Chemistry Research Journal, 2019, 4(6):54-61

Available online www.chemrj.org

Research Article

ISSN: 2455-8990
CODEN(USA): CRJHA5

Bivariate analysis and Ratio Subtraction coupled with extended ratio subtraction methods for simultaneous determination of Paracetamol and Diclofenac sodium in their combined pharmaceutical dosage forms

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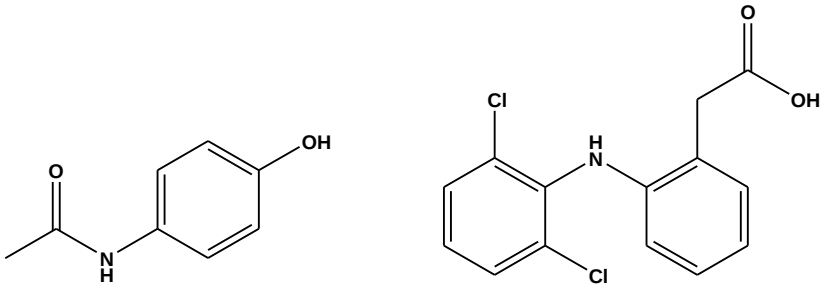
Abstract Three simple, specific, accurate and precise spectrophotometric methods have been developed for simultaneous determination of Paracetamol and Diclofenac sodium in pure form and in their pharmaceutical formulation. Bivariate and Ratio Subtraction coupled with extended ratio subtraction have been used in which bivariate was used for simultaneous determination of both drugss while ratio subtraction method was used for determination of Diclofenac sodium then use of extended ratio subtraction for determination of Paracetamol. Bivariate analysis and Ratio subtraction coupled with extended ratio subtraction methods are validated according to ICH guidelines in which accuracy, precision, repeatability and robustness were found in accepted limits. Advantages and limitations of each method are showed. Statistical comparison between the proposed methods was performed.

Keywords Paracetamol; diclofenac sodium; bivariate; ratio subtraction; extended ratio subtraction

Introduction

Paracetamol (PAR); N-(4-Hydroxyphenyl)acetamide (Fig. 1) is related to NSAID (non-steroidal anti-inflammatory drugs) which can act both centrally and peripherally for the treatment of non-inflammatory conditions in patients having gastric symptoms [1].



PAR **DCL**

Figure 1: Chemical structures of Paracetamol (PAR) and Diclofenac (DCL)

Diclofenac sodium (DCL); 2-(2,6-dichloroamino)phenylacetic acid (Fig. 1) [2], is an analgesic and anti-inflammatory agent which can act by the inhibition of the synthesis and the release of leukotrienes and prostaglandins [3]. Diclofenac is used in combination with paracetamol as the PAR can provide a basic relief before DCL and enhances its analgesic and antipyretic effect. PAR & DCL combination can be used in traumatic and surgical pain, high fever, pyrexia of unknown origin, acute painful conditions like renal, intestinal and other visceral colic and soft tissue injuries like sprain, in symptomatic treatment of acute and chronic inflammatory conditions like orchitis, bursitis, fibrositis, myositis, osteoarthritis, etc, [4].

The literature revealed that several methods have been carried out for the analysis of PAR and DCL in their mixture form or in their combination with other drugs. PAR & DCL have been determined by spectrophotometric methods [5-8], HPLC methods [9–12], TLC methods [13,14], capillary zone electrophoresis method [15], Voltammetric method [16], a method based on poly (diallyldimethylammonium chloride) functionalized graphene [17] and a method based on Au nanoparticles – functionalized graphene/poly (L-Arginine) glassy carbon electrode [18].

The aim of work is to develop more spectrophotometric methods which are accurate, fast and non-complicated for determination of PAR & DCL combination without the interference of their additives or their excipients in pharmaceutical formulations.

Experimental

Apparatus

JASCO dual beam (Japan) UV-visible spectrophotometer model V-630, connected to an ACER compatible computer with spectra manager II software was used. The spectral slit width is 2 nm at speed up can be increased up to 8000 nm/min. All the measurements have been carried out in 1 cm quartz cell. The wavelength ranges were 200 – 400 nm at room temperature.

Materials and Reagents

Pure Standards

Paracetamol and Diclofenac sodium were obtained from EIPICO (Egyptian International Pharmaceutical Industries Co.), located in 10th of Ramadan city, Egypt. Their purity was reported to be 99.50% and 99.80 %, respectively.

Pharmaceutical Formulations

Diclocin® tablets can be obtained from the market (label claim: Paracetamol 250 mg and Diclofenac Sodium 50 mg) manufactured by Cipcopharmaceuticals, India.

Solvents

HPLC grade Methanol is obtained from LiChrosolv, Merck KGaA, Germany. All of measurements have been carried out by using 90% Methanol (HPLC grade methanol: Distilled water 9:1).

Standard Solutions

Preparation of PAR and DCL stock standard solutions of 1 mg/mL in 90% methanol. PAR working standard solutions of 40 µg/mL were prepared in 90% methanol while DCL working standard solutions of 50 µg/mL were prepared by dilution from the stock solution with 90% methanol.

Laboratory Prepared Mixtures

Preparation of different ratios of PAR & DCL by transferring aliquots from their standard solutions to volumetric flasks (10 mL) and then dilution was carried out with 90% methanol.

Procedures

Construction of Calibration Curves

For PAR: Working solutions equivalent to (4-22 µg/mL) were prepared by addition of aliquots (1, 1.50, 2, 2.50, 3, 3.50, 4, 4.50, 5, 5.50 mL) of PAR working standard solution (40 µg/mL) to 10 mL volumetric flasks then dilution with 90% methanol.

For DCL: Working solutions equivalent to (5-45 µg/mL) were prepared by adding aliquots (1, 1.50, 2, 2.50, 3, 3.50, 4, 4.50, 5, 6, 7 mL) of DCL working standard solution (50 µg/mL) to 10 mL volumetric flasks then dilution with 90% methanol.

Measurements of the absorption spectra were carried out at room temperature over the wavelengths (200-400 nm).

For Bivariate Method

The linear calibration regression function for the spectrophotometric determination of any Analyte A at wavelength (i) can be given by:

$A_{Ai} = m_{Ai}. C_A + e_{Ai}$

Where m is the slope of the linear regression, C is the concentration and e is the intercept value. If the measurements were carried out for the binary mixture (A, B) at two selected wavelengths (λ_1, λ_2), then we will have a two equations set:

$A_{AB1} = m_{A1}. C_A + m_{B1}. C_B + e_{AB1}$

$A_{AB2} = m_{A2}. C_A + m_{B2}. C_B + e_{AB2}$

where e_{AB1} and e_{AB2} are the sum of the intercepts at the selected two wavelengths ($e_{ABi} = e_{Ai} + e_{Bi}$) then the values of C_A & C_B can be determined as following:

$C_B = m_{A2} (A_{AB1} - e_{AB1}) + m_{A1} (e_{AB2} - A_{AB2}) / m_{AB1} - m_{A1} m_{B2}$

$C_A = A_{AB1} - e_{AB1} - m_{B1}. C_B / m_{A1}$

These simple algorithms can allow the resolution of the binary mixture by selecting two wavelengths and using the parameters of the linear regression to determine each compound at the same wavelengths. The optimum wavelengths can be determined by using Kaiser method (Table 1). A series of sensitivity matrices K are calculated for each binary mixture and for every pair of pre-selected wavelengths:

$$K = \begin{bmatrix} m_{A1} & m_{B1} \\ m_{A2} & m_{B2} \end{bmatrix}$$

Where $m_{A1,2}$ are the slopes (sensitivity parameters) of component A and $m_{B1,2}$ are the slopes (sensitivity parameters) of component B. The resolution and determinants of these matrices were calculated. The wavelengths set was selected in which the highest absolute matrix determinant is obtained [19]. Bivariate method was used to determine PAR & DCL in presence of each other at the same wavelengths (250 and 270 nm) (Fig. 2).

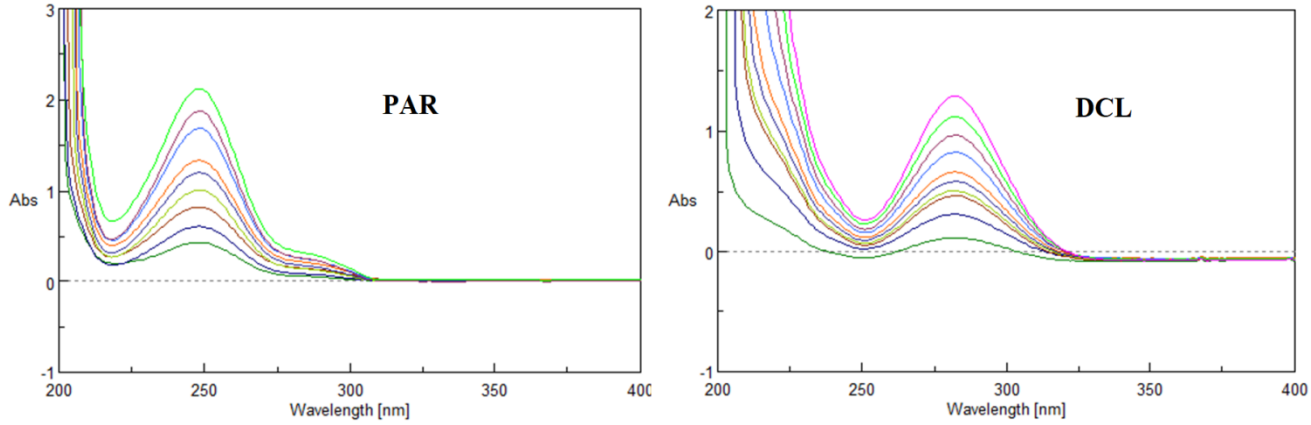


Figure 2: Zero absorption spectra of PAR and DCL

Table 1: Application of the Kaiser method for the selection of wavelength set for the mixture of PAR and DCL

λ_1 / λ_2	245 nm	250 nm	255 nm	260 nm	265 nm	270 nm
245 nm	0					
250 nm	-227	0				
255 nm	-29	171	0			
260 nm	410	588	387	0		
265 nm	1006	1164	912	507	0	
270 nm	1620	1765	1454	1011	478	0

For Ratio Subtraction Coupled

Scanning of Zero order absorption spectra of the solutions which have been prepared were carried out in the range of 200-400 nm for PAR & DCL against 90% methanol as blank (Fig. 2). Measurements were carried out at 282 nm for DCL in which the calibration curve was then constructed and the determination of the drug concentration then the regression equations were computed.

For application in mixtures and tablets, this method involve three steps in which 8ug PAR was used as a divisor then subtraction of the constant value in plateau region (at 305 nm) and lastly multiplication of the resulted spectrum with the same divisor which was used first (8ug PAR).

For Extended Ratio Subtraction Methods

Scanning of Zero order absorption spectra of the solutions which have been prepared were carried out in the range of 200-400 nm for PAR & DCL against 90% methanol as blank (Fig. 2). Measurements were carried out at 248 nm for PAR, respectively in which the calibration curve was then constructed and the determination of the drug concentration then the regression equations were computed.

For application in mixtures and tablets, the obtained spectrum of DCL was divided by a known concentration of DCL' (20 µg/ml) as divisor to get the constant (DCL/DCL') in the plateau region (305 nm). The previously scanned zero order absorption spectra of the laboratory prepared mixtures (DCL + PAR) were then divided by the same divisor DCL' then subtraction of the constant from the obtained curve followed by its multiplication b the divisor DCL', the original spectra of PAR were obtained.

Analysis of Laboratory Prepared Mixtures

The spectra of the mixtures were measured after preparation of different ratios of the laboratory prepared mixtures then treated in the same way as described under each method.

Application to Pharmaceutical Formulation

10 Tablets of Diclocin® Tablets were weighed and crushed then an amount equivalent to 50 mg PAR and 10 mg DCL in each tablet was transferred into a volumetric flask (50 mL) and diluted with 90% methanol as follow: First, 30 mL of 90% methanol were added and sonicated then dilution was carried out to the mark and filtered. Second, 10 mL of the dilution was transferred into a 100 mL volumetric flask to give a concentration equivalent to 100 µg/mL PAR and 20 µg/mL DCL. Third, any further dilutions were carried out in volumetric flasks (10 mL) and treated in the same way as described under each method.

Results and Discussion

Method Optimization

There are two major problems which were found during the analysis of PAR & DCL binary mixture; first, the overlapped spectra between the absorptivity of both drugs, and second, PAR, the major constituent, had unfortunately very high absorbance, while DCL, the minor component, had low absorbance value. As such, Sample enrichment technique [20] was used in which the concentration of DCL (the minor component) in their binary mixtures was increased to facilitate its determination. This was carried out by adding a fixed amount of standard

DCL to each experiment when combined with PAR, then subtraction of its concentration before the calculation of the claimed concentration of DCL. Sample enrichment technique has been used for solving the same problem in the analysis of other drug mixtures of different drug ratios [21,22]. PAR & DCL can be determined by Bivariate and Ratio subtraction coupled with extended ratio subtraction methods.

For Bivariate Method

250 and 270 nm absorbance were used for determination of PAR & DCL in presence of each other at the same wavelengths. The calibration curves revealed accepted linear relationships between concentrations and the bivariate in a range of 4-22 µg/mL for PAR and 7.5-45 µg/mL for DCL with a correlation coefficients of 0.9990 & 0.9996 for PAR and 0.9991 & 0.9994 for DCL. The accuracy of the method illustrated accepted values with 99.65% ± 0.65 for PAR and 100.54% ± 0.37 for DCL. The specificity of the methods demonstrated accepted values with 100.39% ± 1.23 for PAR and 100.56% ± 1.52 for DCL. The results are recorded in Table 2. Bivariate analysis is very easy and simple as it depends on zero absorption spectra without the need of extra processing but it has 2 limitations which are the need for some specific calculations before the application of this method and the need of performing Kaiser method to select the best two wavelengths.

Table 2: Assay parameters and validation results obtained by applying the bivariate spectrophotometric method

Method Parameters	**DCL**		**PAR**	
Wave length (nm)	250	270	250	270
Linearity range (µg/mL) (n=3)	7.5-50	7.5-50	4-22	4-22
Intercept	-0.0730	-0.0590	0.078923	0.019452
Slope	0.0075	0.0218	0.090268	0.02717
Correlation coefficient (r)	0.9991	0.9994	0.9990	0.9996
Accuracy (Mean ± SD)	100.54 ± 0.37	100.54 ± 0.37	99.65 ± 0.65	99.65 ± 0.65
Precision (±%RSD)				
Repeatability	100.26 ± 0.93		98.70 ± 0.33	
Intermediate precision	98.81 ± 0.29		98.83 ± 0.36	
Specificity (Mean ± SD)	100.39 ± 1.23		100.56 ± 1.52	

For Ratio Subtraction Coupled with Extended Ratio Subtraction Methods

248 and 282 nm absorbance were used for determination of PAR & DCL, respectively. The calibration curves revealed accepted linear relationships between concentrations and the bivariate in a range of 4-22 µg/mL for PAR and 7.5-45 µg/mL for DCL with correlation coefficients of 0.9990 for PAR and 0.9995 for DCL. The accuracy of the method illustrated accepted values with 99.88% ± 1.01 for PAR and 99.60% ± 0.91 for DCL. The specificity of the methods demonstrated accepted values with 100.05% ± 0.86 for PAR and 100.16% ± 1.18 for DCL. The results are recorded in Table 3. Ratio subtraction coupled with extended ratio subtraction methods are very easy but they require extra processing before determination of the concentration of each drug.

Table 3: Assay parameters and validation results obtained by applying the ratio subtraction coupled with extended ratio subtraction spectrophotometric methods

Method Parameters	**DCL**	**PAR**
Wave length (nm)	282	248
Linearity range (µg/mL) (n=3)	7.5-45	4-22
Intercept	-0.0930	0.0800
Slope	0.0303	0.0911
Correlation coefficient (r)	0.9995	0.9990
Accuracy (Mean ± SD)	99.60 ± 0.91	99.88 ± 1.01
Precision (±%RSD)		
Repeatability	99.75 ± 1.07	99.72 ± 0.99
Intermediate precision	99.46 ± 1.12	99.50 ± 0.92
Specificity (Mean ± SD)	100.16 ± 1.18	100.05 ± 0.86

Method Validation

All methods were validated according to ICH guidelines [23]. The linear regression data for the calibration curves showed good linear relationships (Tables 2, 3).

The accuracy was calculated by analyzing the standard addition where satisfactory results were obtained as shown in Tables 2, 3.

The specificity of the methods was calculated by assaying the laboratory prepared mixtures of PAR & DCL within the linearity range and good results are obtained (Tables 2, 3).

The intra- and inter-day precisions were calculated by the analysis of 3 different concentrations of the drugs 3 times on the same day and on 3 successive days (Tables 2, 3).

Application to Pharmaceutical Formulation

Bivariate analysis and ratio subtraction coupled with extended ratio subtraction were successfully applied for determination of PAR & DCL in its pharmaceutical formulation (Diclocin® tablets). The results were acceptable and with sufficient agreement with the labeled amounts. The standard addition technique was applied and showed that no interference of the excipients was observed (Table 4).

Table 4: Analysis of the pharmaceutical preparation (Diclocin® tablets) by applying the Bivariate and Ratio subtraction coupled with extended ratio subtraction spectrophotometric methods

	Bivariate method								**Ratio subtraction coupled with extended ratio subtraction methods**							
	DCL				**PAR**				**DCL**				**PAR**			
			Recovery%				**Recovery%**				**Recovery%**				**Recovery%**	
	Tablet Taken (µg/mL)	**Standard Added (µg/mL)**	**Tablet**	**Added**	**Tablet Taken (µg/mL)**	**Standard Added (µg/mL)**	**Tablet**	**Added**	**Tablet Taken (µg/mL)**	**Standard Added (µg/mL)**	**Tablet**	**Added**	**Tablet Taken (µg/mL)**	**Standard Added (µg/mL)**	**Tablet**	**Added**
		8	101.27	100.59		9	101.51	100.24		8	100.76	99.47		9	98.67	100.98
	2	9.50	99.88	100.89	10	10	101.24	98.95	2	9.50	99.07	98.76	10	10	101.07	99.01
		10	100.58	100.15		11	100.45	99.77		10	98.42	100.56		11	99.42	99.64
Mean			100.58	100.54			101.07	99.65			99.42	99.60			99.72	99.88
SD			0.70	0.37			0.55	0.65			1.21	0.91			1.23	1.01

Statistical Analysis

Statistical comparison between the proposed methods was performed through One-way ANOVA method by using PASW statistics 18® software program. The calculated F values were less than the theoretical ones indicating that there was no significant difference between them (Table 5).

Table 5: Statistical comparison of the results obtained by the proposed methods using One-way ANOVA

Tablets	**Drugs**		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Diclocin® tablets	PAR	Between Groups	2.720	1	2.720	3.004	0.158
		Within Groups	3.622	4	0.905		
		Total	6.342	5			
	DCL	Between Groups	2.018	1	2.018	2.079	0.223
		Within Groups	3.884	4	0.971		
		Total	5.903	5			

Conclusion

Bivariate and Ratio subtraction coupled with extended ratio subtraction methods were applied for the determination of paracetamol and Diclofenac sodium in their binary mixtures and in their pharmaceutical formulation. All of these proposed methods are simple, sensitive and accurate and could be used for routine analysis by using simple technology or instruments. By comparison of the previous methods it was concluded that bivariate analysis doesn't

require extra processing but Ratio subtraction and extended ratio subtraction methods require extra processing. Statistical comparison showed that there was no significant difference between the proposed methods.

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