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Simultaneous Spectrophotometric Determination of Diflunisal and Diclofenac Sodium in Pharmaceutical Dosage Form Using Chemometric Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Author IHIH designed the study, wrote the protocol, the draft of the manuscript and managed the experimental process and the statistical analysis. Author RTEE managed the literature searches, performed the spectroscopy analysis. Authors MR, DM and SM managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Is the simultaneous determination of Diflunisal and Diclofenac sodium in pharmaceutical preparations.
Study Design: Four spectrophotometric methods (Classical Least Squares, Iterative Target Transformation Factor Analysis, Principal Component Regression and Partial Least Squares) will

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be described and evaluated for the simultaneous determination of the two drugs. In these techniques, the concentration data matrix was prepared according to the full factorial experimental design of three levels and two components in mixtures.
Place and Duration of Study: Microanalytical Chemistry Laboratory, Applied Organic Chemistry Department, National Research Centre, Dokki, Giza, Egypt, between January 2016 and Marsh 2016.
Methodology: Using 2.5 mM NaOH solution as solvent, the corresponding absorbance data matrix was measured over the wavelength range of 220 – 400 nm with the intervals of Δλ= 1 nm, then regression was obtained by using the absorbance data matrix and the concentration data matrix for the prediction of the unknown concentrations of Diflunisal and Diclofenac sodium in their mixture. The procedure did not require any chemical separation step or prior graphical treatment of the overlapped spectra.
Results: The calibration range was found linear over 2.5 – 20 µg/mL for Diflunisal and Diclofenac sodium through the Partial Least Squares method.
Conclusion: The Partial Least Squares method was selected and validated on both authentic mixtures and pharmaceutical preparations. The accuracy and precision of the methods were assessed and compared with each other.

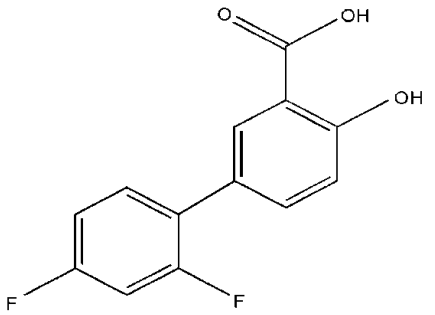
Keywords: Diflunisal; diclofenac sodium; chemometric.

1. INTRODUCTION

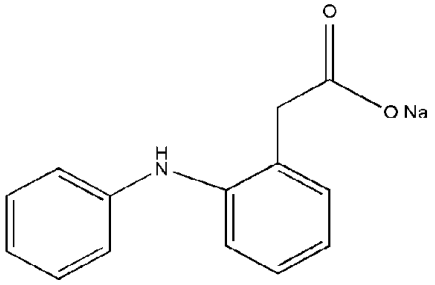
Diflunisal (DIF) (Fig. 1a) is chemically known as 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid official in United State pharmacopeia [1]. Diflunisal, a salicylate derivative, is a nonsteroidal anti-inflammatory agent (NSAID) with pharmacological actions like other prototypical NSAIDs. Diflunisal possesses anti-inflammatory, analgesic and antipyretic activity. Diflunisal is used to relieve inflammatory pain and in the symptomatic treatment of rheumatoid arthritis and osteoarthritis [2]. Several methods have been reported describing the determination of DIF in different matrices including difference spectrophotometry [3-5], Chemometric spectrophotometry [6], spectrofluorimetry [4,7-9], chromatography [1,4,10-12] TLC method [13], capillary electrophoresis with luminescence detector [14] and electrochemical [15] methods.

Diclofenac sodium (DIC) (Fig. 1b), 2-(2,6-Dichloranilino) phenylacetic acid monosodium salt it, is official in United Stated pharmacopeia [1]. It is a nonsteroidal anti-inflammatory drug (NSAID) taken or applied to reduce inflammation and as an analgesic for reducing pain in certain conditions as musculoskeletal and joint disorders such as rheumatoid arthiritis and osteoarthiritis and in other painful conditions such as renal colic, acute gout, dysmenorrheal and migraine [2]. The quantification of DIC in its various drug formulations and/or biological samples was addressed in many reports., including spectrophotometric methods [16-23], chemometric spectrophotometry [24], spectrofluorimetry [25,26], potentiometric [27-32], voltammetric [33-39], liquid chromatographic techniques [1,40-44], GC-MS [45], HPTLC method [46], and capillary electrophoresis [47].

Diflunisal is usually accompanied with Diclofenac Sodium in suppository form under trade name Rheumafen Forte suppositories (GlaxoWellcome, Egypt) containing 200 mg DIF and 100 mg DIC.



a. Diflunisal



b. Diclofenac sodium

Fig. 1. Chemical structures of a. Diflunisal and b. Diclofenac sodium

A literature review revealed that the two cited components were simultaneously determined in mixture either by third order derivative and ratio spectra derivative spectrophotometry [48], different spectrophotometric and TLC-densitometric methods [49] and HPLC methods [50,51].

And from the previous literature, there have been no reports for the simultaneous determination of both drugs using chemometric techniques (multivariate calibration techniques). The advantage of these techniques is the simultaneous analysis of the mixture components with strongly overlapping spectra without graphical pre-treatment such as derivative and ratio spectra derivative. They also require shorter time, less costs and the errors of calibration model are diminished by measuring the absorbance values at many points in the wavelength range of the zero-order spectra.

2. EXPERIMENTAL

2.1 Apparatus

A JASCO V-570 double beam UV-VIS spectrophotometer with a quartz cuvette, 10-mm path at a scan speed of 1200 nm/min and fixed slit width of 2 nm was used for measuring the light absorption in the ultraviolet (UV) region (200-400 nm). It is connected to a computer loaded with Spectra Manager Program (JASCO) used for the spectral acquisition and elaboration of the data obtained in ASCII format to subsequent manipulation by Multivariate length, was used for measuring the light absorption in ultraviolet region (200-400 nm). Analysis Program Add-in Microsoft Excel, written in Macros according to algorithms described by Haaland, Martens and Geladi [52,53] for Classical Least Squares (CLS), Principal Component Regression (PCR), Partial Least Squares (PLS-1) methods and Gemperline [54,55] for Iterative Target Transformation Factor Analysis ITTFA method.

2.2 Materials and Reagents

All experiments were performed using pharmaceutical-grade authentic standards of Diflunisal (Rameda Company, Sixth of October, Egypt) and Diclofenac sodium (Sigma Pharmaceutical Industries company, Mubarak Industrial Zone, First Quarter, Quesna, Egypt), which were of purity 99.9 and 99.8% (w/w), respectively, on a dried basis. The combined dosage form was purchased from the local market known as Rheumafen Forte suppositories labeled to contain 100 mg DIC and 200 mg DIF and manufactured by GlaxoWellcome Egypt (El-Salam City, Cairo, Egypt). Sodium hydroxide (Merck, Darmstadt, Germany) was used. All chemicals were used without further modification or purification.

2.2.1 Standard solutions and calibration curves

Stock solutions were prepared by dissolving 25 mg of each DIF and DIC; separately; in 5 mL 0.05 M NaOH and then completed to 100 mL with distilled water to obtain finally a concentration of 250 µg/mL in 2.5 mM NaOH.

To a series of 10-ml volumetric flasks, aliquots of DIF and DIC solutions were added and then diluted to 10 ml with 2.5 mM NaOH. Four sets of standard solutions were prepared, two series of individual solutions containing 2.5, 5.0, 10.0, 15.0 and 20 µg/mL and two series of 9 mixtures. The composition of the 9 mixtures were prepared twice according to the full factorial experimental design (L^n) of three levels of concentrations and two components as summarized in (Table 1). The first one of mixture data set will be used for calibration and another for validation method. UV spectra of the solutions were then recorded in the range of 220-400 nm against a blank of 2.5 mM NaOH solution.

2.2.2 Pharmaceutical sample preparation

Rheumafen Forte suppository containing 100 mg of DIC and 200 mg of DIF was transferred to a100 mL beaker and 5 ml of 0.05 M NaOH solution was added and the solution was warmed with shaking for 15 minutes, cooled to solidify the paraffin wax, decanted into a 100 mL volumetric flask and completed to the mark with washings of distilled water. Three different aliquots of the solution as 25, 37.5 and 50 µL were transferred into three 10-mL volumetric flasks and then diluted to the mark with 2.5 mM NaOH. UV spectra of the solutions were then recorded in the range of 200-400 nm against a blank of 2.5 mM NaOH solution. The method reproducibility was studied by performing the method on the same day (intra-day precision) and three different days (inter day precision).

Table 1. Composition of diflunisal and diclofenac sodium in mixtures and individual solutions

	Concentration µg/mL			
	Mixture solutions		Individual solutions	
	DIC	DIF	DIC	DIF
1	2.5	2.5	2.5	-
2	2.5	5	5	-
3	2.5	10	10	-
4	5	2.5	15	-
5	5	5	20	-
6	5	10	-	2.5
7	10	2.5	-	5
8	10	5	-	10
9	10	10	-	15
10	-	-	-	20

3. RESULTS AND DISCUSSION

As shown in (Fig. 2), the absorption spectra of DIF and DIC in aqueous 2.5 mM NaOH solutions were severely overlapped obstructing the resolution of this binary mixture to direct absorbance measurements. Such a determination could be theoretically facilitated by the use of chemometric methods. The spectra of DIF and DIC scanned over the wavelength range 220–400 nm with 1 nm intervals were selected for chemometric analysis. Wavelengths below 220 nm were rejected due to the noise appeared on replicating the same sample. It is noted experimentally that using 2.5 Mm NaOH solution as solvent for DIF and DIC gave better reproducible and stable spectra than traditional solvent of methanol.

With the aim of improving the recovery of these compounds, four different chemometric approaches were evaluated, namely Classical Least Squares (CLS), Principle Component Regression (PCR), Partial Least Squares Regression (PLS) and Iterative Target Transformation Factor Analysis (ITTFA). Haaland and Thomas [52] made a comparison of the different multivariate calibration methods for quantitative spectral analysis. They concluded that it is very difficult to generalize about the superiority of one method over each other, because their relative performance is often dependent on the particular data set to analyze. In the calibration step, two types of calibration data sets were tested which is either individually or in mixture as demonstrated in (Table 1). The results found showed significant difference between these two data sets and between chemometric methods as demonstrated later.

The theory and application of the chemometrics in spectroscopy have been discussed by several workers and here we will describe them briefly.

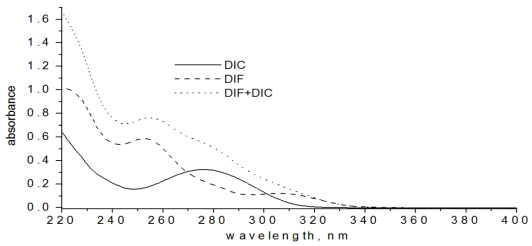


Fig. 2. Overlapped absorption spectra of 10 ug/mL diflunisal and diclofenac sodium

3.1 Classical Least Squares (CLS)

This method assumes Beer's law model with the absorbance at each wavelength being proportional to the component concentration.

$$A = KC + E$$

The training set (calibration set) of absorptivity was used for constructing CLS model or (K) matrix with dimension 2 components at 180 different wavelengths. The CLS method needs that all the components in the samples of calibration must be known. The absorbance matrix***(A)*** of the calibration samples of mixtures (9x180) and their corresponding concentration matrix***(C)*** (9x2) were used to find the absorptivity matrix **(*K*-matrix)** by the following equation:

$$K = (C^t.C)^{-1}.C^t.A$$

Where the superscript ^{t} is transport of the matrix, then the obtained *K*-matrix was further used for the prediction of unknown concentration C_u of the two components in both the validation and pharmaceutical formulation samples:

$$C_u = (K.K^t)^{-1}.K.A_u$$

Where A_u is spectra matrix of the unknown samples.

3.2 Principal Component Regression (PCR) and Partial Least Squares (PLS)

PCR and PLS methods could be used in determination of the components under the search even in the presence of unknown components (interfering substance) which gave these two methods an advantage over CLS. Unlike CLS method based on direct regression of the concentrations onto the spectroscopic

responses, the PCR method consists of two steps; in the first step, it does decomposition of spectral matrix*(A)*either by principal component analysis PCA or by singular value decomposition SVD methods into a minimum number of linear independent products of two smaller matrices of what is so called principal component scores (eigenvalues) and loadings (eigenvectors) and then regress the obtained scores against the concentrations as a calibration step [56]. PLS resembles PCR, but the PLS method decomposes simultaneously the spectroscopic data matrix *(A)* and the chemical data matrix *(C)* into a minimum number of eigenvectors and their eigenvalues containing the most relevant information in *A* and *C*.

The main advantage arise from this decomposition is that each eigenvalue represents the relative importance of the associated eigenvector. A large eigen value indicates a major factor and contains meaningful information, whereas a very small eigenvalue indicates an unimportant factor containing mainly noise and can be deleted. This minimum number of eigenvectors/eigenvalues is called factor number. The remaining eigenvectors are the result of experimental error. If the factor number is greater or smaller than the true one i.e. either data is deficiently or excessively introduced, under fitting or over fitting concentration data is risked. The regression coefficients K was obtained by regression the optimal number of chemical eigenvectors on the spectral eigenvectors and then used in the prediction step to estimate unknown concentrations of a mixture.

In order to evaluate the factor number without under/over fitting the concentration data, the raw data of the calibration samples were mean centered [57] as a preprocessing step (the centering makes the following computations numerically well conditioning, i.e. no colinearity, low noise and no constant background) and then the cross-validation method [52], leaving out one sample at a time and the prediction error sum of squares (PRESS) of concentrations was calculated which is a measure of the efficiency for a calibration fit model and the optimum number of factors would be the number that yielded the minimum PRESS.

$$\mathbf{PRESS} = \sum_{i=1}^{m} (\hat{c}_i - c_i)^2$$

Where $\hat{c}_i$ is the calculated concentration and c_i is the actual concentration for the i^{th} sample left out of the calibration during cross validation.

Other method to evaluate factor number is eigenvalue ratio ER which was evaluated by principal component analysis PCA. In our particular case, based on spectral data and their corresponding concentration data of 9 mixture samples, 2 factors were obtained as optima values for determining DIF and DIC components as shown in (Fig. 3).

3.3 Iterative Target Transformation Factor Analysis (ITTFA)

The spectral data matrix *(A)* is first decomposed by principal component analysis PCA or by singular value decomposition SVD into minimum number of two independent matrices of scores (T) and loadings (P). Target transformation matrix R is computed from projection of T matrix onto concentrations of unknowns which were initialized with best guesses and these are then refined by iterative calculations of the target transform.

$$R = (T.T^t)^{-1}.T.c^o$$
$$c' = T.R$$

The iteration is repeated until concourse $\|C' - Co$ is reached. Different from classical factor analysis technique [58] in ITTFA the absorbance spectrum data of the unknown as well as those of the reference samples are used for calibration. Moreover, the iterative techniques are employed for prediction and better results can generally be obtained [54].

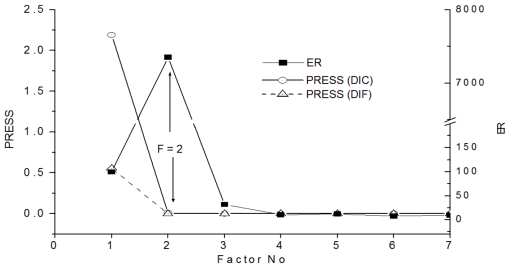


Fig. 3. Optimal factor number using prediction error sum of squares PRESS or eigenvalue ratio ER for diflunisal and diclofenac sodium

3.4 Validation of Training Set

Two data sets for the calibration either of individual or mixture solutions were applied to identify which set will give more accurate and precise on determining the two components in their mixtures. As depicted in Table 2, using CLS

method, DIC gave higher accurate and precise results by mixture data set than individual data set while no significant difference was observed between the two data sets on determining DIF. This was assessed by the comparative statistics of *t*- and *F*-tests to compare between two unpaired groups (independent) with equal variances [59]. It is clear that calculated *t* values for unpaired two-tailed data distribution of DIC and DIF were higher than the tabulated *t* value (t_{cal}= 3.585 for DIC and 3.158 for DIF > t_{tab}= 2.201). This suggested that at 95 % confidence level differences between the recoveries obtained by the two data sets were statistically significant and it is preferably to use mixture data set as training set. This is confirmed by the *F*-test which revealed that there is no difference between the precision of the two data sets. The calculated value of *F* were less than the critical value (F_{cal}= 1.94 for DIC and 1.54 for DIF < $F_{8,8}$= 3.483).

Table 2. Results obtained by CLS for determining diflunisal and diclofenac sodium using either individual or mixture data sets

Taken, µg/ml		Found, %			
		Individual data set		Mixture data set	
DIC	**DIF**	**DIC**	**DIF**	**DIC**	**DIF**
2.5	2.5	113.86	116.76	107.36	107.06
2.5	5	107.12	105.33	97.87	101.53
2.5	10	115.54	105.03	99.73	102.04
5	2.5	105.72	112.79	101.46	103.37
5	5	110.75	109.92	104.79	103.97
5	10	106.86	101.96	98.01	98.18
10	2.5	101.44	114.13	98.20	97.89
10	5	104.72	108.76	100.69	99.34
10	10	102.99	102.94	97.43	97.40
Accuracy (bias), RE %		7.67	8.62	0.61	1.60
Precision RSD, %		4.79	5.20	3.44	4.18
Confidence interval*		3.13	3.39	2.25	2.73
t-test **= 2.120		3.585	3.158		
F-test ***=3.438		1.94	1.54		

** P=0.05 and n=9, ** P=0.05 and df=16, *** P=0.05 and df=8*

Confidence Interval CI is used to detect whether the source of error is due to systematic error (due to chemical interference or faulty calibration and expressed as accuracy, bias= $\bar{x} - x_t$) or random error (express as RSD). The CI for the mean $\bar{x}$ is the interval (above and below) around true value x_t , with a given degree of certainly (or confidence level) [59]:

$$\bar{x} - x_t = \pm \frac{ts}{\sqrt{n}}$$

Where **t** is critical t-test value at confidence level and degrees of freedom (n-1) and **s** is the standard deviation. If the left side is greater than the right; the error is systematic while if it is reverse the error is random. The confidence interval CI revealed that at 95% confidence, the source of error is likely systematic on determining DIC and DIF by individual data set (for DIC bias 7.67 > CI 3.13 and for DIF 8.62 > CI 3.39) and it is random by mixture data set (for DIC bias 0.61 < CI 2.25 and for DIF 1.6 < CI 2.73) as presented in (Table 2).

3.5 Validation of Chemometric Methods

Using the selected calibration (training) data set, all models of CLS, ITTFA, PCR and PLS1 were applied for analysis of DIF and DIC in individual samples (Table 3) and in mixture samples (Table 4). The accuracy expressed by bias (relative error RE, %), and precision expressed by relative standard deviation (RSD, %) values are summarized.

It was clear from (Tables 3 and 4) that PLS had superiority over CLS, ITTFA and PCR in the analysis of individuals and mixtures as indicated by best precision and accuracy. The proposed method PLS1 was then applied for the simultaneous determination of the two components in formulation samples as given in (Table 5).

The results were compared with that which was analysed by HPLC as reported method [51] and found that there was no signefecance deffirence at 95% confedince level (Table 6). The repeatability of the PLS method was determined by performing the assay three replicates of three different concentrations, on the same extract of the prepared dosage form, on the same day (intra-day precision) while the intermediate precision was determined the same but over three different days (inter day precision). Both intra- and inter-day RSD range from 0.45-1.35 % for all analytes, confirming good precision (Table 5).

Table 3. Recoveries of diflunisal and diclofenac sodium individually obtained by different chemometric methods

	Taken µg/mL	Found, %			
		CLS	ITTFA	PCR	PLS
DIC	2.5	106.98	107.17	106.96	97.19
	5	100.07	100.21	100.06	95.33
	10	97.13	97.21	97.11	95.97
	15	98.30	98.35	98.29	98.42
	20	97.17	97.21	97.15	97.86
Accuracy (bias), %		-0.07	0.03	-0.08	-0.34
Precision (RSD), %		4.12	4.17	4.12	1.29
Confidence interval*		3.61	3.66	3.61	1.13
DIF	2.5	105.23	105.15	105.12	97.92
	5	103.71	105.70	105.60	99.13
	10	100.39	100.36	100.29	98.90
	15	98.07	98.06	97.97	98.30
	20	96.96	96.95	96.86	98.04
Accuracy (bias), %		0.87	1.24	1.17	-0.54
Precision (RSD), %		3.45	3.79	3.80	0.90
Confidence interval*		2.14	2.35	2.36	0.56

** P=0.05, n=5*

Table 4. Recoveries of diflunisal and diclofenac sodium in mixtures obtained by different chemometric techniques

Taken, µg/mL		Found, %							
		CLS		ITTFA		PCR		PLS1	
DICL	DIF	DIC	DIF	DIC	DIF	DIC	DIF	DIC	DIF
2.5	2.5	107.36	107.06	107.61	105.61	107.28	106.62	98.63	98.05
2.5	5	97.87	101.53	98.06	101.47	97.75	101.44	95.44	96.86
2.5	10	99.73	102.04	99.69	102.05	99.51	101.94	99.06	101.77
5	2.5	101.46	103.37	101.56	103.25	101.41	103.31	98.29	98.26
5	5	104.79	103.97	104.79	103.97	104.73	103.88	103.21	101.62
5	10	98.01	98.18	98.03	98.17	97.90	98.09	98.75	98.72
10	2.5	98.20	97.89	98.25	97.76	98.17	97.87	97.85	96.21
10	5	100.69	99.34	100.65	99.39	100.65	99.28	101.15	100.69
10	10	97.43	97.40	97.43	97.40	97.37	97.31	99.10	99.85
Accuracy (bias), %		0.61	1.20	0.68	1.01	0.53	1.08	-0.95	-0.88
Precision (RSD), %		3.44	3.27	3.48	2.97	3.45	3.18	2.15	2.00
Confidence interval *		2.25	2.14	2.28	1.94	2.25	2.08	1.41	1.30

**P=0.05, n=9*

Table 5. Recoveries of diflunisal and diclofenac sodium in (Rheumafen suppositories)obtained by PLS on the same day and on the three successive days

Taken, µg/mL		Found, mean ± SD, %			
		Intra-days[1]		Inter-days[2]	
DIC	DIF	DIC	DIF	DIC	DIF
2.5	5	100.81±0.17	100.71±0.34	100.48±0.37	100.88±0. 18
3.75	7.5	101.28±0.41	101.17±0.19	100.55±0.30	100.23±1.49
5	10	100.13±0.27	100.02±0.56	100.87±1.23	100.66±1.6 1
Accuracy, bias,%		0.59	0.87	0.29	-0.11
Precision, RSD		1.19	0.66	1.35	0.96
CI at α=0.05		1.35	0.71	0.85	0.60

1 mean of three determinations in the same first day, 2 mean of nine determinations over three days

Table 6. Analysis of DIC–DIF mixture in its pharmaceutical preparation (Rheumafen suppositories) by the proposed chemometric method and the reported method

	Proposed method		Reported method [51]	
	DIC	DIF	DIC	DIF
% Recovery ± SD	100.13±0.27	100.02±0.56	100.79±1.11	100.97±1.20
RSD	0.26	0.56	1.14	1.19
n	3		5	
t -calculated	0.98	1.26		
F-test	18.22	4.59		

Theoretical values for t and F at P = 0.05 are 2.44 and 19.24, respectively

4. CONCLUSION

Four different chemometric techniques; Classical Least Squares, Iterative Target Transformation Factor Analysis, Principal Component Regression and Partial Least Squares were studieded for the determination of Diflunisal and Diclofenac sodium. The proposed multivariate calibration method; Partial Least-Squares showed the best results regarding accuracy and precision, in comparison to the other methods, in the analysis of individuals and mixtures in comparison with other methods. Partial Least-Squares was simple, rapid, sensitive and precise and could be easily applied successfully for the simultaneous determination of DIC and DIF in dosage form without any preliminary separation step.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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