**DRUG FORMULATIONS**

**Novel Spectrophotometric Approaches for the Simultaneous Quantification of Ternary Common Cold Mixture Containing Paracetamol with a Challenging Formulation Ratio: Greenness Profile Evaluation**

Rabab M. Soliman 1, Nadia M. Mostafa 2, Yasmin M. Fayez 2, Hany H. Monir 2, and Yasmin Rostom 2,*

1Ministry of Health and Population, Directorate of Health Affairs, Ismailia Health Administration, Ismailia, Egypt, 2Cairo University, Faculty of Pharmacy, Analytical Chemistry Department, Kasr El-Aini Street, Cairo 11562, Egypt

*Corresponding author’s e-mail: yasmin.rostom@pharma.cu.edu.eg

---

**Abstract**

**Background:** Common cold and cough preparations represent a huge segment of the global pharmaceutical market. Recently, cold/cough formulations containing paracetamol (PAR) have attracted significant attention as PAR has been implemented into the supportive treatment of mild cases of COVID-19 as the first-line antipyretic. From a literature review, no method has been reported yet for simultaneous estimation of PAR, pseudoephedrine hydrochloride (PSE) and carbinoxamine maleate (CRX) in any matrix. Thus, there is an urgent need for smart and green methods that would enable quantification of the cited components in their challenging ratio.

**Objectives:** The aim of this work is to develop and validate the first UV spectrophotometric methods for simultaneous determination of the selected drugs taking into consideration the list of challenges including the highly overlapping features and spectral interferences in the cited mixture.

**Methods:** Namely, the proposed methods are: direct spectrophotometry, dual wavelength, first derivative, derivative ratio, ratio difference, constant center coupled with spectrum subtraction, and the constant multiplication method paired with spectrum subtraction.

**Results:** These methods were linear over the concentration range of 2.5–35 µg/mL, 1.5–20 µg/mL, and 4.5–35 µg/mL for PAR, PSE and CRX, respectively. These methods fulfill the validity parameters according to International Conference on Harmonization (ICH) guidelines. The results obtained were statistically benchmarked to the official ones where no significant difference was noticed.

**Conclusion:** The developed methods are successfully applied for concurrent quantification of the studied components in the marketed dosage form without interference from matrix excipients. The impact on the environment was assessed by five green metrics, namely a recent Analytical greenness (AGREE) metric algorithm based on the green analytical chemistry framework, Green Analytical Procedure Index (GAPI), Eco-Scale, Assessment of Green Profile (AGP), and National Environmental Methods Index (NEMI).
Highlights: Eco-friendly and successive spectrophotometric methods were firstly developed in this work, for the simultaneous quantification of PAR, PSE and CRX. These approaches incorporate a simple enrichment-aided technique to augment their spectrophotometric signals, facilitating the accurate quantitation of the minor component in the cited mixture.

Introduction

The common cold is a viral infection of the upper respiratory tract caused by viruses such as rhinoviruses, adenoviruses and coronaviruses and they have many overlapping symptoms (1). Since it is highly contagious, the complete prevention of infection does not exist so far and it is impossible to attain immunity against all serotypes (2). There is an overwhelming demand for cold and cough medications for the management of mild symptoms especially after the recent outbreak of a novel coronavirus COVID-19 (3).

Medication Review

Paracetamol (PAR), N-(4-hydroxyphenyl)acetamide, is popular analgesic and major ingredient in numerous cold and flu therapies (4–6). Recently, PAR has been integrated into the supportive therapy of COVID-19 and described as the first-line antipyretic in the symptomatic relief of mild cases (7, 8).

Pseudoephedrine hydrochloride (PSE), (1S, 2S)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride, is a nasal decongestant which acts by reducing inflammation of mucous membranes; it is also used for bronchodilation (4, 5).

Carboxinomaleine maleate (CRX), 2-[(4-chlorophenyl)-2-pyridinylmethyl]o-N,N-dimethyl-ethanamine maleate, is a first-generation antihistaminic drug with sedative effects that acts primarily by competing with histamine in binding to the H1 receptor. It is used as monotherapy or in combination with PSE and/or PAR in the management of the symptoms of the common cold, hay fever, and allergic conjunctivitis (4, 6). Chemical structures of the three components are presented in in Suplemental Figure S1. These medications are commonly combined in one formulation for treating common cold- and cough-associated symptoms.

A literature survey reveals that numerous analytical methods have been developed for the assay of PAR and PSE in combination with other antihistaminic drugs (9–11).

To the best of our knowledge, no method has yet been reported for the analysis of the three investigated drugs in any matrix. Therefore, there is an urgent need for simple, smart, economic, and green methods for the simultaneous quantification of the cited components.

Spectral Challenges

Quantification of such pharmaceutical preparations is usually an interesting analytical challenge as these preparations are composed of a complex matrix comprising numerous active constituents and a wide range of additives. In addition, they are present in extremes of low and high concentrations.

Analysis of the studied ternary mixture suffered from multiple problems: there was severe overlap in the spectra of the cited mixture and there was no clear PSE peak as shown in (Figure 1). The studied drugs differ widely in their absorptivities, and so have different linearity ranges; 2.5–35 \( \mu \)g/mL, 1.5–20 \( \mu \)g/mL and 4.5–35 \( \mu \)g/mL for PAR, PSE and CRX, respectively. Adding to the list of challenges; for their effective pharmacological action they are co-formulated in the commercial pharmaceutical formulation (Micheloon® tablet) in a desperate ratio 100:15:1 for PAR, PSE, and CRX, respectively. Thus, PAR represents the major component in the pharmaceutical dosage form, while CRX represents the minor component and consequently a sample enrichment technique was required for the analysis of CRX concentration.

In order to meet the challenges of simultaneous determination of the cited mixture, the present work couples both a successive resolution technique for eliminating the overlapped spectra to get less complicated spectra via simple mathematical filtration and versatile classical spectrophotometric methods for quantification of the active ingredients in their marketed tablets.

Objectives

The aim of this work is to develop and validate the first UV spectrophotometric methods for simultaneous determination of PAR, PSE, and CRX in their pure form and in marketed combination using integrated spectrophotometer software. Recently, a global trend has been to move towards applying the principles of green analytical chemistry (GAC; 12–15), hence, these methods are separation-free and environmentally sustainable; distilled water has been used as a solvent which is considered the greenest one as it is perfectly safe for the environment and operators alike (16). Determination of each drug in the cited mixture was achieved by more than one method via different approaches.

The applied methods are direct method (\( D^0 \)), dual wavelength (\( D^W \)), first derivative (\( D^1 \)), derivative ratio method (\( D^1 \)), ratio difference (RD; 19), besides the fingerprint resolution techniques; constant center coupled with spectrum subtraction (CC-SS), and constant multiplication method paired with spectrum subtraction (CM-SS; 11, 19). These methods are well established and successfully applied for the quantification of PAR, PSE, and CRX in their authentic form and in pharmaceutical formulation. Additionally, the greenness profile was evaluated using the most recent Analytical Greenness Metric software (AGREE; 20) based on the 12 principles of GAC (16), the Green Analytical Procedure Index (GAPI; 21), the analytical Eco-Scale (22), Assessment of Green Profile (AGP; 23), and the National Environmental Method Index (NEMI; 24).

Experimental

Apparatus and Software

Spectrophotometric measurements were performed on a Shimadzu UV-1800 double-beam spectrophotometer (Tokyo, Japan), using matched 1.00 cm quartz cells. Scans were carried out in the range from 200.0 to 400.0 nm at 0.1 nm intervals. Spectra were automatically obtained by Shimadzu UV-Probe 2.43 system software. AGREE (\( \gamma \)0.5 2020) was used as an analytical greenness calculator.

Chemicals and Reagents

(a) Pure samples.—PAR, PSE, and CRX were kindly supplied by Amoun Pharmaceutical Co. (El-Obour City, Cairo, Egypt). Their purities were found to be 100.25 ± 0.71, 99.45 ± 0.63,
and 99.23 \pm 0.88 for PAR, PSE, and CRX, respectively, by the BP method (5) for PSE and by USP methods (6) for CRX and PAR.

(b) Market sample.—Michaelon® tablets, batch number 182020 (each tablet is claimed to contain 400 mg, 60 mg, and 4 mg PAR, PSE, and CRX, respectively), was manufactured by Amoun Pharmaceutical Co. and purchased from the local market.

(c) Solvents.—Double-distilled water, (Merck, Darmstadt, Germany).

Standard Solutions
Stock solutions of PAR, PSE, and CRX (1 mg/mL, each) were prepared by dissolving the compounds in double-distilled water in three separate 100 mL volumetric flasks and then the volume was completed to the mark and stored at the refrigerator. Ten milliliters of each of the prepared stock solutions was further diluted with double-distilled water to a final volume of 100 mL. The diluted solutions were used as the working solutions for PAR, PSE, and CRX (each, 100 \(\mu\)g/mL).

Procedures
(a) Analyte spectral characteristics.—Zero-order (\(D^0\)) absorption spectra of the three analytes were recorded in the range of 200–400 nm using double-distilled water in the blank cell (Figure 1).

(b) Construction of calibration graphs.—Aliquots equivalent to 25–350 \(\mu\)g PAR, 15–200 \(\mu\)g PSE, and 45–350 \(\mu\)g CRX were accurately transferred from their working solutions into three separate series of 10 mL volumetric flasks then completed to the mark with double-distilled water. The UV absorption spectra of the prepared solutions were recorded from 200–400 nm and saved to PC.

(c) Methods manipulating the absorbance of zero-order absorption spectra.—
(1) Direct spectrophotometric determination (\(D^0\)) and Constant multiplication (CM).—Zero-order (\(D^0\)) absorption spectra PAR were recorded and saved to the computer. Calibration graph was made by relating the absorbance of \(D^0\) spectra of PAR at \(\lambda_{max}\) 244 nm against the corresponding concentrations from which the regression equations were deduced.

(2) Dual wavelength method (DW).—Two calibration graphs were plotted relating the difference in absorbance of the stored spectra at 255.5 and 263 nm for PAR, and at 232.2 and 252.2 nm for CRX against the corresponding drug concentrations. Then, the regression equation of each drug was computed.

(d) Methods manipulating the amplitude of derivative spectra.—
(1) First derivative method (\(D^1\)).—The first derivative of the stored \(D^0\) absorption spectra of PSE, 1.5–20 \(\mu\)g/mL, was recorded using \(\Delta\lambda\) 4 nm and scaling factor 10. The calibration graph was obtained by plotting the peak amplitude at 217 nm versus its corresponding concentration and the regression equation was computed.

(2) Derivative ratio method (DD^1).—The stored \(D^0\) spectra of PSE were divided by the PAR spectrum (17 \(\mu\)g/mL) then the obtained ratio spectra of PSE were derived to their first order using \(\Delta\lambda\) 4 nm and scaling factor 10. The amplitude values of the ratio derivative spectra at 217.4 nm were plotted versus the corresponding PSE.
concentrations to get the calibration graph and the regression equation was computed.

(e) Methods manipulating the amplitudes of ratio spectra.—
(1) Ratio difference method (RD).—The previously stored D⁰ absorption spectra of PSE were divided by the spectrum of PAR (17 μg/mL) and the differences between amplitudes of ratio spectra at 212.5 and 218 nm were recorded. Construction of the calibration graph was based on plotting the amplitude differences of PSE at (A_P212.5–218 nm) versus the concentrations of PSE (1.5–20 μg/mL).

(2) Constant center method (CC).—The stored D⁰ spectra of PAR were divided by the absorption spectrum of CRX (30 μg/mL) then the ratio spectra obtained were recorded. Subsequently, two calibration curves were constructed, one of them relating the difference between the amplitudes of the ratio spectra at 244 nm and 257 nm against the amplitudes at 244 nm, while the other one relating the absorbance values of the zero-order curves of CRX at λ_{max} 261 nm versus their corresponding concentrations.

Synthetic Blends Analysis

Accurate aliquots of PAR, PSE, and CRX were transferred from their working standard solutions into a series of 10 mL volumetric flasks to give laboratory-prepared mixtures containing different ratios of the cited drugs. Volumes were completed with double-distilled water. The D⁰ spectra of the laboratory-prepared mixtures were recorded from 200–400 nm and stored on computer. The scanned spectrum of each synthetic mixture was manipulated as described below.

PAR Quantification

PAR can be determined either in the presence of the scanned ternary mixture or the resolved binary mix with PSE after resolution of the CRX spectrum via constant center followed by spectrum subtraction (CC-SS).

(a) Determination of PAR in the presence of co-formulated drugs.—
(1) Dual wavelength method (DW).—The stored D⁰ spectra of each laboratory-prepared mixture was measured at 255.5 and 263 nm, then the difference between these two wavelengths was calculated. The concentrations of PAR were computed by substituting in the corresponding regression equation.

(2) First derivative method (D¹).—The first derivative spectrum of PAR is extended beyond the D¹ spectra of PSE and CRX, so the amplitude of the first derivative spectra of the scanned ternary mixture is recorded at 290 nm where both PSE and CRX show no contribution. PAR can be determined efficiently without the interference of the other two components.

(b) Determination of PAR in the resolved binary mixture after elimination of CRX.—
(1) Direct spectrophotometry (D⁰).—PAR could be determined via its λ_{max} 244 nm without any contribution from PSE and its concentration in the resolved spectrum of each mixture was calculated using its corresponding regression equation.

(2) Constant multiplication coupled with spectrum subtraction (CM-SS).—Each laboratory-prepared mixture absorption spectrum was divided by the spectrum of PAR (17 μg/mL), then the constant was obtained as a straight line parallel to the wavelength axis (plateau zone) from 224 to 300 nm. The whole D⁰ spectrum originally present in the mixture was recovered by multiplying the value of the measured constant by the divisor used. The concentration of PAR was calculated using the corresponding regression equation at its absorption maximum 244 nm. Finally, spectrum subtraction could be used to eliminate PAR from the mixtures to allow mathematical filtration and get the D⁰ spectrum of PSE as a single drug.

CRX Quantification via a Sample Enrichment Technique

(a) Determination of CRX in the presence of co-formulated drugs.—
(1) Dual wavelength method (DW).—For scanned absorption spectra of different laboratory-prepared mixtures, absorbance difference values were calculated at 232.2 and 252.2 nm. The concentration of CRX in each mixture was obtained by substitution in the corresponding regression equation.

(2) Constant center coupled with spectrum subtraction method (CC-SS).—The scanned absorption spectrum of each synthetic mixture was divided by the absorption spectrum of standard CRX (30 μg/mL) to get the ratio spectrum then the amplitudes at 244 and 257 nm were recorded. A linear correlation equation was established by plotting the difference between the recorded amplitude values at the selected wavelengths (A_{P244-257 nm}) versus the amplitude at 244 nm to get the postulated amplitude value of PAR. The constant value of CRX in each mixture was obtained by subtracting the postulated amplitude value from the recorded one at 244 nm. The constant value obtained for each mixture was multiplied by the spectrum of CRX divisor (30 μg/mL), so the original curve of CRX was obtained and the absorbance was recorded at its λ_{max} 261 nm. The concentration of CRX in each mixture was calculated using the corresponding regression equation.

The constant center method can also be used for cancelation of the whole spectrum of CRX if followed by spectrum subtraction. Finally, the obtained D⁰ absorption spectrum of the resolved binary mixture of PAR and PSE was subjected to further manipulation steps which enabled determination of PSE effectively as single resolved drug by mathematical filtration as it was difficult in the beginning to estimate in the presence of CRX in the scanned ternary mixture.

PSE Quantification

(a) Determination of PSE in the resolved binary mixture after CRX elimination.—
(1) Derivative ratio method (DD¹).—The stored, resolved zero-order spectra of laboratory-prepared mixtures were divided by the absorption spectrum of a standard solution of PAR (17 μg/mL) to get the ratio spectra. Then the ratio spectra were transformed into their first derivative using A_λ = 4 nm and scaling factor 10. The concentration of PSE in each mixture was calculated at 217.4 nm using the corresponding regression equation.

(2) Ratio difference method (RD).—This method is based on utilizing the ratio spectrum obtained from each
mixture and calculating the amplitude difference between 212.5 and 218 nm. The concentration of PSE in each laboratory-prepared mixture was obtained using its corresponding regression equation.

(3) Determination of PSE as a single resolved drug after PAR elimination via the first derivative method ($D_1^0$).—After complete resolution of both PAR and CRX, PSE cannot be determined using direct spectrophotometry since the resolved spectrum with indeterminate peak at 207 nm. It could be applied for quantitation of PSE after PAR elimination by CM-SS in the resolved binary mixture of PAR and PSE where the peak amplitude of $D_1^0$ spectrum of PSE at 217 nm was measured using $\lambda_1 = 4\text{ nm}$ and scaling factor 10. The concentration of PSE was calculated using the computed regression equation.

Application to Pharmaceutical Dosage Form

Ten Michaelon tablets were weighed accurately and finely powdered. An amount of the resulting powder equivalent to one fourth of one tablet (containing 100 mg PAR, 15 mg PSE, and 1 mg CRX) was accurately transferred into a 100 mL volumetric flask then 50 mL double-distilled water was added and sonicated for 15 min. The volume was completed to the mark and finally filtered. A 0.2 mL aliquot of this solution was transferred into a 10 mL volumetric flask and spiked with 0.5 mL CRX standard working solution. Finally, the volume was completed to the mark with double-distilled water to get a sample solution satisfying the dosage form ratio (20 mg/mL PAR, 3 mg/mL PSE, and 5.2 mg/mL CRX) within the linearity ranges of each analyte.

The proposed methods were applied for the analysis of prepared pharmaceutical solutions using the same procedures previously mentioned in Synthetic Blends Analysis. Concentrations of the targeted drugs were calculated from the corresponding regression equations after applying the corresponding manipulating steps for each method. The concentration of CRX in the pharmaceutical formulation was calculated after subtraction of the added concentration (5 mg/mL of CRX) by using the same procedure.

Results and Discussion

Till now, no method has been reported for the analysis of this ternary mixture in any matrix. Thus, the goal of this work is to develop and validate the first UV spectrophotometric methods for the simultaneous determination of PAR, PSE, and CRX in their pharmaceutical preparation without prior separation steps. Several challenges were faced in the present work including the completely overlapped spectra of the studied drugs and the PSE spectrum having no significant peak, which hinders direct determination with conventional zero-order spectrophotometry as shown in (Figure 1). This problem was solved upon transformation of the recovered zero-order $D_0^0$ absorption spectrum of PSE into its first derivative. Besides, the cited drugs were present in their formulation in challenging ratio 100:15:1 for PAR, PSE, and CRX, respectively. In order to overcome the obstacle of the analysis of a minor component, a sample enrichment technique has been applied by adding a fixed amount of standard CRX to each experiment then subtracting its concentration before calculating the claimed concentration of the drug.

The coupling of successive resolution steps to resolve the unresolved bands of the ternary mixture by eliminating one or more of the interfering components in the mixture by mathematical filtration to get a simpler, resolved binary mixture of PAR and PSE, followed by data processing of the resolved binary mixture, acts as powerful strategy in the analysis of multicomponent mixtures. The diversity of the spectrophotometric methods allows analysts to choose the most appropriate method of analysis to be applied in QC laboratories lacking hyphenated analytical instruments such as HPLC-DAD (Diode-Array Detection).

This work is designed to develop accurate, reliable, and cost-effective spectrophotometric methods for the estimation of PAR, PSE, and CRX in Michaelon tablets. Initial trials were applied to resolve the ternary mixture through a different strategy using two separate samples; the first sample was used for the determination of PAR and PSE without any contribution from CRX. On the other hand, the second sample involved spiking to determine the minor component accurately.

Both procedures gave satisfactory results, however, the proposed approach that incorporates using only one sample and an enrichment technique is highly advantageous over using two samples via two different pathways to determine the target drugs. The developed spectrophotometric methods are considered eco-friendly utilizing one sample that will lead to a major reduction in the number of samples and waste generation during the whole analytical process and save energy as well. Additionally, these methods did not require any complex algorithm, software programs (like matlab), or sophisticated calculations.

(a) Methods manipulating the absorbance of zero-order absorption spectra.—

(1) Direct spectrophotometric method ($D_0^0$).—The method could be applied for determination of PAR in the resolved binary mixture of PAR and PSE after elimination of CRX by CC-SS. PAR was determined directly through its maximum absorbance at 244 nm without any contribution from PSE up to 100.0 $\mu$g/mL of PSE. At higher PSE concentration, the two spectra became highly overlapped as the PSE spectrum exhibited two maxima at 207 and 257 nm and this spectral overlapping hindered the use of direct UV spectrophotometry. The PAR absorbance at $\lambda_{max}$ (244 nm) was plotted against its corresponding concentration and the regression equation was computed and is shown in Table 1.

(2) Dual wavelength method (DW).—DW facilitates analyzing a component in the presence of an interfering component. The requirements for the DW method are the selection of two wavelengths where the absorbance difference is equal to zero for the interfering compound and the drug of interest shows a marked difference in absorbance with concentration (17). The DW method was used for the determination of CRX and PAR in $D_0^0$ of the scanned ternary mixture without preliminary resolution. For CRX determination, 232.2 and 252.2 nm were selected as the difference in absorbance between them is directly proportional to CRX concentration in contrast to a zero absorbance difference for PAR at these wavelengths, (see Supplemental Figure S2). The calibration curve relating the absorbance differences between 232.2 and 252.2 nm to the corresponding concentration of CRX was constructed.

For PAR determination, 255.5 and 263.0 nm were selected as the difference in absorbance between them is directly proportional to PAR concentration in contrast
to a zero absorbance difference for CRX at these wavelengths, (see Supplemental Figure S2). The calibration curve relating the absorbance differences between 255.5 and 263.0 nm to the corresponding concentration of PAR was constructed. CRX and PAR concentrations were calculated using their corresponding regression equations (Table 1).}

(b) Methods manipulating the amplitude of derivative spectra.—

(1) First derivative method (D1) — D1 is a simple and long-established analytical technique that offers a useful means for extracting both qualitative and quantitative information from the spectra composed of overlapped bands. It is able to enhance the resolution of overlapping absorption bands and to discriminate sharp bands over large bands. It is based on using the first- or higher order derivatives of absorbance with respect to wavelength from parent zero-order spectra (18). This method could be applied for determination of PAR where the peak amplitude of the D1 spectrum of PAR extended beyond the D1 spectra of PSE and CRX, so it can be determined at 290 nm without any contribution of the other two components as shown in (Figure 2). Calculation of the concentration of PAR was achieved by substituting in the corresponding regression equation as shown in Table 1. Moreover, peak amplitude of D1 spectrum of PAR at 217.4 nm (Figure 4a). The PSE concentration was calculated using the corresponding regression equation relating the absorbance of PSE at 217.4 nm to its corresponding concentration (Table 1). The influence of D1 for obtaining the first derivative of the ratio spectra as well as the effect of divisor concentration is fundamental. To optimize DD1, many D1 and scaling factors were tested. Satisfactory results were obtained using D1 = 4, a scaling factor of 10, and a standard PAR spectrum of 17.0 µg/mL as a divisor and demonstrated acceptable S/N and good resolution of spectra.

(c) Methods manipulating the amplitude of ratio spectra.—

(1) Ratio difference method (RD).—RD method is simple, fast, and efficient in solving the problem of severely overlapped spectra and it does not require any complicated computer programs. In this method, the absorption spectrum of the studied mixture was obtained and divided by the absorption spectrum of the standard solution of one of the drugs, obtaining its corresponding ratio spectrum. The difference between the two chosen wavelengths cancels the interfering substance completely. Therefore, the amplitude difference value is corresponding to the drug of interest (19). RD method was applied for the analysis of PSE in the resolved binary mixture. Two pairs of wavelengths, 212.5 and 218 nm were selected for PSE estimation using PAR (17.0 µg/mL) as a divisor (Figure 4b). Concentration of PSE in each mixture was determined using its regression equation.

The two essential factors affecting the RD method are the choice of the divisor and the selection of the two wavelengths. A compromise between maximum sensitivity and minimal noise should be considered when selecting the divisor, while the requirement for the two chosen wavelengths is that the drug of interest should have the highest amplitude difference in the region with the interfering substances.

Table 1. Validation parameters of the proposed spectrophotometric methods for the determination of PAR, PSE, and CRX in pure forms

| Validation parameters | PAR | PSE |
|-----------------------|-----|-----|
| Wavelength            | D0/CM | DW | D1 | DD1 | D1 | DD1 | RD | CC | CRX |
| Linearity, µg/mL      | 1.04 | 0.92 |
| Regression equation   | 0.58 99.79 0.62 99.47 0.45 100.12 0.75 100.04 1.24 99.82 |
| Slope                | 0.0762 | -0.055 |
| Intercept            | 0.0209 | 0.0016 |
| Correlation coefficient (r) | 0.9999 | 0.9999 |
| Mean ± SD            | 99.81 ± 1.06 100.04 ± 1.18 100.13 ± 0.68 99.71 ± 1.24 99.82 ± 0.75 100.01 ± 0.62 99.67 ± 0.80 100.18 ± 1.04 |
| Accuracy: Recovery % ± SD | 100.45 ± 0.58 99.79 ± 0.28 99.47 ± 0.62 100.12 ± 1.29 99.94 ± 0.51 99.78 ± 0.89 99.96 ± 0.45 100.61 ± 0.92 |
| Precision: %RSD       | 0.45 0.714 0.563 0.427 0.839 0.268 0.337 0.950 |

*Accuracy was checked using concentration levels of 7.5, 12, and 22 µg/mL for PAR, 2.0, 6.0, and 8.0 µg/mL for PSE, and 6.5, 14.0, and 22 µg/mL for CRX. n = 9; RSDs of concentrations 5, 10, and 15 µg/mL in triplicate for each drug.

Average of three replicates for each drug for three different concentration levels of PAR, PSE, and CRX (6.0, 9.0, and 12.0 µg/mL).
Figure 2. First derivative of the spectrum of PAR (----) 20.0 μg/mL, PSE (-----) 3.0 μg/mL, and CRX (----) 5.2 μg/mL.

Figure 3. First derivative spectrum $D_1$ of PSE (3.0 μg/mL).
spectrum of the target drug from its co-formulated interfering substances. This method was applied to the ratio spectra using the drug of interest as a divisor (11, 19). It is based on recording ratio spectra amplitude values at two wavelengths existing in the overlapped region. Consequently, no restrictions of the spectral extension of one drug than the other are assigned and could be considered as a universal technique for resolving drug mixtures with partially or completely overlapped spectra.

This method could be applied for the determination of CRX in the scanned ternary mixture in the wavelength region 223–300 nm where PSE has zero contribution (see Supplemental Figure S3a). By applying this method, the absorption spectrum of the scanned ternary mixture was divided by the absorption spectrum of a standard solution of CRX (30 μg/mL) as a divisor, showing the two selected wavelengths 212.5 and 218.0 nm.

The zero-order absorption spectrum of CRX in the mixture was obtained via the constant multiplication method by multiplying this constant value by the spectrum of the divisor CRX (see Supplemental Figure S3c). The concentration of CRX could be calculated using the $D^0$ absorption spectrum of CRX at $\lambda_{max}$ 261 nm directly using the corresponding regression equation (Table 1). Furthermore, CC-SS is used for mathematical filtration to get the less complicated binary mixture of PAR and PSE by the stepwise elimination of CRX from the $D^0$ absorption spectrum of the ternary mixture to remove the influence of the interfering component and minimize the degree of overlap by using the spectrum subtraction method (see Supplemental Figure S3d). The resolved binary mixture of PAR and PSE then will be subjected to further manipulation steps for the determination of PSE and to improve the performance of the investigated methods to accomplish the complete resolution of the cited mixture.

(3) Constant multiplication method paired with spectrum subtraction method (CM-SS).—This method is also considered as a fingerprint resolution technique that allows the recovery of the original zero-order absorption spectrum of the drug of interest from its co-formulated drugs. This CM-SS applied for resolving mixtures with partially-overlapped spectra where one analyte is more extended than the other. The major demerit of such method is calculating the constant value at the plateau zone necessitates high signal to noise (S/N) ratio which will be difficult if the extended component in low concentration. This results in inaccurate recovery percentages for both components. When the constant multiplication method is paired with the spectrum subtraction method, it can be applied for elimination of one or more components in the mixture to enhance the resolution power of the spectrophotometric methods (11, 19).

The CM-SS method could be applied for determination and elimination of PAR in the resolved binary mixture of PAR and PSE. Each spectrum in the resolved binary mixture was mathematically regained to its $D^0$ absorption spectrum separately, which acted as spectral profile of each cited drug. The ratio spectrum of the resolved binary mixture was obtained using 17 μg/mL PAR' divisor and the constant PAR/PAR' at the plateau zone 224–300 nm was measured (see Supplemental Figure S3e). Upon multiplication of this constant value by the divisor, the original $D^0$ absorption spectrum of PAR could be obtained and used for its direct determination at 244 nm. The PAR concentration was calculated using the related regression equation. Furthermore, the $D^0$ spectrum of PAR was subtracted from the zero-order absorption spectrum of its corresponding resolved binary mixture to obtain PSE in pure, single drug form. Unfortunately, this method failed to be applied in the initial step to remove the PAR contribution from the $D^0$ spectrum of the scanned ternary mixture as the ratio spectra of the ternary mixture were tried, and it was found that 244 and 257 nm gave a good correlation coefficient. The postulated amplitude value was calculated via substitution in the regression equation representing a linear relationship between the amplitude difference of PAR at ($A_{P244-257nm}$) against the corresponding ratio amplitudes at $P_{244nm}$. The constant value was calculated by subtracting the postulated value of $P_{244nm}$ from the recorded value.

Figure 4. (a) First derivative of the ratio spectrum $DD_1$ of PSE (3.0 μg/mL) using the spectrum of PAR (17.0 μg/mL) as a divisor, showing the selected wavelength 217.4 nm. (b) Ratio spectrum of PSE (3.0 μg/mL) using the spectrum of PAR (17.0 μg/mL) as a divisor, showing the two selected wavelengths 212.5 and 218.0 nm.
mixture using \( \text{PAR}' \) as a divisor resulted in a noisy constant that gave unsatisfactory results.

**Methods Validation**

The developed methods were validated according to International Conference on Harmonization (ICH) guidelines (25) as summarized in Table 1.

(a) **Linearity and range.**—Linearity ranges were determined for each component and regression parameters were calculated (Table 1). The calibration graphs were constructed within the concentration ranges of PAR, PSE, and CRX that were present in the pharmaceutical formulation and achieved adherence to Beer’s law. The linearity of the calibration graphs was judged by the high values of the correlation coefficients.

(b) **Accuracy.**—Accuracy of the suggested methods was assessed by analyzing different blind samples of PAR, PSE, and CRX within the linearity range. The concentrations levels were obtained from the corresponding regression equations, as shown in Table 1. Good percentage recoveries were obtained, which indicates the high accuracy of the proposed methods.

(c) **Precision.**—Repeatability and intermediate precision for the investigated methods were checked by analyzing three different concentrations (5, 10, and 15 \( \mu \)g/mL) of each drug, within the linearity range, repeated on the same day or on three successive days using these methods. The results are expressed as RSD (Table 1). The results did not exceed 2%, proving the high precision of the methods. This good level of precision is suitable for QC analysis of PAR, PSE, and CRX in their pharmaceutical formulation.

(d) **Selectivity.**—Selectivity of the proposed methods was investigated by the analysis of different ternary synthetic mixtures containing different ratios of the cited drugs within the linearity range. Satisfactory results were obtained (Table 2).

(e) **Robustness.**—The cited analyte working solutions in double-distilled water were kept in a refrigerator for enough time to examine method validation without any significant variation in their concentrations. These solutions showed no spectrophotometric changes up to 4 weeks when stored in a refrigerator at 4°C (Table 1).

**Application to Pharmaceutical Formulation**

The suggested methods were used for the determination of the cited drugs in their combined formulation, and satisfactory results were obtained in good agreement with the label claim. Furthermore, the validity of the proposed procedures was evaluated by applying the standard addition technique where different known concentrations of pure standard PAR, PSE, and CRX were added to the pharmaceutical formulation before proceeding with the previously mentioned methods. Satisfactory results were obtained, showing no interference from excipients and additives (Table 2).

**Statistical Analysis**

The results of the analysis of the pure drugs obtained from these methods were benchmarked to those obtained by applying the official methods (5, 6) for pure powdered forms. There was no significant difference observed from the calculated \( t \) and \( F \)-values as listed in Table 3. Additionally, statistical analysis using one-way analysis of variance (ANOVA) was

---

**Table 1.** Table of data for determination of the studied drugs in their laboratory-prepared mixtures, co-formulated tablet, and application of the standard addition technique.

| Drug | PAR | PSE | CRX |
|------|-----|-----|-----|
| Method | CM | DW | DW | CM | DW | CRX |
| D₀ | 100.15 ± 0.98 | 99.94 ± 0.86 | 99.57 ± 0.74 | 100.15 ± 0.98 | 99.94 ± 0.86 | 99.57 ± 0.74 |
| D₁ | 100.04 ± 0.82 | 99.83 ± 0.65 | 99.44 ± 0.53 | 100.04 ± 0.82 | 99.83 ± 0.65 | 99.44 ± 0.53 |
| D₂ | 100.12 ± 0.91 | 99.91 ± 0.79 | 99.52 ± 0.67 | 100.12 ± 0.91 | 99.91 ± 0.79 | 99.52 ± 0.67 |

---

**Table 2.** Assay results for determination of the studied drugs in their laboratory-prepared mixtures, co-formulated tablet, and application of the standard addition technique.

| Drug | PAR | PSE | CRX |
|------|-----|-----|-----|
| Method | CM | DW | CRX |
| D₀ | 100.15 ± 0.98 | 99.94 ± 0.86 | 99.57 ± 0.74 |
| D₁ | 100.04 ± 0.82 | 99.83 ± 0.65 | 99.44 ± 0.53 |
| D₂ | 100.12 ± 0.91 | 99.91 ± 0.79 | 99.52 ± 0.67 |

---

*Six sets for each method, average of three determinations.*

---

*Each tablet was labeled to contain 400 mg PAR, 60 mg PSE, and 4 mg CRX.*
performed on the results obtained by both the proposed and the official methods, where calculated $F$-values were always less than tabulated $F$-values for the studied drugs proving that there was no significant difference between them (see Supplemental Table S1).

Assessment of the Analytical Methods Greenness

Not all the analytical procedures have the same level of greenness; therefore, the greenness of analytical methods should be evaluated. Five different greenness evaluation techniques were used to estimate the eco-friendly nature of the developed methods.

(a) **Analytical greenness metric (AGREE).**—The most recent analytical greenness metric (AGREE) approach was applied in this work. It is novel, downloadable, greenness assessment software proposed in June 2020 by Pena-Pereira et al. (20). The AGREE algorithm is an excellent metric for the comprehensive sustainability assessment that relies on the dozen principles of GAC (16), so the output is a clockwise circular diagram with numbers from 1 to 12 around the edge. The final score of each segment of the 12 principles is a fraction of unity, from zero to one with the inputs provided together with their weights. Each segment has a specific color range from deep green ($\frac{1}{12}$) to deep red ($\frac{0}{12}$). The overall score appears in the center of the circular pictogram as shown in Table 4. The score obtained for the proposed method is 0.86, close to the maximum score of 1 and hence proving the greenness of our procedures. The striking advantage of AGREE is the clarification of strong and weak sections among the principles of GAC. Moreover, its net score is reliable and informative as regards GAC bases. AGREE is strongly recommended in terms of simplicity and flexibility for control of each section width according to their importance. Besides, it is an automated tool where conclusions and reports of the method greenness profile can be obtained with minimum effort.

(b) **Green Analytical Procedure Index (GAPI).**—In 2018, Płotka-Wasylyka (21) introduced the combination of qualitative and quantitative greenness calculation methods known as the green analytical procedure index (GAPI). It is a reliable tool, which provides a comprehensive ecological assessment of the entire analytical procedure, starting from the sample collection and passing through sample preservation, transport, and preparation until the final determination. It consists of 15 different parameters, represented in five pentagrams with a three-level color scale for each stage including green, yellow, or red ranging from high, medium, and low environmental impact. The GAPI pictogram for the developed methods is illustrated in Table 4. The score obtained for the proposed method is 0.86, close to the maximum score of 1 and hence proving the greenness of our procedures.

The striking advantage of AGREE is the clarification of strong and weak sections among the principles of GAC. Moreover, its net score is reliable and informative as regards GAC bases. AGREE is strongly recommended in terms of simplicity and flexibility for control of each section width according to their importance. Besides, it is an automated tool where conclusions and reports of the method greenness profile can be obtained with minimum effort.

(c) **Analytical Eco-Scale.**—This is a quantitative approach and is based on assigning penalty points to various factors included in the developed method and subtracting them from 100. The result of the calculation is ranked on a scale, where scores above 75 are considered excellent green analysis, above 50 represent acceptable green analysis and below 50 represent inadequate green analysis (22). The proposed method was found to be an excellent green analytical method and the details of Eco-Scale scoring for the proposed methods are given in Table 4.

(d) **Assessment of Green Profile (AGP).**—This method is a semi-quantitative method developed by Raynie et al. in 2009 (23). Assessment of green profile (AGP) is represented by a

| Parameters   | Mean, % | SD | n | Variance | Student’s t-test | F-test | D²  | D³  | Official method [6]<sup>a</sup> | D²  | D³  | Official method [6]<sup>a</sup> | D²  | D³  | Official method [6]<sup>a</sup> | D²  | D³  | Official method [6]<sup>a</sup> |
|--------------|---------|----|---|----------|-----------------|--------|-----|-----|-------------------------------|-----|-----|-------------------------------|-----|-----|-------------------------------|-----|-----|-------------------------------|
| PAR          | 100.25  | 0.71| 6 | 0.50     | 0.87 (2.179)    | 0.39 (2.101)| 3.91 (2.242)| 2.79 (2.38) |
| PSE          | 99.81   | 1.18| 8 | 0.12     | 1.12 (2.349)    | 0.10 (2.101)| 0.80 (2.242)| 1.10 (2.38) |
| CRX          | 100.13  | 0.68| 8 | 0.46     | 0.68 (2.179)    | 0.32 (2.101)| 0.58 (2.242)| 0.98 (2.38) |

Table 3. Statistical comparison of the results obtained by the proposed spectrophotometric methods and those obtained by the official ones for the determination of PAR, PSE, and CRX in pure powdered form.
pentagram divided into five risk potentials: health, safety, environmental, waste, and energy. Each potential has three possible shading levels: green, yellow, or red as traffic signals. Distilled water is a safe solvent in terms of health, safety, and environment hazards. The instrument used was a spectrophotometer, hence, the energy utilized by these methods are safe and the waste generated is less than 50 g. The proposed methods are completely eco-friendly and fulfilled all the above criteria, so that all the segments are shaded green as shown in Table 4.

(e) National Environmental Methods Index (NEMI).—The National Environmental Methods Index (NEMI) is the oldest tool used for the greenness assessment of analytical procedures (24). The profile criteria for NEMI are represented by a pictogram consisting of four fields. Water is not hazardous, not corrosive, not PBT (persistent, bio-accumulative, and toxic), and does not produce any hazardous waste. Thus, it passes the four quadrants of acceptance criteria and is considered a completely green solvent (Table 4).

**Conclusion**

Spectrophotometry is considered as the heart of QC laboratories; it offers several powerful analytical solutions for different complex mixtures. These opportunities can be advantageous in QC laboratories for drug analysis and can provide optimum sensitivity without the need for expensive instrumentation. The proposed spectrophotometric methods are considered to be smart, sensitive, selective, accurate, and precise. These methods succeeded in quantitative determination of PAR, PSE, and CRX in their pure form and pharmaceutical formulation without interference from excipients. To the best of our knowledge, there are no analytical reports describing the analysis of this ternary mixture indicating the novelty of the proposed approaches. The advantages of each method as well as the essential conditions for applying each one are summarized (see Supplemental Table S2). All the developed methods were completely validated in accordance with the ICH guidelines. It is noteworthy that the greenness profile was evaluated via five different green metrics. Hence, it is evident that these methods are potential green nominees for the analysis of the cited mixture in QC laboratories and pharmaceutical factories.

**Acknowledgment**

The authors express their sincere thanks to Amoun Pharmaceutical Co. (El-Obour City, Cairo, Egypt) for supplying gift samples of pure paracetamol, pseudoephedrine hydrochloride, and carbinoxamine maleate. The authors also would like to thank the staff and colleagues in Kialy Laboratory for their help and assistance.

**Conflict of Interest**

The authors of the manuscript declared that they do not have any conflict of interest.

**Supplemental Information**

Supplemental information is available on the J. AOAC Int. website.
References
1. Ismail, H., & Schellack, N. (2017) S Afr. Fam. Pract. 59, 5–22. doi:10.4102/safp.v59i3.4704
2. Wilson, M., & Wilson, P.J. (2021) Close Encounters of the Microbial Kind. Springer, Cham, pp 159–173. doi:10.1007/978-3-030-56978-5_10
3. Binns, C., Low, W.Y., & Kyung, L.M. (2020) Asia Pac. J. Public Health. 32, 140–144. doi:10.1177/1010539520929223
4. Sweetman, S.C. (2009) Martindale: The Complete Drug Reference. Pharmaceutical Press, London
5. British Pharmacopoeia (2013) The Stationary Office on behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA)
6. U.S. Pharmacopeial Convention (2007) The U.S. Pharmacopoeia (USP), National Formulary (NF 25), Rockville, MD
7. Pergolizzi, J.V., Varrassi, G., Magnusson, P., LeQuang, J.A., Paladini, A., Taylor, R., Wollmuth, C., Breve, F., & Christo, P. (2020) Pain Ther. 9, 353–358. doi:10.1007/s40122-020-00173-5
8. Casalino, G., Monaco, G., Di Sarro, P.P., David, A., & Scialdone, A. (2020) Eye (Lond) 34, 1235–1236. doi:10.1038/s41433-020-0909-x
9. Youssef, S.H., Hegazy, M.A., Mohamed, D., & Badawey, A.M. (2018) Chem. Cent. J. 12, 1–14. doi:10.1186/s13065-018-0436-z
10. Youssef, S.H., Hegazy, M.A.M., Mohamed, D., & Badawey, A.M. (2017) WJPPS. 6, 1644–1659. doi:10.20959/wjpps2017-9410
11. Lotfy, H.M., Fayz, Y.M., El-Hanboushy, S., Shokry, E., & Abdelkawy, M. (2016) Anal. Chem. Lett. 6, 718–737. doi:10.1080/22297928.2016.1245628
12. Gamal, M., Naguib, I.A., Panda, D.S., & Abdallah, F.F. (2021) Anal. Methods 13, 369–380. doi:10.1039/D0AY02169E
13. Almalki, A.H., Naguib, I.A., & Abdallah, F.F. (2021) Separations 8, 46. doi:10.3390/separations8040046
14. Kokilambigai, K.S., & Lakshmi, K.S. (2021) Green Chem. Lett. Rev. 14, 99–107. doi:10.1080/17518253.2020.1862311
15. Elbordiny, H.S., Elonsy, S.M., Daabees, H.G., & Belal, T.S. (2022) Sustain. Chem. Pharm. 25, 100580. doi:10.1016/j.scp.2021.100580
16. Gałuszka, A., Migaszewski, Z., & Namieśnik, J. (2013) TrAC Trends Anal. Chem. 50, 78–84. doi:10.1016/j.trac.2013.04.010
17. Lotfy, H.M., & Saleh, S.S. (2016) Int. J. Pharm. Pharm. Sci. 8, 40–56. doi:10.22159/ijpps.2016v8i10.13537
18. Lotfy, H.M., Hegazy, M.A., Mowaka, S., & Mohamed, E.H. (2016). Acta - Part A Mol. Biomol. Spectrosc. 153, 321–332. doi:10.1016/j.saa.2015.07.106
19. Lotfy, H.M., Ahmed, D.A., Abd El-Rahman, M.K., & Weshahy, S.A. (2019) Acta - Part A Mol. Biomol. Spectrosc. 223, 117322.doi:10.1016/j.saa.2019.117322
20. Pena-Pereira, F., Wojnowski, W., & Tobiszewski, M. (2020) Anal. Chem. 92, 10076–10082. doi:10.1021/acs.analchem.0c01887
21. Plotka-Wasylika, J. (2018) Talanta 181, 204–209. doi:10.1016/j.talanta.2018.01.013
22. Gałuszka, A., Konieczka, P., Migaszewski, Z.M., & Namieśnik, J. (2012) TrAC - Trends Anal. Chem. 37, 61–72. doi:10.1016/j.trac.2012.03.013
23. Raynie, D., & Driver, J. (2009) Green Assessment of Chemical Method, 13th Annual Green Chemistry & Engineering Conference, ASC, Maryland, USA, pp 34
24. Keith, L.H., Gron, L.U., & Young, J.L. (2007) Chem. Rev. 107, 2695–2708. doi:10.1021/cr068359e
25. ICH Guidelines (2005), Validation of Analytical Procedures: Text and Methodology, Q2 (R1)