The laboratory prepared mixture was transferred to a 50 ml volumetric flask, dissolved in 30 ml methanol, and sonicated for 15 minutes, and then, the solution was made up to the required volume using methanol. The solution was filtered and the first 10 ml of the filtrate was discarded. An aliquot equivalent to 1 ml of the filtrate was transferred to a 50 ml volumetric flask and made up to final volume with distilled water to obtain a solution containing; 20 μg.ml⁻¹ TPH, 130 μg.ml⁻¹ PCM and 4 μg.ml⁻¹ povidone. A volume equivalent to 1 ml of the previous solution was transferred to a 10-ml volumetric flask, spiked with 1 ml of TPH working standard solution (200 μg.ml⁻¹), and then completed to volume with distilled water. This solution contains 22μg.ml⁻¹ TPH, 13μg.ml⁻¹ PCM and 0.4 μg.ml⁻¹ povidone. The solution was analyzed using the proposed method. The concentrations of both drugs were calculated in the presence of povidone from their corresponding regression equations. The claimed concentration of TPH in laboratory prepared mixture was calculated after subtraction of the added concentration (20 μg.ml⁻¹ previously analyzed by the method developed by Mokhtar et al. [13]).

RESULTS AND DISCUSSION

Salinas et al. [21] proposed a new spectrophotometric method, ratio-derivative spectrophotometry, for the simultaneous determination of two compounds in binary mixtures. This method is based on the derivative of the ratio spectra for a binary mixture. The absorption spectrum of the mixture is divided by the absorption spectrum of a standard solution of one of the compounds and the first derivative of the ratio spectrum is obtained. Compared to derivative spectrophotometry at which measurements are done only at zero-crossing points, ratio derivative method permits the use of the wavelength of highest value of analytical signals with several maxima and minima, which give an opportunity for the determination of active compounds in the presence of other compounds and excipients which could possibly interfere in the assay [22]. Berzas Nevado et al. [23] developed a new method for the analysis of ternary mixtures by ratio-spectra zero-crossing derivative method. In this method, the simultaneous determination of three compounds in ternary mixtures is realized by measuring amplitudes at the zero-crossing points in the ratio-derivative spectra.

The aim of this work is to develop a simple and accurate ratio-first derivative spectrophotometric method for the simultaneous determination of TPH and PCM in their fixed dose combination without interference from povidone which is used as excipient. This method is based on measurement of amplitudes of first derivative of ratio spectra at zero-crossing points of povidone.

The UV zero order spectra of TPH, PCM and povidone are shown in Fig. 2. The spectra display moderate overlap, so the application of direct UV spectrophotometry seems to be difficult for the simultaneous determination of TPH and PCM in presence of povidone. The study of the first derivative of the ratio spectra at zero-crossing points of povidone may overcome this difficulty. To optimize the simultaneous determination of TPH and PCM, it is necessary to test the influence of two variables: divisor concentration and Δλ needed to calculate the first derivative. For selection of the most suitable variables affecting the method; a binary mixture containing 20μg.ml⁻¹ TPH and 13μg.ml⁻¹ PCM was prepared. The UV spectrum of the binary mixture was scanned and stored in the computer. The stored zero order UV spectrum of either TPH or PCM as divisor, and the ¹D of the ratio spectra was calculated. On the other hand, standard solutions containing 20μg.ml⁻¹ TPH and 13μg.ml⁻¹ PCM were treated similarly for comparison. For the determination of TPH, different concentrations of PCM (2, 4, 6, 8, 10, 12 μg.ml⁻¹) were tried as divisors. The computer stored UV spectrum of the binary mixture was divided by spectra of various concentrations of PCM smoothed at Δλ = 2 nm, and then first derivative of the spectra obtained at Δλ=8 were calculated. Smoothing of the divisor's spectrum is useful in eliminating noise or fine-scale structures in the spectrum, so facilitating division process needed to prepare ratio spectra. ¹D spectra of the binary mixture and 20μg.ml⁻¹ TPH standard solution using the same concentration of divisor were compared. ¹D of both solutions were only superimposed when 10μg.ml⁻¹ PCM was used as a divisor. This means that 10μg.ml⁻¹ standard solution of PCM was considered as the most suitable divisor as it produces good % recovery. The influence of Δλ for plotting the first derivative of the ratio spectra was also tested to obtain the optimum wavelength interval. Different Δλ values were tried (Δλ: 2, 4, 8, 16). Δλ affects shape, intensity, and position of peaks of the analyzed compound. Δλ = 4 nm was selected as optimum value as it produced maximum peak heights with minimum noise. Ratio and ¹D spectra of PCM are shown in Fig. 3. Povidone spectrum is divided by the spectrum of 10μg.ml⁻¹ PCM and the first derivative of its ratio spectrum was calculated at
Even the presence of more than one peak (two maxima and one minima) in the obtained $^1{D_0}$ spectra of TPH; only that at $\lambda$ 280.4 nm (minima) gave good linearity, % recovery and reproducibility which may be attributed to zero-crossing of povidone. The concentration of TPH was proportional to the amplitude at 280.4 nm in the concentration range 20–100 $\mu$g.ml$^{-1}$.

Similarly for PCM determination; the computer stored zero order UV spectrum of the binary mixture was divided by spectra of various concentrations of TPH (20, 40, 60, 80, 100 $\mu$g.ml$^{-1}$) smoothed at $\delta_0$ = 2 nm, and then first derivative of the spectra obtained at $\delta_0$ = 8 were calculated. $^1{D_0}$ spectra of the binary mixture and 13 $\mu$g.ml$^{-1}$ PCM standard solution using the same concentration of divisor were compared. $^1{D_0}$ of both solutions were superimposed with all divisors' concentrations, however, increasing the divisor concentration produced low peak intensity in both ratio and derivative spectra. So, 20$\mu$g.ml$^{-1}$ TPH was selected as the most suitable divisor's concentration. The influence of $\delta_0$ for plotting the first derivative of both solutions were compared. Different $\delta_0$ values were tried ($\delta_0$ = 2, 4, 8, 16). It was found peak intensity increases in the following order; $\delta_0$ = 2>$\delta_0$= 4>$\delta_0$= 8>$\delta_0$ = 16. On the other hand $\delta_0$ = 2 produces the highest noise. $\delta_0$ = 4 nm was selected as optimum value as it produced maximum peak heights with minimum noise. The obtained ratio and $^1{D_0}$ spectra of PCM are represented in Fig. 5. Povidone spectrum is treated in a similar manner and the overlay $^1{D_0}$ spectra of PCM and povidone are shown in Fig. 4b. Povidone $^1{D_0}$ spectrum shows a zero-crossing point at 300 nm at which PCM can be successfully determined. The concentration of PCM was proportional to the $^1{D_0}$ amplitude measured at 300 nm in the concentration range 2–14 $\mu$g. ml$^{-1}$.

**METHOD VALIDATION:**
The developed methods were validated according to the ICH guidelines [20]. The following validation parameters were addressed.

**Linearity:** The linearity of the methods was evaluated by analyzing nine concentrations of TPH and six concentrations of PCM. Calibration curves were obtained in the linearity range of 20-100 $\mu$g.ml$^{-1}$ and 2-14 $\mu$g.ml$^{-1}$ for TPH and PCM, respectively. The quantitative statistical parameters for the determination of TPH and PCM were summarized in Table 1. The high values of correlation coefficients (r) with negligible intercepts indicate the good linearity of the calibration curves.

**Detection and quantitation limits:** Detection (DL) and quantitation (QL) limits can be calculated depending on standard deviation of the response and the slope. They may be expressed as: 

$$DL = 3.3\sigma/S$$

$$QL = 10 \sigma/S$$

where; "$\sigma$" is the standard deviation of y-intercept of regression line and "S" is the slope of the calibration curve. DL and QL were found to be 1.93µg.ml$^{-1}$ and 5.84µg.ml$^{-1}$ for TPH and 0.32µg.ml$^{-1}$ and 0.97µg.ml$^{-1}$ for PCM (Table 1).

**Accuracy:** The accuracy of analytical procedure is the closeness of test results obtained by that procedure to the true value. Accuracy should be established across its range.

The accuracy of the proposed method was evaluated by analyzing three different laboratory prepared mixture of TPH; PCM (40:8, 60:6, and 80:4) within the linearity range "three replicates". The concentrations of TPH and PCM were obtained from the corresponding regression equations, from which the percentage recoveries were calculated (Table 2). Mean percentage recovery $\pm$ S.D. were 100.813 $\pm$ 0.518 and 100.313 $\pm$ 0.001 for TPH and PCM, respectively.

**Precision:** The precision of an analytical method gives information on the random errors. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed condition. Precision was carried out by analyzing three different laboratory prepared mixture of TPH and PCM within the linearity range in the same day (repeatability, intra-day precision) or on three consecutive days (intermediate, inter-day precision). Standard deviation (S.D.) and percentage relative standard deviation (% R.S.D.) values of the results obtained were calculated (Table 3). The % R.S.D was not more than 0.95 % for TPH and not more than 1.55 % for PCM.

**Specificity:** According to ICH[20] specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. For testing specificity of the proposed method; % recovery of TPH and PCM was determined in mixtures containing both drugs with the possible excipients present in dosage form by the proposed
method as shown in (Table 4). Accepted mean % recovery ± S.D. indicates specificity of the method.

**Analysis of laboratory prepared mixture:** Because combination dosage form of TPH and PCM is not available in Egypt, a laboratory prepared mixture containing the two drugs with all excipients that present in tablet was prepared. Two major problems were encountered upon the analysis of this laboratory prepared mixture: the overlapped spectra with a large difference between the absorptivities of the two drugs; and the ratio of drugs in dosage form [PCM: TPH is 13:2] which make it difficult to determine TPH accurately. On the analysis of mixtures of compounds of largely variable absorptivities, there is a need to use higher concentrations of the components of the mixture solution to analyze those with low absorptivity (TPH) which may lead to a deviation from Beer’s law (concerning PCM) due to interaction between particles of the analyte and variation of refractive index of the sample which results in changing absorptivity [24]. To facilitate the determination of the minor component (TPH), its concentration in the laboratory prepared mixture should be increased using sample enrichment technique. This is done by adding fixed amounts of standard TPH (equivalent to 20 µg) to each experiment to increase its concentration to be within the linearity range of the method. This technique has been used to solve the same problem for analyzing other drug mixtures [25, 26]. Assay solution of the laboratory prepared mixture was analyzed triplicate using the proposed method. The concentrations of both drugs were successfully calculated in the presence of povidone from their regression equations. Recovery data for TPH and PCM were shown in Table 4.

**CONCLUSION**

The proposed method is simple, and rapid. It permits direct determination of TPH and PCM in binary mixtures and laboratory prepared mixture containing all possible excipients present in dosage form without previous separations with good accuracy and precision. Moreover, it has many advantages over separation techniques such as HPLC methods that need more sophisticated instruments. The developed method is also inexpensive and non-polluting to the environment. It is suitable for routine quality control analysis of TPH and PCM in pharmaceutical preparations.

Table 1: Quantitative Parameters for Determination of TPH and PCM With The Proposed 1D_2 Method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity</th>
<th>λ (nm)</th>
<th>r</th>
<th>a</th>
<th>B</th>
<th>S_y/x</th>
<th>S_a</th>
<th>S_b(10^-3)</th>
<th>DL</th>
<th>QL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH</td>
<td>20-100</td>
<td>280.4</td>
<td>0.9997</td>
<td>0.0014</td>
<td>0.0036</td>
<td>0.0025</td>
<td>0.0021</td>
<td>0.033</td>
<td>1.93</td>
<td>5.84</td>
</tr>
<tr>
<td>PCM</td>
<td>2-14</td>
<td>300</td>
<td>0.9997</td>
<td>0.0024</td>
<td>0.0325</td>
<td>0.0035</td>
<td>0.0031</td>
<td>0.335</td>
<td>0.32</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Linearity (µg.ml⁻¹), λ (nm), r: correlation coefficient, a: intercept, b: slope, S_y/x: residual standard deviation of the regression line, S_a: standard error of intercept, S_b: standard error of slope, DL: detection limit µg.ml⁻¹(calculated), QL: quantitation limit µg.ml⁻¹(calculated).

Table 2: Evaluation of Accuracy for the Determination of TPH and PCM.

<table>
<thead>
<tr>
<th>Concentration taken TPH : PCM</th>
<th>Mean concentration Found*</th>
<th>% Recovery</th>
<th>Mean % Recovery ± S.D.</th>
<th>Mean concentration Found*</th>
<th>% Recovery</th>
<th>Mean % Recovery ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 : 8</td>
<td>40.545</td>
<td>101.363</td>
<td>100.813 ± 0.518</td>
<td>7.937</td>
<td>99.217</td>
<td>100.331 ± 1.001</td>
</tr>
<tr>
<td>60 : 6</td>
<td>60.201</td>
<td>100.335</td>
<td></td>
<td>6.037</td>
<td>100.620</td>
<td></td>
</tr>
<tr>
<td>80 : 4</td>
<td>80.593</td>
<td>100.741</td>
<td></td>
<td>4.046</td>
<td>101.156</td>
<td></td>
</tr>
</tbody>
</table>

*: n=3 (concentration in units of µg.mL⁻¹).
Table 3: Evaluation of the Intra-day and Inter-day Precision for the Determination of TPH and PCM.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration taken</th>
<th>Mean concentration Found*</th>
<th>S.D.</th>
<th>% R.S.D.</th>
<th>Mean concentration Found*</th>
<th>S.D.</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH</td>
<td>40</td>
<td>40.545</td>
<td>0.385</td>
<td>0.950</td>
<td>40.434</td>
<td>0.143</td>
<td>0.353</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60.201</td>
<td>0.245</td>
<td>0.407</td>
<td>60.363</td>
<td>0.372</td>
<td>0.616</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>80.593</td>
<td>0.257</td>
<td>0.319</td>
<td>80.310</td>
<td>0.276</td>
<td>0.343</td>
</tr>
<tr>
<td>PCM</td>
<td>8</td>
<td>7.937</td>
<td>0.007</td>
<td>0.086</td>
<td>8.063</td>
<td>0.109</td>
<td>1.346</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.037</td>
<td>0.002</td>
<td>0.028</td>
<td>6.015</td>
<td>0.088</td>
<td>1.456</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.046</td>
<td>0.026</td>
<td>0.646</td>
<td>4.033</td>
<td>0.063</td>
<td>1.550</td>
</tr>
</tbody>
</table>

*: n=3 (concentration in units of μg.mL⁻¹).

Table 4: Recovery Data of TPH and PCM from Laboratory Prepared Mixture.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration Taken*</th>
<th>Concentration found*</th>
<th>Mean concentration Found*</th>
<th>Mean % Recovery ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH</td>
<td>2</td>
<td>2.037</td>
<td>1.990</td>
<td>1.979</td>
</tr>
<tr>
<td>PCM</td>
<td>13</td>
<td>13.169</td>
<td>13.135</td>
<td>13.184</td>
</tr>
</tbody>
</table>

*: Concentration in units of μg.mL⁻¹.

*Fig.1. Chemical structures of TPH, PCM and povidone.*
Fig. 2. Overlain zero order UV absorption spectra of 20 μg.ml⁻¹ TPH (…….), 12 μg.ml⁻¹ PCM (—), and 0.4μg.ml⁻¹ povidone (- - - -).

Fig. 3. Overlain (a); ratio and (b); first derivative ratio (1Dₜ) spectra of TPH (20, 30, 40, 50, 60, 70, 80, 90, 100 μg.ml⁻¹) using 10 μg.ml⁻¹ PCM as a divisor.

Fig. 4. Overlain first derivative ratio (1Dₜ) spectra of 0.4 μg.ml⁻¹ povidone (—) with (a); 20 μg.ml⁻¹ TPH (…..) and (b); 12 μg.ml⁻¹ PCM (…..).
Fig.5. Overlain (a); ratio and (b); first derivative ratio ($D_1$) spectra of PCM (2, 4, 8, 10, 12, 14 μg.ml$^{-1}$) using 20μg.ml$^{-1}$ TPH as a divisor.