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Sequential Injection Spectrophotometric Determination of Diclofenac in Urine and Pharmaceutical Formulations

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Abstract

This paper reports a sequential injection spectrophotometric method for determining the concentration of diclofenac (DCF) in urine samples and pharmaceutical formulations. The method is based on the reaction of DCF with potassium permanganate in an alkaline medium. The influence on the analytical signal of the reactor length, the permanganate volume, the sample volume and the flow rate was investigated using a Taguchi parameter design. Under optimal conditions, the linear range of the calibration curve varied from 10.0 to 100.0 mg l^{-1}, with a detection limit of 5.0 mg l^{-1}. A sampling rate of 15 determinations h^{-1} was achieved; the relative standard deviation of analytical repeatability was less than 3.0% in all cases. The method was validated by comparing the results obtained against those provided by RP-HPLC; no significant differences were seen (p<0.05)

Keywords Diclofenac, Spectrophotometry; Sequential Injection Analysis, Pharmaceutical, Urine

1. Introduction

Diclofenac sodium (DFC) (2-[(2,6-dichlorophenyl)amino]-benzene-acetic acid monosodium salt) is a non-steroidal anti-inflammatory agent with analgesic, anti-inflammatory and antipyretic activity. Its exact mechanism of action remains unknown, but many of its main properties appear to be associated with the inhibition of prostaglandin synthesis [1].

The concentration of this agent in biological fluids and pharmaceutical formulations can be determined by ultraviolet and visible spectroscopy [2-7], fluorimetry [8], gas and liquid chromatography [9-10], and flow techniques with spectrophotometric [11-12] and electrochemical detection [13-14]. Spectrophotometric methods afford a number of economic advantages over chromatographic techniques while their sensitivity, precision and accuracy are similar. In most cases DFC is determined indirectly by means of either the formation of coloured species with other reagents (methylene blue, copper(II) acetate, ferric chloride/1,10-phenantroline) or via oxidation (with $KBrO_3$, ceric ammonium sulphate or sodium nitrite).

The present work proposes a sequential injection system with spectrophotometric detection to determine the concentration of DFC in pharmaceutical preparations and urine samples. This is based on the oxidation of the drug by potassium permanganate in a strongly basic medium and the measurement of the green product formed. The influence of the injection volume (sample and oxidising agent), flow rate and the reaction coil length on detection sensitivity was investigated employing a Taguchi parameter design (TPD). The method was validated by comparing the results obtained with those provided by a reference method [15].

2. Experimental

2.1. Reagents and solutions

All solutions were prepared by dissolving the corresponding analytical grade reagent in filtered, distilled water; these solutions were used without further purification.A stock solution (1000 mg l^{-1}) of diclofenac sodium was prepared weekly by dissolving the salt in water. Standard solutions ranging from 1 to 100 mg·l^{-1} were prepared daily by dilution of this stock solution. Potassium permanganate ($1.0x10^{-3}$ mol l^{-1}) was used as an oxidising agent and NaOH (1.0 mol l^{-1}) as the carrier solution.

2.2. Sample preparation

Four types of samples were analysed: tablets, ampoules, a topical gel and urine. Tablet powder (0.5 g) was dissolved in an appropriate amount of water in an ultrasonic bath for 3 min; this solution was then diluted with water in a 50.0 ml calibration flask. Suitable aliquots (0.1-0.5 ml) were then diluted 1:20 with distilled water and filtered through a 0.45 µm membrane filter (Millipore).

The content of one ampoule was dissolved in 50.0 ml of water in a calibrated flask. Suitable aliquots (0.1-0.5 ml) of this solution were diluted 1:20 with distilled water.

A 1.0 g sample of the topical gel was dissolved in 25.0 ml of water and the mixture heated at 40-45°C for 5 min. The aqueous solution was diluted to 100 ml with water in a calibrated flask. Suitable aliquots (0.1-0.5 ml) of this solution were filtered and diluted 1:2 with distilled water.

Urine samples were diluted (1:10) and filtered through a 0.45 µm membrane filter (Millipore).

2.3. Apparatus

The proposed sequential injection system (Fig.1) consists of a programmable speed burette multisyringe (MicroBu 2030, Crison) used to aspire and dispense the reagent solutions, an eight-way selection valve (Crison), and a UV-visible spectrophotometer (Lambda 40, Perkin-Elmer) with a Hellma 178.712QS 18 µl flow-through detector cell. Omnifit PTFE

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tubing (0.8 mm i.d.) connects the different components of the flow system. The instrument devices are controlled by Autoanalysis 5.0 software.

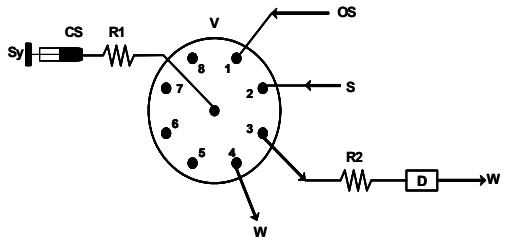

Figure 1. Diagram of the proposed system used to determine DFC. Sy, syringe; CS, carrier solution (NaOH 1.0 mol l^{-1}); R1, loading reactor; V, selection valve; OS, oxidising solution ($KMnO_4$, $1x10^{-3}$ mol l^{-1}); S, sample; R2, mixing reactor; D, detector (445 nm); W, waste

2.4. Reverse phase HPLC comparisons

The concentration of DFC in the analysed samples was also determined using reverse phase HPLC (RP-HPLC), the standard technique used in pharmaceutical analysis. The apparatus used was a PerkinElmer Series 200 liquid chromatograph (PerkinElmer MA, USA) equipped with a UV–Vis detector at 254 nm and a manual injector connected to a 50 µl external loop. Chromatographic separation was achieved with a Microsorb 100-5C18 column (5µm; 150mm×4.6mm i.d.) (Varian, Palo Alto, CA, USA). The mobile phase was methanol-phosphate buffer (2:1), pH 2.5, 0.01 mol l^{-1}. A flow rate of 1.0 ml min^{-1} was established at a constant temperature of between 23 and 25°C [15].

2.5. Analytical cycle

A 0.5 ml sample aliquot was aspirated from port 2 into the holding coil (Fig. 1). This was then directed towards the waste port (port 4) to clean the system. These steps were repeated when the sample was changed (Fig. 1).

Once the sample tube was filled, 0.12 ml of the content was aspirated towards the reactor (R1) along with 0.12 ml of $1x10^{-3}$ mol l^{-1} permanganate solution (from port 1) (both aspiration rates 30 ml min^{-1}); mixing occurred in reactor 2 (R2). The mixture was transported via port 3 towards the detector at a flow rate of 1.0 ml min^{-1} for 2 min. The signal was recorded at 445 nm.

3. Results and Discussion

3.1. Optimisation of flow system variables

DFC is oxidised in acidic media by several oxidising reagents, such as Ce(IV), BrO_3^- or Fe(III). Some of these reactions have been used in the determination of DFC in pharmaceutical formulations and biological fluids [6.7]. DFC is oxidized by MnO_4^- in alkaline media, and the green product formed (manganate ions) shows an absorption maximum at 445 nm. The proposed mechanism is shown in Figure 2. In this mechanism, the stabilization of the quinone-imide structure depends mainly on the pH of the medium. The colour is stable in alkaline solution but disappears in acidic and neutral media.

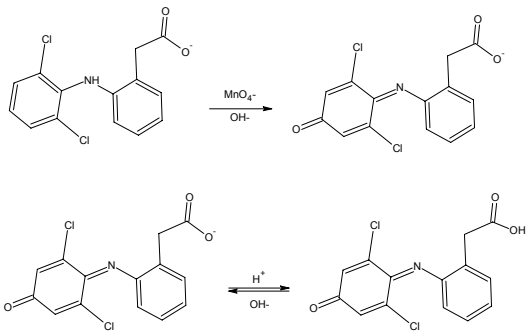

Figure 2. Estimated reaction between diclofenac and permanganate

Several factors affecting the proposed system required optimisation. A TPD was selected as the optimisation method since this provides the necessary information with the minimal experimentation. TPD discriminates between control factors, uncontrollable factors and experimental noise, treating them separately by means of special design matrices (orthogonal arrays) in which the columns (corresponding to factors) and rows (corresponding to trials) are arranged in a conveniently fixed manner. These matrices indicate the combination of factor settings in each experiment and allow the simultaneous evaluation of several variables with the minimum number of trials. The results obtained were analysed statistically to adjust each variable to its optimum. The main advantage of the TPD is that it introduces a noise factor such that the obtained optimum configuration is insensitive to noise and therefore the analytical signals obtained of greater reproducibility.

Optimisation of the system with TPD involves 5 steps, I) identifying the output variable to optimise, II) identifying and selecting factors that affect the system, III) selecting the appropriate orthogonal array and assigning adequate settings to the chosen factors, IV) analysing the data and determining the optimum settings, and V) conducting a confirmatory experiment under the optimal conditions obtained.

In analytical flow techniques, the desired response is the maximum signal height (absorbance, arbitrary units). The flow variables optimised in sequential injection systems are the flow rate of the carrier solution, the aspirated volume (sample and oxidant solutions) and the reactor length from the valve to the detector. The selected orthogonal array must have a number of columns equal to or higher than the number of degrees of freedom of the system; thus an $L_9(3^4)$ array was used. The three settings selected for each factor were chosen bearing in mind preliminary analyses.

The reactor length had to be sufficiently long for the samples and oxidising solution to react and successfully form the green product. The aspirated volumes are critical in flow systems; therefore, those selected had to be large enough to allow the correct dispersion of the sample and oxidising agent. With respect to the flow rate, it has been shown in flow injection analysis that the oxidising reaction of DFC is slow, requiring the use of flow rates of <0.5 ml min^{-1} [12]. In addition, the hydroxylation reaction of DFC preceding the oxidation reaction and the effect of storage time on the DFC standard solution have to be taken into account. The storage of DFC aqueous solutions is reported to be associated with the formation of 4'-hydroxydiclofenac [16]. This compound was therefore selected as a noise factor. Table 1 shows the settings for each control and noise factor used in the optimisation experiments.

Table 1. Control factor settings for the optimisation experiment

Factors		Level		
		1	2	3
Control	Reactor length R2 (cm) **RL**	60.0	90.0	120.0
	Oxidant volume (µl) **OV**	60.0	90.0	120.0
	Sample volume (µl) **SV**	60.0	90.0	120.0
	Flow rate (ml min^{-1}) **Q**	0.50	0.75	1.00
Noise	Storage time (h) **t**	0.0	24.0	36.0

Table 2 shows the factorial design matrix and the absorbance results obtained for each trial. All experiments were performed in triplicate in order to calculate the residual error; the total number of experiments was therefore 81 (9 experiments x 3 noise levels x 3 replicates). Measurements were performed with solutions containing 50.0 mg l^{-1} of DFC held for different storage times to provide three noise factor settings.

Table 2. $L_9(3^4)$ orthogonal array with three levels of noise (time), and absorbance in each case (each value is the mean of three readings).

Exp	Control factors and levels				Absorbance (a.u.)		
	RL	OV	SV	Q	t1*	t2*	t3*
1	1	1	1	1	0.141	0.292	0.282
2	1	2	2	2	0.172	0.165	0.177
3	1	3	3	3	0.591	0.545	0.536
4	2	1	2	3	0.007	0.279	0.340
5	2	2	3	1	0.101	0.219	0.228
6	2	3	1	2	0.013	0.300	0.297
7	3	1	3	2	0.362	0.493	0.465
8	3	2	1	3	0.168	0.210	0.246
9	3	3	2	1	0.013	0.463	0.403

* mean values (n=3)

The results were analysed statistically to adjust each variable to its optimum with the least variability possible. All calculations were made using ANOVA-TM v2.5 software. Table 3 shows the results for these analyses. The values of the variance ratio (*F*) and the critical variance ratio (3.17 for a 95% confidence level) show that all the factors taken into account (control and noise) were critical ($F_{calculated} > F_{critical}$). The factor with the greatest influence on the response was the sample volume, which accounted for 25.41% of the total variance of the results, followed by storage time (20.82%). The contribution of the residual error was 1.11%; this indicates the correct selection of experimental parameters.

Water is not an ideal medium for storing DCF since hydrated DFC species are generated, especially hydroxyl species [16]. Storage time can therefore strongly affect the results obtained with the proposed system. To minimize this effect, all standard and sample solutions were prepared moments before their injection into the flow system.

Figure 3 shows the effects of the control factors on the output variable (the absorbance of the green compound), among which the sample volume is the most important. The combination of settings that allowed the highest output peak was RL_1-OV_3-SV_3-Q_3 (see Fig. 3). The flow rate used to aspirate the solutions (30.0 ml min^{-1}) helps to promote a turbulent flow, which favours the mixing of the sample and reagents. This in turn reduces the required reactor length and increases the flow rate of the carrier solution. This is recommended since it avoids problems associated with pressure drops.

Table 3. Pooled ANOVA for absorbances in Table 2.

Variance source	Variance	Variance ratio (*F*)[a]	Pooled sum of squares	Influence (%)[b]
RL	0.13	448.88	0.26	12.39
OV	0.19	633.75	0.37	17.51
SV	0.27	919.09	0.54	25.41
Q	0.05	178.17	0.10	4.90
t	0.22	753.40	0.44	20.82
Interaction	0.02	81.67	0.38	17.86
Residual	$3.0X10^{-4}$		0.02	1.11
Total	0.03		2.11	100.00

[a] The critical variance ratio for a 95% confidence level is 3.17 (2.54 d.f.)
[b] Contribution is defined as 100 x (pooled sum of squares/total sum of squares

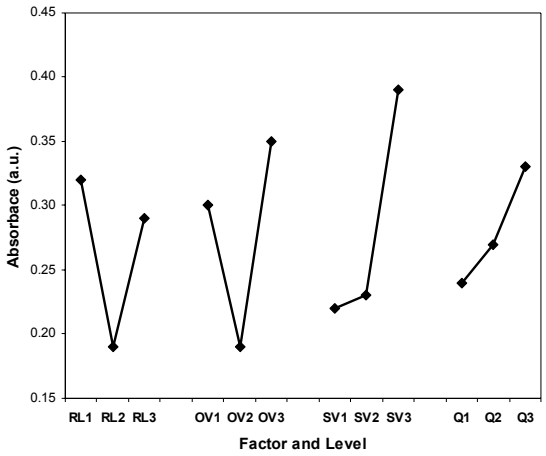

Figure 3. Effect of control factors on the mean DFC values recorded. RL, reactor length; OV, oxidant volume SV, sample volume; Q, flow rate

3.2. Analytical properties of the procedure

Using the SIAgram obtained under optimal conditions (Fig. 4), a standard curve for DFC was constructed using the mean peak height (absorbance) values of each solution. Table 4 shows the regression parameters taken from this standard curve. The limits of detection were calculated according to IUPAC criteria [17], i.e., three times the value of s_e/b_1, where s_e is the square root of the residual variance of the standard curve, and b_1 is the slope. The intermediate precision of the procedure, expressed as the relative standard deviation (RSD), for six determinations (made on different days) using synthetic samples with analyte concentration of 10.0 mg l^{-1} was 1.80%. When synthetic samples with an analyte concentration of 60.0 mg l^{-1} were used, the intermediate precision was 0.75%.

The SIAgram was used to calculate the repeatability of the determinations; the RSD was below 5% for all standard solutions and samples. Under optimum conditions 15 samples per hour can be analysed. The accuracy of the technique was further investigated by determining the recovery of DFC form a spiked urine sample. DFC was determined before and after the addition of different concentrations, and the recoveries calculated. Table 5

shows the results, which indicate good recovery was achieved. The proposed method may therefore be used to determine DFC in urine samples.

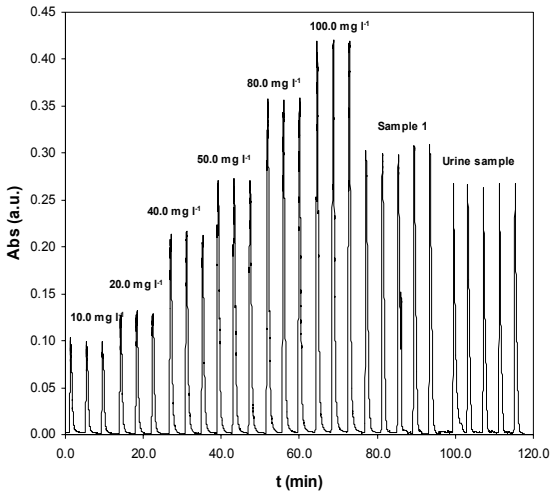

Figure 4. Recorder output obtained with the proposed system, providing a DFC calibration plot between 10 and 100 mg ml^{-1}. The peaks for a tablet and a urine sample are shown.

Table 4. Regression values for the calibration plot (peak height [absorbance] vs DFC concentration [mg l^{-1}]).

Parameter	Value
Square root of residual variance, s_e	0.0067
Intercept confidence interval, $b_0 \pm t\ s(b_0)$	-0.0171±0.0145
Slope confidence interval, $b_1 \pm t\ s(b_1)$	0.0036±0.0002
Linear range (mg l^{-1})	10-100
Detection limit (mg l^{-1})	5.0
Repeatability (%RSD, n=3, 10.0 mg l^{-1})	2.90
Intermediate precision (%RSD, n=3, 60.0 mg l^{-1})	0.75

The effect of the constituent pharmaceutical excipients (sucrose, sorbitol, sodium benzoate, glycerol and calcium phosphate) present in the pharmaceutical samples was studied. Solutions containing 20.0 mg l^{-1} of DCF and the foreign compound at higher concentrations (maximum 100:1) were analysed. The interfering concentration of each compound was considered that which caused a variation in the response greater than or equal to ± 5% compared to the response obtained in its absence. The results showed that, at the concentrations in which they were present in the samples tested, none of these excipients interfered in the determination of DCF.

Table 5 Recovery of DFC in a spiked urine sample. Concentration units are mg l^{-1}. In parenthesis: RSD% for n=5.

Sample	Added concentration	Found concentration	Recovery (%)
Urine	0.0	< L.D.	--
	20.0	21.01 (1.29)	105.1
	50.0	49.61 (0.72)	99.2

3.3. Sample analysis

The proposed method was used to determine DFC in current commercial pharmaceutical formulations (tablets, ampoules and topic gel) and in urine samples from patients prescribed them. Table 6 shows the results obtained. For comparative purposes, DFC in the samples was also determined by the reference method.

For each sample type, the mean DFC concentrations (n=5) obtained with the two methods were compared using the Student t test, assuming comparable variances (confirmed by an F test). The values of $t_{calculated}$ were then compared to a $t_{tabulated}$ value for 4 degrees of freedom and a 95% confidence interval (t=2.78). No significant differences were seen between the results provided by each method.

Table 6. Contents (mean and RSD%, n=5) of diclofenac determined in pharmaceutical formulations and urine by the proposed SIA system, and comparison between concentrations determined by SIA and HPLC. Concentration units (mg $Unit^{-1}$)

Sample	SIA	HPLC	t_{value} [(a)]
Tablet 1	60.6(0.6)	61.2(0.9)	2.19
Tablet 2	50.6(1.5)	49.6(1.5)	2.08
Ampoule	52.79(1.2)	52.2(0.5)	2.09
Topic gel	36.58(1.4)	37.1(0.6)	2.04
Urine	50.9(2.4)	50.8(1.4)	0.12

[a] Calculated Student t-value. Tabulated value 2.31 (p=0.05).

4. Conclusions

The method proposed for the determination of DFC has all the advantages of flow analysis techniques (simplicity, speed and inexpensive equipment), and is just as accurate yet less labour intensive than the standard method of RP-HPLC. It is suitable for the analysis of DCF in pharmaceutical preparations since there is no interference from the excipients they normally contain. The proposed procedure allows DCF to be determined in human urine samples in the physiological concentration range obtained after the administration of the usual therapeutic doses of DCF.

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