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DRUG FORMULATIONS AND CLINICAL METHODS

Novel Approach for the Simultaneous Determination of Carbinoxamine Maleate, Pholcodine, and Ephedrine Hydrochloride Without Interference from Coloring Matter in an Antitussive Preparation Using Smart Spectrophotometric Methods

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The presence of coloring matters in syrups usually interferes with the spectrophotometric determination of active pharmaceutical ingredients. A novel approach was introduced to eliminate the interference of sunset yellow (coloring matter) in Cyrinol syrup. Smart, simple, accurate, and selective spectrophotometric methods were developed and validated for the simultaneous determination of a ternary mixture of carbinoxamine maleate, pholcodine, and ephedrine hydrochloride in syrup. Four of the applied methods used ratio spectra: successive derivative subtraction coupled with constant multiplication, successive derivative of ratio spectra, ratio subtraction coupled with ratio difference, and ratio spectra continuous wavelet transforms zero-crossing. In addition, a method that was based on the presence of an isosbestic point, the amplitude summation method, was also established. A major advantage of the proposed methods is the simultaneous determination of the mentioned drugs without prior separation steps. These methods were successfully applied for the determination of laboratory-prepared mixtures and a commercial pharmaceutical preparation without interference from additives, thus proving the selectivity of the methods. No significant difference regarding both accuracy and precision was observed upon statistical comparison of the results obtained by the proposed methods with each other and with those of official or reported ones.

Pholcodine {PHL; 7,8-didehydro-4,5α-epoxy-17-methyl-3-[2-(morpholin-4-yl)ethoxy] morphinan-6α-ol} is a centrally acting cough suppressant. Its actions and uses are similar to those of dextromethorphan. It helps in the suppression of unproductive coughs in addition to its mildly sedative effect (1). However, it has little or no analgesic effects (1). Carbinoxamine maleate {CAR; 2-[(4-chlorophenyl)(2-pyridinyl)methoxy]-*N*,*N*-dimethylethanamine (2*Z*)-2-butenedioate} is an antihistamine with anticholinergic and sedative properties. It is administered mainly for the relief of allergic conditions such as rhinitis. It is a common component in preparations used for the symptomatic treatment of coughs and the common cold (1). Ephedrine hydrochloride [EPH; 2-(methylamino)-1-phenyl-1-propan-1-ol hydrochloride] is a sympathomimetic amine that is frequently used as decongestant and bronchodilator. It produces its action by reducing swelling, constricting blood vessels in nasal passages, and widening lung airways, thus permitting easier breathing (1). The chemical structures for these drugs are shown in Figure 1. The combination of CAR, PHL, and EPH is usually used for the relief of nonproductive coughs and upper respiratory symptoms associated with allergy and the common cold.

Several methods were found in the literature for the determination of CAR, PHL, and EPH either alone or in combination with other drugs. Various analytical methods were established for the analysis of CAR, such as spectrophotometry (2–5), HPLC (6–9), capillary electrophoresis (CE) (10), GC (11, 12), and LC-MS (13). Concerning PHL, it was determined by HPLC (14–19), CE (20), GC (21, 22), and LC-MS (23). With regards to the analysis of EPH, numerous methods were developed, such as spectrophotometry (24–26), HPLC (27, 28), CE (29–32), GC (33, 34), and LC-MS (35).

To date, only one separation method has been reported for the analysis of the combination of CAR, PHL, and EPH, utilizing HPLC (36). The presence of coloring matter in the pharmaceutical dosage form hinders the determination of these pharmaceutical ingredients using a spectrophotometric technique. However, the use of the hyphenated instrumentation, such as HPLC–UV, GC-MS, or HPLC-MS for the determination of multicomponents is of high cost and consumes much time for method development and optimization, which are considered disadvantages in QC laboratories in which

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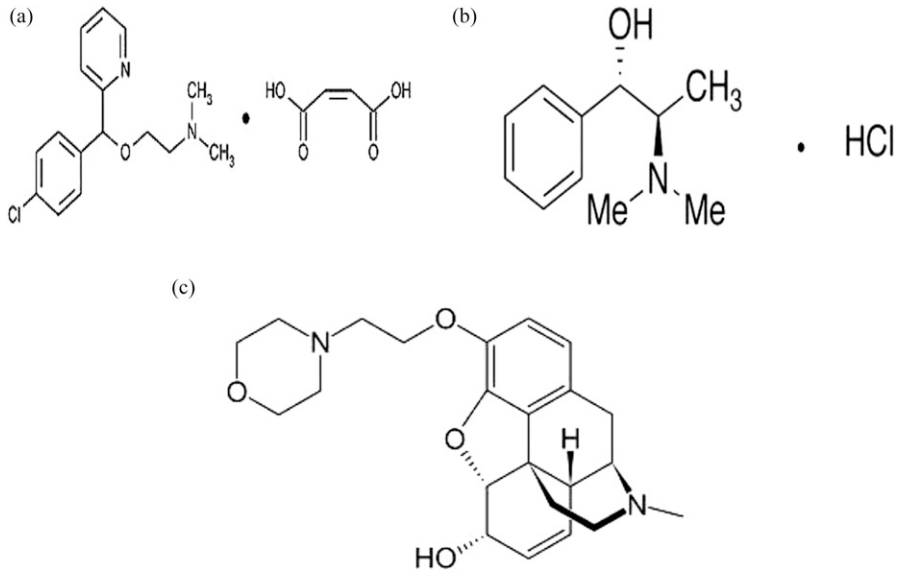

Figure 1. The chemical structures of (a) CAR, (b) EPH, and (c) PHL.

speedy analysis is required. Mathematical spectrophotometric methods as well as chemometric techniques are considered more reliable, can solve the above drawbacks, and provide alternative resolutions for the complex mixtures of analytes without needing prior separation or extraction. More significantly, these techniques eliminate the use of harmful chemicals to both health and environment because the main solvents used are usually water, ethanol, or methanol, which are considered less hazardous than the buffers and solvents used in chromatographic methods. Thus, mathematical spectrophotometric and chemometric methods could be considered green analytical methods of analysis.

From this point of view, the main issue of the proposed work was to develop smart spectrophotometric methods that could determine the three components without interference of the coloring matter present in the syrup. These methods are successive derivative subtraction coupled with constant multiplication (SDS-CM), successive derivative of ratio spectra (SDR), ratio subtraction coupled with ratio difference (RS-RD), ratio spectra continuous wavelet transforms zero-crossing (RSCWT-ZC), and amplitude summation (A-Sum) for the determination of CAR, PHL, and EPH. The used methods were very simple, accurate, and precise and did not require any sophisticated apparatus or computer programs.

Experimental

Apparatus and Software

A Shimadzu UV 1800 double-beam UV-Vis spectrophotometer (Japan) with matched 1 cm quartz cells in the 200–400 nm range was used for all absorbance measurements. Spectra were automatically obtained with Shimadzu UVProbe 2.32 system software. For CWT calculations, Matlab Version 7.9 was used.

Chemicals and Solvents

(a) *Pure samples.*—CAR, PHL, and EPH were kindly supplied by Amoun Pharmaceutical Co. (El-Obour City, Cairo, Egypt). Their purities were found to be 100.92 ± 1.212, 99.05 ± 1.089, and 101.23 ± 0.956% for PHL, CAR, and EPH, respectively, by the reported method (36) for CAR and by the *British Pharmacopoeia* (BP) method (37) for PHL and EPH. Sunset yellow (SUN) was kindly supplied by GlaxoSmithKline (Fifth District, New Cairo, Egypt).

(b) *Market sample.*—Cyrinol syrup (Batch No. 135275) manufactured by Amoun Pharmaceutical Co. was purchased from a local market. Each 5 mL syrup contained 2 mg CAR, 4 mg PHL, 7 mg EPH, and 0.5 mg SUN.

(c) *Solvents.*—Distilled water was used.

Solutions

(a) *Stock solutions.*—Stock solutions at a concentration of 1 mg/mL for PHL and CAR and 4 mg/mL for EPH were prepared using distilled water as the solvent.

(b) *Working solutions.*—Solutions were freshly prepared by dilution with stock solutions of the same solvent to obtain a concentration of 400 μg/mL PHL, 200 μg/mL CAR, 2000 μg/mL EPH, and 40 μg/mL SUN.

Procedure

(a) *Spectral characteristics and linearity.*—Into a series of 10 mL volumetric flasks, aliquots of the working solutions of 400 μg/mL PHL, 200 μg/mL CAR, and 2000 μg/mL EPH were separately transferred to obtain final concentrations equivalent to 20–240 μg/mL PHL, 8–100 μg/mL CAR, 80–1000 μg/mL EPH, and 0.4–40 μg/mL SUN. The absorption spectra were scanned for the prepared solutions in the range of 200–400 nm and were stored in the computer.

(b) *SDS-CM.*—From the stored zero-order absorption spectra of the three drugs, the first derivative (D^1) was calculated. Calibration curves were constructed by plotting the D^1 peak-to-peak amplitude of PHL at $P_{248.5}$–$P_{271.8}$, CAR at P_{254}–$P_{269.5}$, and EPH at $P_{250.5}$–$P_{267.5}$ versus the corresponding concentrations of PHL, CAR, and EPH.

(c) *SDR.*—The zero-order absorption spectra of different concentrations of PHL were divided by the spectrum of 10 μg/mL CAR; thus, ratio spectra were obtained. D^1 of the ratio spectra were attained with $\Delta\lambda = 8$ and a scaling factor of 10.

These vectors (D^1 of the ratio spectra) were further divided by d/dλ (400 μg/mL EPH ÷ 10 μg/mL CAR), corresponding to the derivative of the ratio of the spectra of EPH and CAR; thus, second ratio spectra were obtained. D^1 of these vectors were obtained with $\Delta\lambda = 8$ and a scaling factor of 10. The calibration curve of PHL was constructed by plotting the amplitude of the resulting spectra at 251.7 nm against its corresponding concentration. The same steps were accomplished for CAR using the spectrum of 40 μg/mL PHL as the first divisor, followed by the spectrum of d/dλ (400 μg/mL EPH ÷ 40 μg/mL PHL) as the second divisor, and for EPH, using the spectrum of 10 μg/mL CAR as the first divisor, followed by the spectrum of d/dλ (40 μg/mL PHL ÷ 10 μg/mL CAR) as the second divisor. The calibration curves of CAR and EPH were constructed by plotting the amplitude of the resulting spectra at 254.4 and 251 nm, respectively, against their corresponding concentrations.

(d) *RS-RD.*—The second derivative (D^2) of PHL was calculated for the stored zero-order absorption spectra. A calibration curve was plotted relating the amplitude of the D^2 spectra of PHL at 295 nm ($\Delta\lambda = 8$ and a scaling factor of 10) against its corresponding concentrations, and the regression equation calculated.

The stored zero-order spectra (D^0) of CAR were divided by the spectrum of 200 μg/mL EPH, whereas the D^0 spectra of EPH were divided by the spectrum of 20 μg/mL CAR. Calibration curves for CAR and EPH were plotted, relating the amplitude difference of the ratio spectra between 250.5 and 260 nm for CAR and 253.5 and 262 nm for EPH against their corresponding concentrations, and the regression equations computed.

(e) *RSCWT-ZC.*—The data points of the scanned D^0 of PHL were transferred to Matlab and processed by using Daubecies CWT family with a scale value of 40 ($\alpha = 40$). The calibration curve was constructed by measuring the CWT amplitude of the maxima at 295.5 nm versus PHL's corresponding concentrations, followed by computing of the regression equation.

To determine CAR and EPH, the D^0 of the CAR and EPH compounds were divided by the spectrum of 80 μg/mL PHL (divisor), followed by processing the obtained ratio spectra data vectors by Daubecies CWT family with a scale value of 40 ($\alpha = 40$). CWT calibration equations were constructed by measuring the CWT amplitudes at 261.7 nm for CAR and 258 nm for EPH versus their corresponding concentrations, followed by computing of the regression equations.

(f) *A-Sum.*—The zero-order absorption spectra of PHL, CAR, and EPH were derivatized in the first order, with $\Delta\lambda = 8$ and a scaling factor of 1000, followed by recording the D^1 amplitudes. The amplitude factors of PHL were calculated at wavelengths $D_{284}/D_{294.5}$ and $D_{246.3}/D_{294.5}$ and were found to be 0.30 and 2.72, respectively. The D^1 amplitude of PHL at 294.5 nm was multiplied by the previously calculated absorption factor, $D_{284}/D_{294.5}$, to get its amplitude at 284 nm. The calibration curves were constructed by plotting the amplitudes of PHL and CAR at 284 nm and EPH at 246.3 nm against their corresponding concentrations, followed by computing of the regression equations.

(g) *Analysis of laboratory-prepared mixtures.*—Aliquots of PHL, CAR, and EPH were transferred into a series of 10 mL volumetric flasks from their corresponding standard working solutions of 400 μg/mL for PHL, 200 μg/mL for CAR, and 2000 μg/mL for EPH and diluted to volume with distilled water. Consequently, mixtures comprising different ratios of the three drugs were prepared. The absorption spectra of the laboratory-prepared mixtures were scanned in the range of 200–400 nm and stored. The different procedures corresponding to each method were performed as described above. The concentrations of PHL, CAR, and EPH were calculated by substitution in the corresponding regression equations.

(h) *Analysis of the pharmaceutical dosage form.*—Five milliliters of Cyrinol syrup were transferred into a 10 mL volumetric flask and diluted to volume with distilled water to form a sample stock solution with a concentration of 400 μg/mL PHL, 200 μg/mL CAR, and 700 μg/mL EPH. From this solution, 2 mL were further diluted in 10 mL distilled water to obtain a working solution with a final concentration of 80 μg/mL PHL, 40 μg/mL CAR, and 140 μg/mL EPH.

To remove the interference of SUN, before carrying forward the general procedure described under each method, the spectrum of SUN was eliminated using the RS method. The zero-order absorption spectra of the working solution of Cyrinol syrup were divided by the standard spectrum of 12 μg/mL SUN (divisor) to obtain the ratio spectra. Absorbances in the plateau region, 305–400 nm (the constant), were subtracted from the ratio spectra, and the obtained curves were multiplied by the divisor. Thus, spectra representing the ternary mixture of PHL, CAR, and EPH were obtained. The general procedures previously described under each method were followed to determine the concentration of PHL, CAR, and EPH in the prepared dosage form solution. The concentrations of the cited drugs were calculated using the corresponding regression equations.

The standard addition technique was applied to further assess the validity of the proposed procedures. Thus, different known concentrations of pure standard PHL, CAR, and EPH were added to the pharmaceutical formulation before continuing to the previously mentioned methods.

Results and Discussion

The use of spectrophotometric methods, such as derivative spectrophotometry, ratio spectra spectrophotometry, and other chemometric spectral calibration techniques, has proven to be beneficial in analytical studies related to the QC and routine analysis of commercial products both in research and industry laboratories. These spectrophotometric methods are preferably used as an alternative to hyphenated analytical instrumentations or techniques, such as LC-MS, GC-MS, and LC-NMR, as the latter methods permanently require prior steps, such as extraction, which are considered to be tedious processes during analysis.

Taking into account all the above arguments, the resolution of binary or ternary mixtures with overlapped spectra using quantitative spectrophotometric methods is an interesting concern in analytical chemistry. These existing spectrophotometric methods have proven to be easily applied, very rapid, and sensitive and, additionally, very cheap for the analysis of mixtures. The necessity for new analytical approaches to obtain accurate, precise, and safe results for resolving complex mixtures has allowed the analytical

chemist to be focused on the use of new mathematical techniques or the combined use of the mentioned approaches with traditional analytical techniques. However, the presence of some additives, e.g., coloring matters and preservatives, sometimes interferes and hinders the spectrophotometric determination of the studied compounds.

So, the main task of this work was to find an approach that could remove the interference of the present coloring matter and apply smart methods that could determine active ingredients without any interference. Simple, sensitive, and accurate analytical methods for the simultaneous determination of PHL, CAR, and EPH in their bulk powders and pharmaceutical dosage form with satisfactory precision and accuracy were established. In addition, construction of a statistical comparison between the abilities of the proposed methods for the determination of the cited drugs was carried out.

By scanning the absorption spectra of PHL, CAR, EPH, and SUN in distilled water, severely overlapped spectral bands were observed in the wavelength region of 200–300 nm, which hindered their simultaneous determination (Figure 2a), so different methods were applied for achieving the best resolution and the quantitative determination of each drug without any interference from the other. No spectrophotometric methods were found in the literature for the determination of PHL, CAR, and EPH in a ternary mixture.

SDS-CM

This method was presented for resolving complex mixtures with severely overlapped zero-order absorption spectra by using their derivative spectra (38). D^1 was calculated for the laboratory-prepared mixtures so PHL could be determined without interference from CAR and EPH. The first step was achieved in which D^1 spectra for the laboratory-prepared mixtures were divided by the D^1 spectrum of 40 µg/mL PHL as the divisor; the constant amplitude values of PHL/PHL′ **(I)** were recorded in the plateau region at 282–292 nm and subtracted.

In the second step, multiplication of the obtained spectra by the D^1 spectrum of the divisor 40 µg/mL PHL was carried out to obtain the D^1 spectrum, demonstrating the binary mixture of CAR and EPH. In the third step, determination of the constant amplitude value of CAR/CAR′ was performed by dividing the obtained spectra from the previous step (CAR + EPH) using the D^1 spectrum of 10 µg/mL CAR as the divisor, followed by recording of the constant amplitude values of CAR/CAR′ **(II)** in the plateau region at 277–288 nm, which were then subtracted. In the fourth step, multiplication of the obtained spectra by the D^1 spectrum of the divisor 10 µg/mL CAR was carried out to obtain the D^1 spectrum of EPH present in each of the laboratory mixtures, and its concentration was determined.

A final step was carried out to obtain the D^1 spectra of PHL and CAR through multiplication of the previously recorded constants values **(I)** and **(II)** by the D^1 spectra of the divisors 40 µg/mL PHL and 10 µg/mL CAR, respectively. With the aim of optimizing the D^1 method, different smoothing and scaling factors were tried. A smoothing factor of $\Delta\lambda = 8$ and a scaling factor of 10 displayed an appropriate S/N, and the spectra exhibited good resolution. The divisor was selected to compromise between minimal noise and maximum sensitivity, and it presented the best average recovery percentage when used for the analysis of the laboratory-prepared mixtures.

PHL was determined peak-to-peak at $P_{248.5}$–$P_{271.8}$, CAR at P_{254}–$P_{269.5}$, and EPH at $P_{250.5}$–$P_{267.5}$.

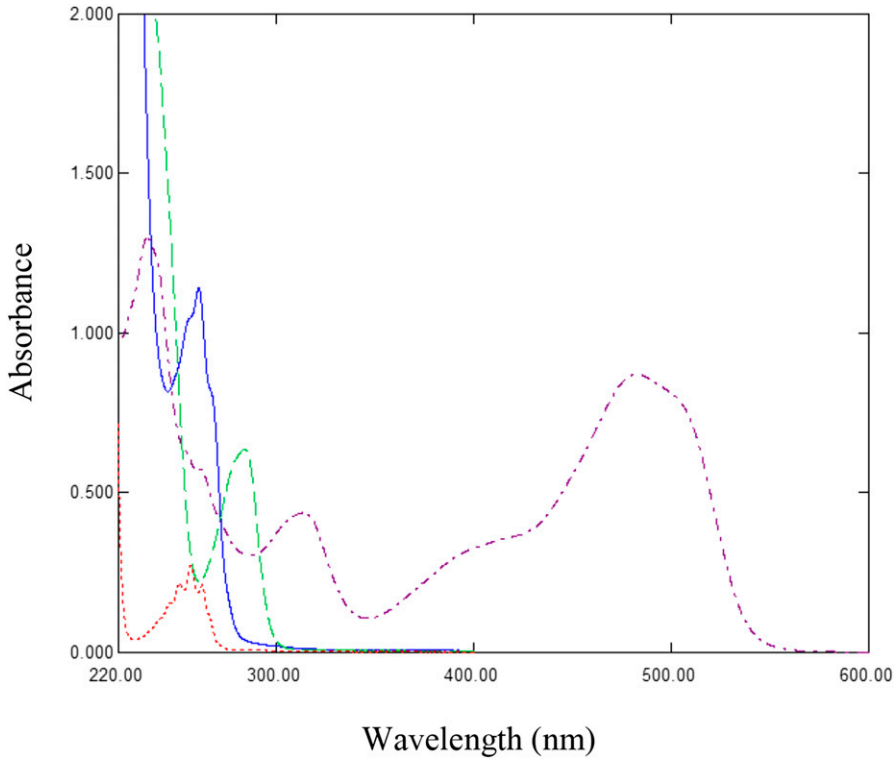

Figure 2. Zero-order absorption spectra of 160 µg/mL PHL (dashed line), 80 µg/mL CAR (solid line), 280 µg/mL EPH (dotted line), and 20 µg/mL SUN (dash–dot line).

This method was only applied for the resolution of ternary mixtures of *X*, *Y*, and *Z*, in which the spectrum of *Z* was more extended than *Y*, which was in turn more extended than *X*, as demonstrated in Figure 3.

SDR

This method was established for the simultaneous determination of ternary mixtures, without prior separation steps (39). The method relied on the successive derivative of ratio spectra in two steps for each component. For the determination of PHL and EPH, the absorption spectra of the laboratory-prepared mixtures were divided by the spectrum of 10 µg/mL CAR, and the D^1 was calculated for the ratio spectra, V1. For PHL, the vectors, V1, were divided by the D^1 spectrum of 400 µg/mL EPH ÷ 10 µg/mL CAR; thus, the second ratio spectra were obtained (V2). Finally the D^1 was calculated for these vectors (V2) in which the concentration of PHL was determined by measuring the maximum amplitude at 251.7 nm, as shown in Figure 4a. For EPH, vectors V1 were divided by the D^1 spectrum of 40 µg/mL PHL ÷ 10 µg/mL CAR, in which the second ratio spectra were obtained (V3). The D^1 was calculated for these vectors (V3), and the concentration of EPH was determined by measuring the maximum amplitude at 251 nm, as shown in Figure 4b. For the determination of CAR, the absorption spectra of the laboratory-prepared mixtures were divided by the spectrum of 40 µg/mL PHL, followed by calculating the D^1 for these ratio spectra. The obtained derivative of ratio spectra was then divided by the D^1 spectrum of 400 µg/mL EPH ÷ 40 µg/mL PHL; thus, the second ratio spectra were obtained. Finally, the concentration of CAR was determined by measuring the maximum amplitude at 254.4 nm, as shown in Figure 4c. The divisors and smoothing and scaling factors were chosen as in SDS.

RS-RD

PHL was directly determined by the D^2 at 295 nm ($\Delta\lambda = 8$ and scaling factor of 10), as shown in Figure 5, in which its concentrations were calculated from the computed regression equation. To eliminate PHL, the RS method (40, 41) was used. RS is based on the extension of the spectrum of PHL over the spectra of CAR and EPH in their ternary mixture. For the determination of CAR and EPH in mixtures, the zero-order absorption spectra of the laboratory-prepared mixtures were scanned and divided by a carefully chosen concentration of standard PHL′ (40 µg/mL) as the divisor. Thus, new ratio spectra were produced that represent CAR + EPH/PHL′ + constant. The values of these constants PHL/PHL′ in the plateau region 282–292 nm were subtracted. The obtained spectra were then multiplied by the spectrum of the divisor PHL′ (40 µg/mL). Thus, the original spectra of CAR–EPH were obtained, which were used for the direct determination of CAR + EPH by the RD method.

To determine CAR and EPH by the RD method, the D^0 of the different laboratory-prepared mixtures were divided by the absorption spectra of standard 200 µg/mL EPH and standard 20 µg/mL CAR. Calibration curves were obtained by plotting the difference in the amplitudes at 250.5 and 260 nm for CAR and the difference in the amplitudes at 253.5 and 262 nm for EPH

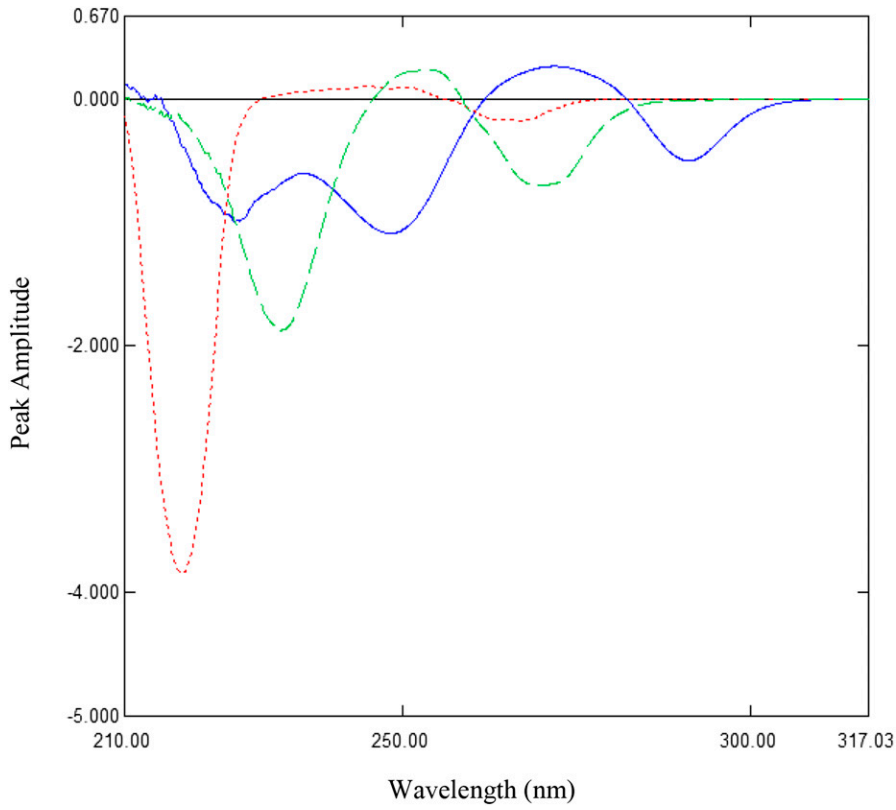

Figure 3. ^{1}D spectra of the mixture of 160 µg/mL PHL (solid line), 80 µg/mL CAR (dashed line), and 280 µg/mL EPH (dotted line).

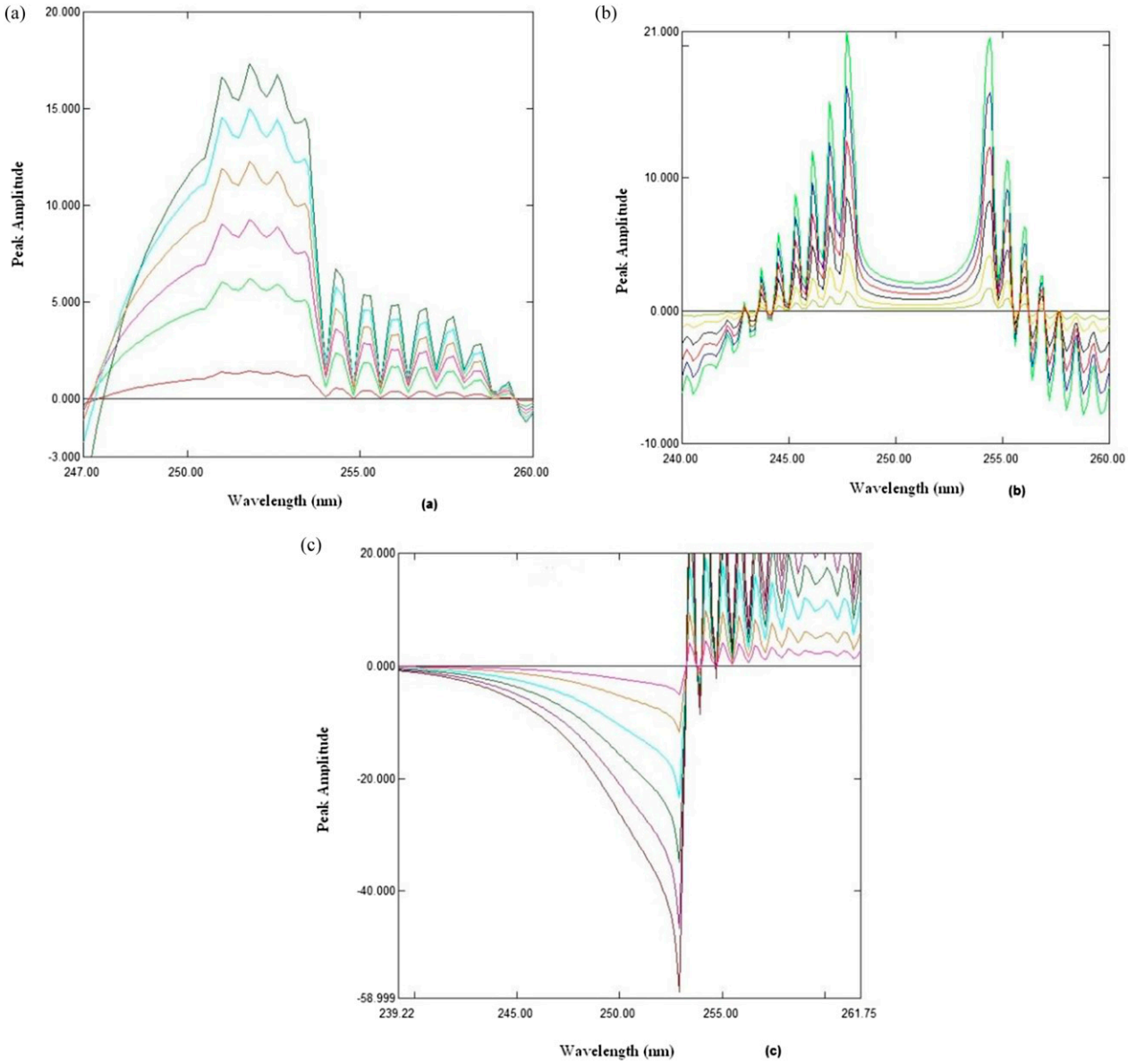

Figure 4. The vectors of the ^{1}D of the second ratio spectra obtained with $\Delta\lambda$ = 8 nm and a scaling factor of 10 for (a) PHL, (b) EPH, and (c) CAR within their linearity ranges.

against their corresponding concentrations, and the regression equations were computed (Figure 6).

RSCWT-ZC (42, 43)

For the determination of PHL, CWT spectra were obtained using Daubecies family for the ternary mixture by using the second order and a scaling factor of 40, and PHL was measured at the CWT amplitude at 295.5 nm in which CAR and EPH showed no contribution, as shown in Figure 7.

For the determination of CAR and EPH in their ternary mixtures, the absorption spectra of different concentrations of CAR and EPH compounds were divided by the standard spectrum of 80 μg/mL PHL and the CWT spectra obtained by using the second order and a scaling factor of 40. CWT calibration equations were obtained by measuring the CWT amplitudes at 261.7 nm for CAR (zero-crossing of EPH) and 258 nm for EPH (zero-crossing of CAR).

It was observed that the CWT method in combination with the zero-crossing technique and spectral ratio procedure (43, 44) is a new hybrid analytical approach with very simple application for the higher resolution of the overlapping signals and ratio signals, despite the difficult mathematical theory of these wavelet families.

A-Sum

A-Sum (45) is based on the isosbestic point present in derivative absorption spectra. The spectrum of the PHL, CAR, and EPH ternary mixture exhibited two isosbestic points, the first one at 284 nm for PHL and CAR, in which EPH showed no contribution, whereas the second was at 246.3 nm for CAR and EPH with a certain contribution of

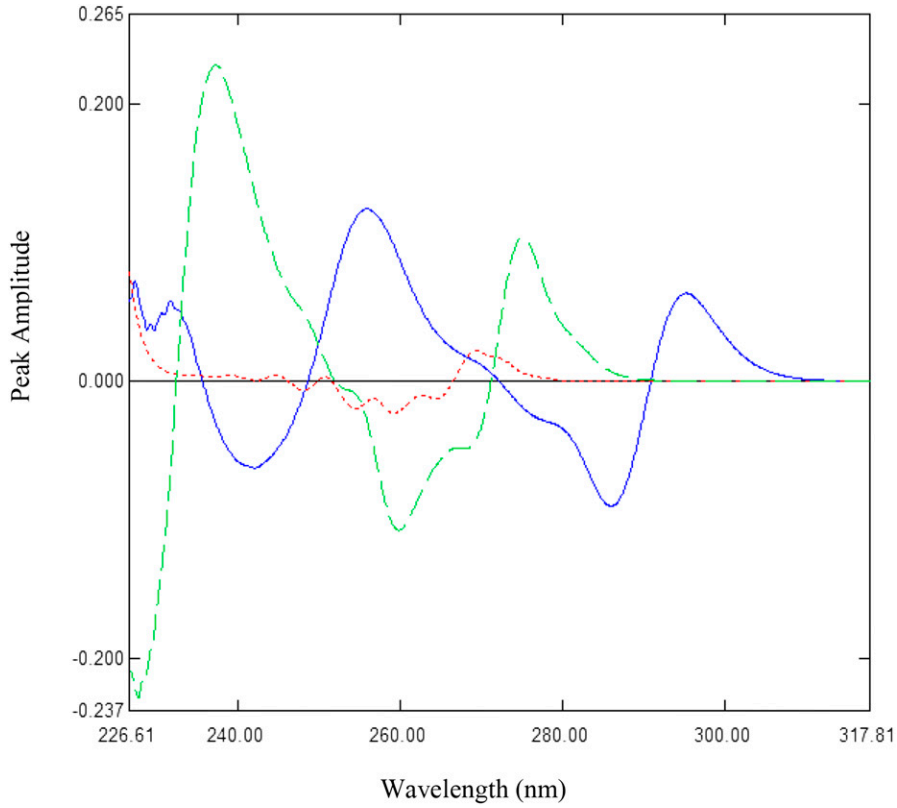

Figure 5. The ^{2}D spectra of mixture of 160 µg/mL PHL (solid line), 80 µg/mL CAR (dashed line), and 280 µg /mL EPH (dotted line).

PHL. The two components exhibiting the isosbestic point could be determined via a unified regression equation at a zero-crossing point or zero contribution of other interfering substances. For the determination of PHL and CAR, their isosbestic point at 284 nm was used (Figure 8). The D^1 amplitude of PHL at 284 was calculated using its amplitude factor between 284 and 294.5 nm, which was equal to 0.30, whereas the corresponding amplitude of CAR was obtained by subtraction. The D^1 amplitude of PHL and CAR was used to calculate each of their concentrations using the unified regression equation at λ_{iso1} = 284 nm, in which EPH exhibited zero contribution, even at higher concentrations.

$$D_{PHL} \text{ at } 284\,\text{nm} = [D_{284}/D_{294.5}] \cdot (D_{mix} \text{ at } 294.5\,\text{nm})$$

$$D_{CAR} \text{ at } 284\,\text{nm} = D_{mix} \text{ at } 284\,\text{nm} - D_{PHL} \text{ at } 284\,\text{nm}$$

where D_{mix} = D^1 amplitude value of the ternary mixture; D_{PHL} and D_{CAR} = D^1 amplitudes of PHL and CAR, respectively; and

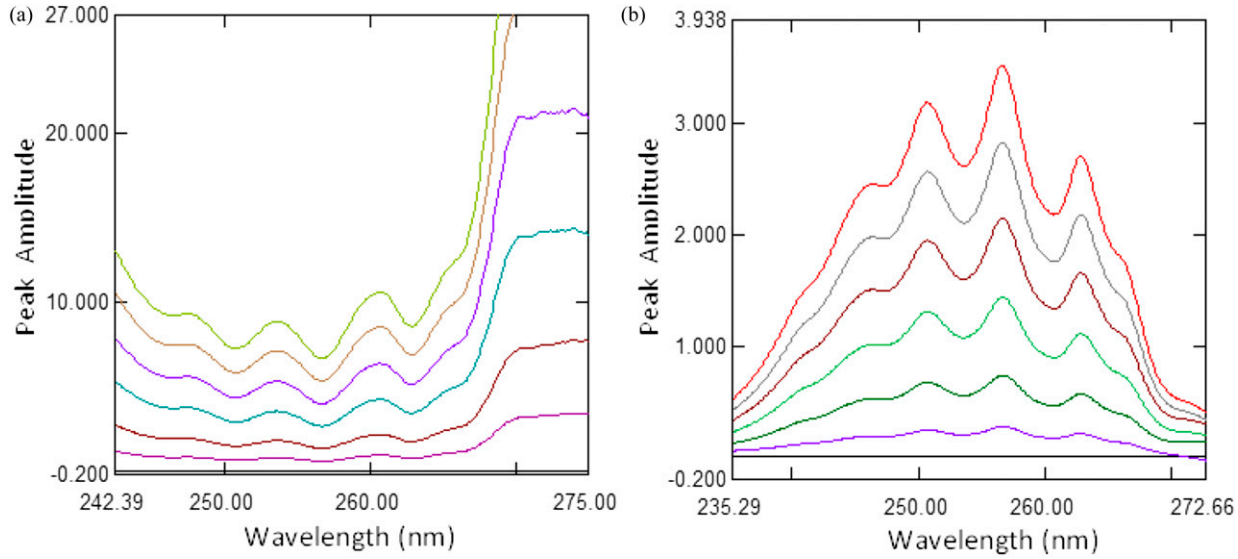

Figure 6. (a) Ratio spectra of CAR (8–100 µg /mL) using EPH 200 µg/mL as the divisor and (b) the ratio spectra of EPH (80–1000 µg /mL) using CAR 20 µg/mL as the divisor.

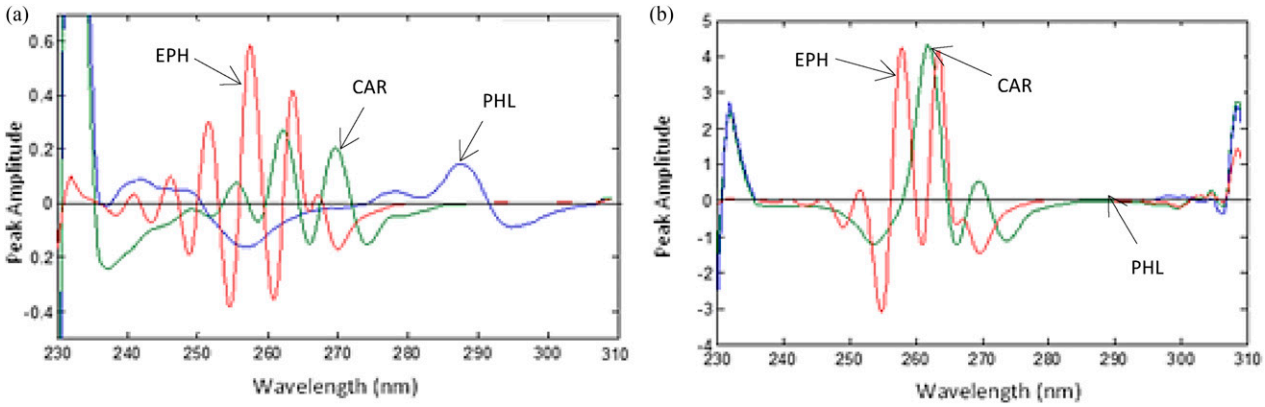

Figure 7. (a) CWT spectra of PHL, CAR, and EPH and (b) CWT ratio spectra of PHL, CAR, and EPH using 80 µg/mL of PHL as a divisor.

$D_{284}/D_{294.5}$ = ratio of the D^1 amplitudes of pure PHL at 284 nm to those at 294.5 nm.

For the determination of EPH, the isosbestic point of CAR and EPH at λ_{iso2} = 246.3 nm was used after subtracting the amplitude of the interfering PHL at this point (Figure 8). The D^1 amplitude of PHL at 246.3 was calculated using its amplitude factor between 246.3 and 294.5 nm, which was equal to 2.72. The amplitude of EPH was obtained by subtracting the amplitude corresponding to CAR at the same point.

The amplitude of CAR was calculated by substituting the previously obtained CAR concentration in the unified regression equation at 246.3 nm, and the concentration of EPH was calculated using the unified regression equation at 246.3 nm.

$$D_{PHL} \text{ at } 246.3\text{ nm} = [D_{246.3}/D_{294.5}] \cdot (D_{mix} \text{ at } 294.5\text{ nm})$$

$$D_{EPH} \text{ at } 246.3\text{ nm} = D_{mix} \text{ at } 246.3\text{ nm} - [D_{PHL} \text{ at } 246.3\text{ nm} + D_{CAR} \text{ at } 246.3\text{ nm}]$$

where D_{mix} = D^1 amplitude of the ternary mixture (PHL + CAR + EPH); D_{PHL}, D_{CAR}, and D_{EPH} = D^1 amplitude of PHL, CAR, and EPH, respectively; and $D_{246.3}/D_{294.5}$ = D^1 amplitude factor of pure PHL at 246.3 nm to that at 294.5 nm.

Comparing all the used proposed methods has demonstrated that there are several advantages and some limitations to each method. Regarding the SDS-CM method, the advantage of this method is obtaining the D^1 spectrum of each component in the mixture as that of the pure one without any interference from any other components. The amplitudes of the D^1 spectra of each drug could be measured peak-to-peak, resulting in higher amplitude values and consequently a larger slope and maximum sensitivity. On the contrary are those attained by applying the derivative technique without preliminary resolution in which the amplitudes were critically measured either at zero-crossing or zero contribution of the interfering substances (peak-to-zero baseline). However, the disadvantage of this method is the requirement for a different divisor for each component and using different ratio spectra for the determination of the ternary mixture.

For the SDR method, the advantage of this method is that it has no restrictions for its application as it does not require the

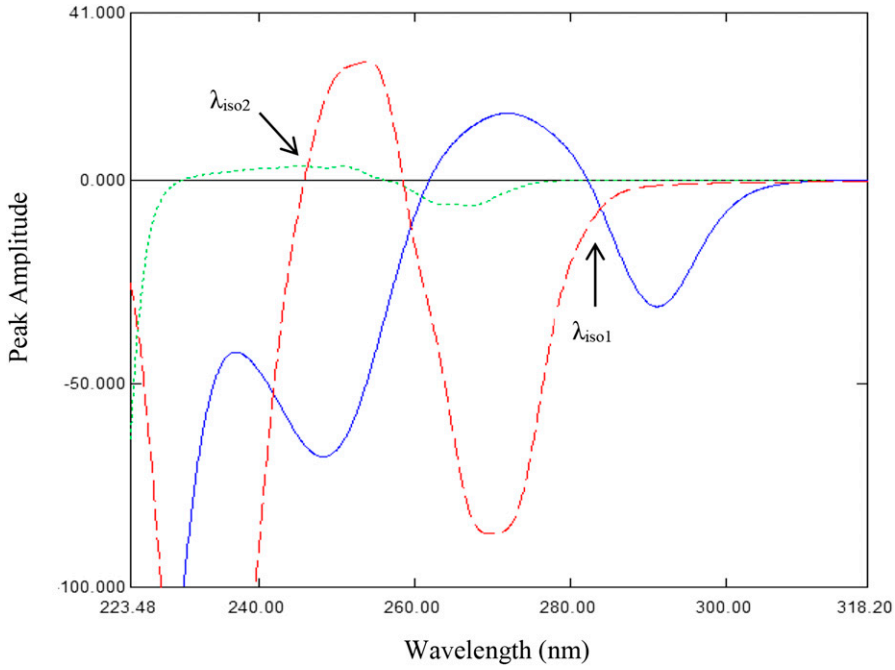

Figure 8. ^{1}D spectra of each of 100 µg/mL PHL(solid line), CAR (dashed line), and EPH (dotted line).

extension of the spectrum of any of the components. However, the disadvantage of this method is the utilization of several derivatization steps using two divisors for the determination of each component, which leads to lower amplitude values and consequently minimum sensitivity if compared with those obtained by SDS.

The RS-RD method has the combined advantages of both the RS method and the RD method. The RS method produces spectra similar to those of the pure form, which settles the spectral profile of each component of interest, although the RD method has the capability of complete removal of the interfering component as a constant, so there is no need for critical measurements, leading to more reproducible results. However, the disadvantage of the RS-RD method is the necessity of the presence of a standard solution of the interfering component to act as a divisor in which its proper choice shows a critical effect on the S/N.

The RSCWT-ZC method is advantageous over the applied derivative ratio methods, as it can be applied for the determination of the three components in a ternary mixture with no limitations. RSCWT-ZC has advantages over the SDR because the denoising and elimination of the derivative steps enhanced the S/N, thus enabling higher sensitivity rather than SDR and the other proposed methods. The only limitation is the need for special software (Matlab) to transform the signals.

The advantage of the A-sum method is that its application does not require the presence of a divisor as in SDR and SDS, but its limitation is the requirement of an isosbestic point in the derivative spectra.

The corresponding concentration ranges and calibration equations for the proposed methods are listed in Table 1.

The selectivity of the proposed procedures was evaluated by analyzing the laboratory-prepared mixtures containing different ratios of the cited drugs, for which acceptable results were achieved, as shown in Table 2.

The proposed procedures were also applied for the determination of Cyrinol syrup. Besides PHL, CAR, and EPH, Cyrinol syrup also contains the azo dye, SUN, whose presence was considered challenging as it hindered the determination of the three drugs because it showed high absorbance in the region of 200–300 nm. Thus, the spectrum of SUN was usually eliminated by RS method before the analysis of the ternary mixture in the dosage form. The validity of the proposed procedures was further assessed by applying the standard addition technique, showing no excipient interference. The results obtained are displayed in Table 3.

Method Validation

Validation was done according to International Conference on Harmonization recommendations (46). Linearity, accuracy, selectivity, range, and precision (repeatability and intermediate precision) were determined, as shown in Table 1. Satisfactory results were obtained within the global validation reference values, as discussed in the following sections.

Range and linearity.—The different calibration curves were processed on 3 different days to evaluate the linearity of the proposed methods. The calibration curves were plotted within concentration ranges that were selected on the basis of the drug concentration during the assay of the dosage form. The analytical data of the calibration graph, including concentration ranges and

Table 1. Results of regression and validation parameters of the proposed spectrophotometric methods for the determination of PHL, CAR, and EPH

	SDS			SDR			RS-RD			CWT			A-Sum		
Validation parameters	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH
Linearity range, μg/mL	20–240	8–100	80–1000	20–240	8–100	80–1000	20–240	8–100	80–1000	20–240	8–100	80–1000	20–240	8–100	80–1000
Slope	0.0081	0.0116	0.0009	0.0687	0.5748	0.0209	0.0004	0.0308	0.0019	0.0009	0.0710	0.0051	–0.0766	–0.0669	0.0338
SE slope	5.26×10^{-5}	2.220×10^{-5}	8.352×10^{-6}	0.0007	0.0016	0.0002	1.722×10^{-6}	1.447×10^{-4}	8.913×10^{-6}	6.332×10^{-6}	0.0002	2.626×10^{-5}	1.556×10^{-4}	0.0004	0.0003
Intercept	0.0366	–0.0024	0.0054	0.6076	0.3996	0.0779	–0.0003	0.0135	–0.0019	0.0045	0.0386	0.0729	0.2091	–0.1081	0.1439
SE intercept	0.0082	0.0013	0.0051	0.1133	0.0986	0.1208	0.0003	0.0087	0.0054	0.0009	0.0137	0.0159	0.0241	0.0263	0.1670
Correlation coefficient, r	0.9999	1.0000	0.9998	0.9998	1.0000	0.9998	0.9999	0.9999	0.9998	0.9999	1.0000	0.9999	1.0000	0.9999	0.9999
SD of residuals	1.1671	0.1513	0.0066	1.9111	0.1285	0.1575	0.0003	0.0114	0.0070	0.0011	0.2514	0.0208	0.0279	0.0341	0.0453
LOD, μg/mL	2.304	0.501	24.200	6.307	0.738	24.868	2.475	1.221	12.158	4.033	0.830	13.433	1.204	1.686	21.264
LOQ, μg/mL	6.983	1.517	73.333	19.112	2.237	75.359	7.500	3.701	36.842	12.222	2.514	40.706	3.649	5.109	64.438
Accuracy: mean ± SD[a]	99.22 ± 0.902	99.86 ± 1.126	99.79 ± 1.533	101.26 ± 0.959	100.72 ± 1.554	99.75 ± 1.365	99.32 ± 1.327	100.10 ± 0.875	101.05 ± 0.527	99.79 ± 0.877	101.05 ± 1.046	99.52 ± 0.920	99.14 ± 1.062	99.83 ± 1.184	99.44 ± 1.413
Precision: RSD, %[b]															
Intraday	0.698	0.895	0.914	0.126	1.032	0.647	0.668	0.553	0.354	0.741	0.235	0.422	0.621	0.385	0.587
Interday	1.042	1.311	1.287	0.898	1.458	1.544	0.981	0.949	0.895	0.936	0.974	1.233	1.155	0.871	0.987

[a] Average of three experiments.

[b] RSD of three concentrations of each drug.

Table 2. Results obtained for the determination of PHL, CAR, and EPH in the laboratory-prepared mixtures by the proposed spectrophotometric methods

Concn, μg/mL[a]			Recovery, %[b]													
			SDS			SDR			RS-RD			RSCWT-ZC			A-Sum		
PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH	PHL	CAR	EPH
160	80	280	102.26	98.64	102.22	101.55	100.21	99.22	100.94	102.22	102.00	100.97	99.97	98.04	101.56	101.37	99.01
80	40	140	99.20	100.09	98.89	100.90	102.69	99.22	101.00	98.08	101.36	98.53	98.11	100.02	102.21	100.28	98.65
100	40	140	100.87	98.58	98.89	100.47	98.78	100.56	102.19	101.92	101.13	100.41	99.37	99.44	100.78	99.40	101.19
160	80	240	101.34	101.88	99.35	98.17	98.54	99.79	100.85	99.85	100.84	101.24	98.19	98.30	99.87	99.23	98.11
120	80	320	100.34	98.21	98.13	100.34	101.42	100.87	98.91	100.10	101.36	98.52	100.32	101.79	99.74	98.86	100.85
Mean ± SD			100.80 ± 1.139	99.48 ± 1.519	99.50 ± 1.587	100.29 ± 1.275	100.33 ± 1.759	99.93 ± 0.762	100.78 ± 1.180	100.44 ± 1.688	101.34 ± 0.428	99.94 ± 1.321	99.19 ± 1.011	99.52 ± 1.506	100.83 ± 1.067	99.83 ± 1.008	99.56 ± 1.375

[a] Ratio of PHL, CAR, and EPH in Cyrinol syrup.

[b] Average of three determinations.

calibration equations and other statistical parameters for the proposed methods, are listed in Table 1.

LOD and LOQ.—The LOD and LOQ were calculated for the studied drugs using the proposed methods with a ratio of 3.3 and 10 times the SDs of the residuals and the slope of the calibration line, respectively (Table 1).

Accuracy.—The proposed spectrophotometric methods were applied for the determination of different blind samples of PHL, CAR, and EPH to check accuracy. The concentrations were obtained from the corresponding regression equations, from which the percent recoveries were calculated (Table 1).

Accuracy of the method was further assured by the use of the standard addition technique. It was achieved by the addition of known amounts of pure PHL, CAR, and EPH to known concentrations of the pharmaceutical formulation. The resulting mixtures were analyzed, and the obtained results were compared with the expected results (Table 3). The good recoveries of the standard addition technique suggested good accuracy of the proposed methods.

Precision

Repeatability.—Three concentrations of PHL (80, 140, and 200 μg/mL), CAR (10, 50, and 80 μg/mL), and EPH (100, 400, and 600 μg/mL) were analyzed three times intradaily using the proposed spectrophotometric methods. The percent recoveries and RSDs were calculated (Table 1).

Intermediate precision.—The previous procedures were repeated interdaily three times on 3 different days for the analysis of the three chosen concentrations. The percent recoveries and RSDs were calculated (Table 1).

Selectivity.—Selectivity of the methods was achieved by the analysis of different laboratory-prepared mixtures of PHL, CAR, and EPH within the linearity range. Satisfactory results are shown in Table 2.

Statistical Analysis

Results obtained from the proposed methods were compared with those obtained by applying the reported method for CAR (36) and the official methods (37) for PHL and EPH, and no significant difference was observed from the calculated *t*- and *F*-values (Table 4). One-way analysis of variance was applied for the purpose of comparison of the developed methods. Table 5 shows that there was no significant difference between them for the determination of PHL, CAR, and EPH.

Conclusions

This work introduced the application of five sensitive, selective, accurate, and precise spectrophotometric resolution techniques for the analysis of ternary mixtures of PHL, CAR, and EPH. These techniques were dependent either on the utilization of the ratio spectra of the drugs or the presence of an isosbestic point. The proposed methods were directly applied for multicomponent determination in laboratory-prepared mixtures and in a pharmaceutical formulation in the presence of the strongly overlapping azo dye, SUN, without the need for any prior chemical treatment, such as derivatization or extraction. The advantages of the proposed methods were

Table 3. Quantitative determination of PHL, CAR, and EPH in its pharmaceutical preparation by the proposed methods and application of the standard addition technique

			Standard addition					
				Recovery, %[b]				
Pharmaceutical formulation	Drug[a]	Claimed amount taken, µg/mL	Added, µg/mL	SDS	SDR	RS-RD	RSCWT-ZC	A-Sum
Cyrinol syrup labeled to contain 4 mg PHL, 2 mg CAR, and 7 mg EPH/5 mL syrup (Batch No. 135275)	PHL	80	60	100.22	100.22	98.63	100.25	100.51
			80	99.32	101.06	100.74	98.47	99.58
			120	100.36	101.63	101.43	99.36	99.52
			Mean ± SD	99.97 ± 0.564	100.97 ± 0.709	100.27 ± 1.459	99.36 ± 0.890	99.87 ± 0.555
	CAR	40	30	99.23	100.11	101.22	98.71	100.49
			40	98.58	98.81	98.67	100.88	98.37
			50	101.55	99.59	100.95	100.92	100.53
			Mean ± SD	99.79 ± 1.561	99.50 ± 0.654	100.28 ± 1.401	100.17 ± 1.265	99.80 ± 1.236
	EPH	140	100	100.67	101.31	100.54	100.89	98.33
			140	98.86	99.86	100.31	98.67	99.52
			200	100.88	101.61	99.22	98.15	100.44
			Mean ± SD	100.14 ± 1.111	100.93 ± 0.936	100.02 ± 0.705	99.24 ± 1.455	99.43 ± 1.058

[a] Ratio of PHL, CAR, and EPH in Cyrinol syrup.

[b] Mean of three determinations.

discussed, and they were applied for the analysis of the ternary mixture in syrup with minimum mathematical manipulating steps. As a final conclusion, the proposed methods could be promising for the routine assay of the studied drugs in their pure bulk powders and pharmaceutical formulations in QC laboratories, with satisfactory accuracy and precision. These

Table 4. Statistical comparison between the reference methods and the proposed multivariate methods for the determination of CAR, PHL, and EPH

Parameter	Mean %[a]	SD	*n*	Variance	*t*-Test	*F*-test
			PHL			
SDS	100.27	1.145	6	1.312	0.311 (2.26)[b]	0.410 (5.19)[b]
SDR	99.72	1.401	6	1.963	0.397 (2.26)[b]	1.904 (6.26)[b]
RS-RD	101.72	0.554	6	0.307	0.561 (2.26)[b]	1.877 (5.19)[b]
RSCWT-ZC	99.87	1.076	6	1.157	0.344 (2.26)[b]	1.112 (5.19)[b]
A-Sum	99.86	0.700	6	0.491	0.781 (2.26)[b]	2.216 (5.19)[b]
Reference method[c]	100.92	1.212	5	1.469		
			CAR			
SDS	100.45	0.685	6	0.469	0.746 (2.26)[b]	2.540 (5.19)[b]
SDR	100.24	1.024	6	1.049	1.874 (6.26)[b]	1.126 (5.19)[b]
RS-RD	99.75	0.926	6	0.858	0.505 (2.26)[b]	1.530 (5.19)[b]
RSCWT-ZC	100.29	1.290	6	1.664	0.335 (2.26)[b]	1.402 (6.26)[b]
A-Sum	100.22	0.892	6	0.796	0.295 (2.26)[b]	1.486 (5.19)[b]
Reference method[c]	99.05	1.089	5	1.186		
			EPH			
SDS	100.94	1.382	6	1.910	1.058 (2.26)[b]	1.497(6.26)[b]
SDR	99.90	1.335	6	1.783	0.155 (2.26)[b]	1.513 (6.26)[b]
RS-RD	99.76	1.674	6	2.803	0.204 (2.26)[b]	1.917 (6.26)[b]
RSCWT-ZC	100.13	1.101	6	1.212	0.215 (2.26)[b]	1.111 (6.26)[b]
A-Sum	99.86	1.226	6	1.504	0.198 (2.26)[b]	1.193 (6.26)[b]
Reference method[c]	101.23	0.956	5	0.914		

[a] Average of six determinations.

[b] Numbers in parentheses represent the corresponding tabulated *t*- and *F*-values at $P = 0.05$.

[c] Reference methods are the reported spectrophotometric method for CAR (36) and the *British Pharmacopoeia* methods for PHL (potentiometric method) and EPH (titrimetric method; 37).

Table 5. One-way analysis of variance testing for the different proposed methods and the reference methods used for the determination of PHL, CAR, and EPH[a]

Source	Sum of squares	df[b]	Mean squares	*F*-value[c]	*P*-value	*F*-critical
			PHL			
Between experiment	5.7470	5	1.1494	0.6883	0.6362	2.5454
Within experiment	48.4303	29	1.6700			
Total	54.1773	34				
			CAR			
Between experiment	1.7085	5	0.3417	0.3709	0.8644	2.5454
Within experiment	26.7190	29	0.9213			
Total	28.42748	34				
			EPH			
Between experiment	5.7470	5	1.1494	0.6883	0.6362	2.5454
Within experiment	48.4303	29	1.6700			
Total	54.1773	34				

[a] Reference methods are the reported spectrophotometric method for CAR (36) and the *British Pharmacopoeia* methods for PHL (potentiometric method) and EPH (titrimetric method; 37).

[b] At the 0.05 level.

[c] The population means are not significantly different.

proposed methods did not require any sophisticated apparatus and can be used as alternative methods to chromatographic methods.

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