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ARTICLES

Determination of Clonazepam in Pharmaceutical Preparations Using Simple High-Throughput Flow Injection System[1]

Hind Hadi* and Marwa Mouayed

Department of Chemistry, College of Science, University of Baghdad, Jadriyah, Baghdad, Iraq

**e-mail: hindhadi13@yahoo.com*

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Abstract—This study was aimed at examining two flow injection-spectrophotometric systems (normal and reverse) for the determination of clonazepam (**CLO**) at the microgram level in pure and pharmaceutical dosage forms. The estimation of CLO has been developed by conjugating a normal (or reverse) flow injection analysis (**nFIA** or **rFIA**) and spectrophotometric detection with phloroglucinol as a coupling reagent. Beer's law was obeyed over a range of 50–400 and 30–400 μg/mL. The limits of detection were 11 and 8 μg/mL and the sampling rates were 51 and 28 samples per hour for nFIA and rFIA respectively. Both systems were successfully applied for the determination of CLO in its commercially available dosage forms. A comparison between the proposed flow systems was also done. These simple and high throughput methods could be utilized for pharmaceutical analysis of CLO.

Keywords: clonazepam, reverse flow injection, phloroglucinol

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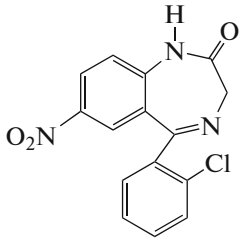
Clonazepam, 5-(O-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one, (Scheme 1) is an anxiolytic, anticonvulsant and muscle relaxant compound. It has been mainly effective in the treatment of typical and atypical absence, myoclonic and kinetic seizures and hence it is used for infantile spasms in the control of epilepsy [1, 2].



Scheme 1. Clonazepam.

Infrared spectroscopy method has been reported for the identification of CLO while potentiometric and HPLC methods were described for the assay of CLO in official pharmacopeias [3–5]. There are several methods for the estimation and determination of CLO in biological fluids and pharmaceutical dosage forms. Among these methods are HPLC [6, 7], electrochemiluminescence sensors [8, 9], voltammetry [10], spectrophotometry [11], gas and liquid chromatography mass spectroscopy [12, 13], and flow injection spectrophotometry [14]. Flow injection analysis method continues to be one of the most popular methods available for rapid trace analysis because it is simple and economical. However, limited information is available on the use of FIA-spectrophotometric methods for the determination of CLO in pharmaceutical analysis. There are few FIA methods available but they have been described as complicated and required a pH control system.

In the present study, we developed a normal and a reverse flow injection methods based on oxidative coupling reaction between CLO and phloroglucinol (**PHG**) in the presence of sodium periodate in a neutral medium. The maximum absorbance of the pink colored product was measured spectrophotometrically at 543 nm. The reaction was carried out in batch and FIA systems and the two approaches were compared.

EXPERIMENTAL

Apparatus. All spectral and absorbance measurements were carried out using a digital double beam spectrophotometer (Shimadzu, UV–Vis 260). A flow cell with 50 μL internal volume and 1 cm bath length was used for measuring the absorbance. A peristaltic pump (Ismatec, Labortechnik Analytik, CH8152, Zurich, Switzerland) was used to transport the solutions through flexible vinyl tubing (0.5 mm i.d.). In addition, an injection valve (Rheodyne, Altex 210, Supelco, USA) was employed to provide appropriate injection volumes of standard solutions and samples.

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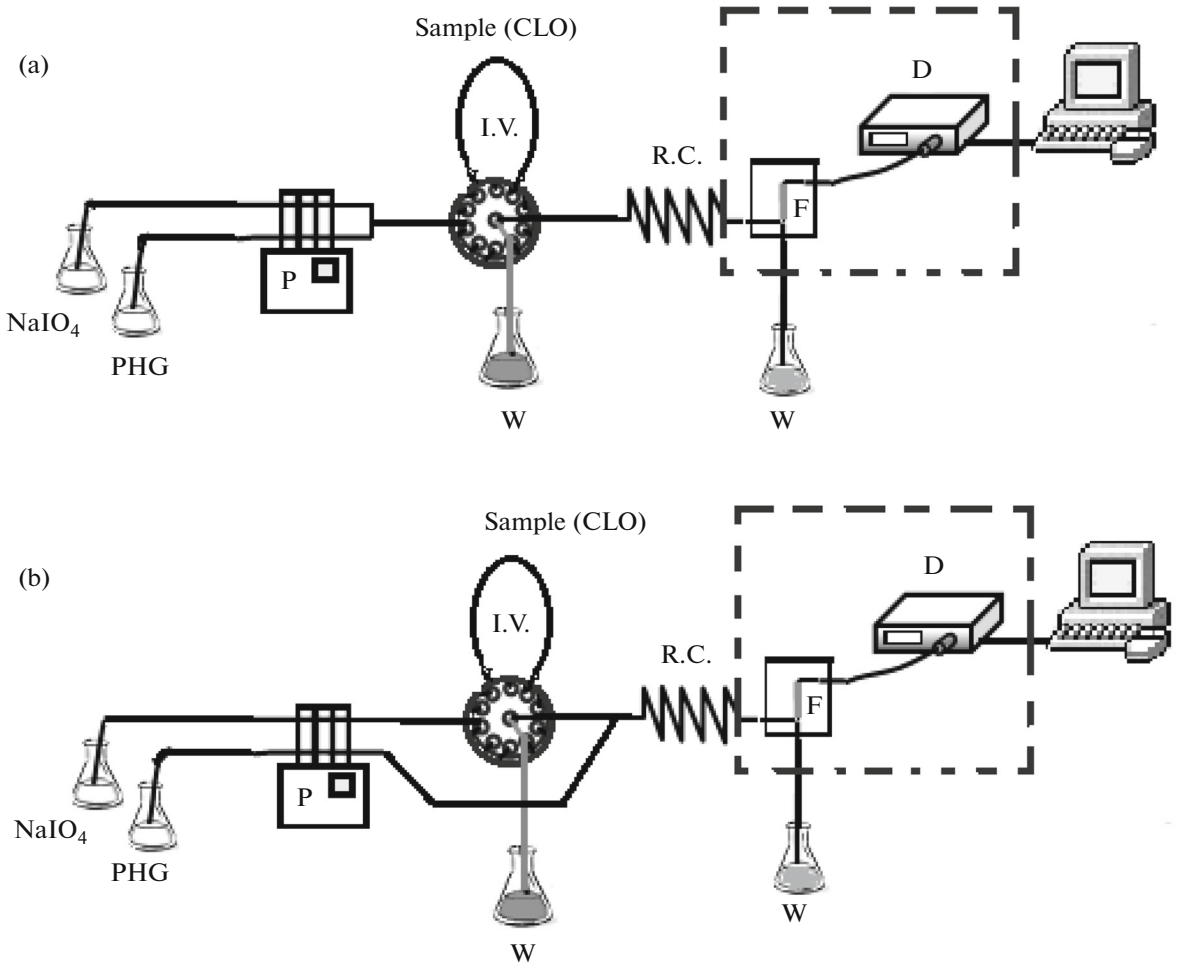


Fig. 1. Manifolds employed for nFIA system (a) and rFIA system (b). Notations: I.V.—injection valve, R.C.—reaction coil, P—peristaltic pump, F—flow cell, D—detector, W—waste.

The injection valve and the flow cell were connected through a teflon reaction coil with an internal diameter of 0.5 mm.

In nFIA, a two-channel manifold (Fig. 1a) was employed for the spectrophotometric determination of CLO. One of these two channels was used to transport PHG while the other one was used to transport sodium periodate. The sample (CLO) was injected into the stream of both reagent solutions through the injection valve and then mixed in the reaction coil. Solutions were propelled by peristaltic pump with individual flow rate of 1.25 mL/min and the absorbance was measured at 543 nm. Similar to nFIA, rFIA manifold (Fig. 1b) had a two-channel manifold design. The reagent (PHG) was injected into the stream of the oxidant solution through the injection valve which was then combined using a Y-shape link with the stream of CLO and mixed in the reaction coil. Finally, the solutions were propelled by the peristaltic pump and the absorbance was measured at 534 nm.

Reagents. The analytical reagent grade chemicals and distilled water were used throughout the experiment. Pharmaceutical grade CLO was received from state company for Drug Industries and Medical Appliance, SDI, Samara, Iraq. The following formulations were obtained from local commercial sources and were subjected to analysis:

—Rivotril® 2 mg (Hoffman LaRoche, Switzerland),

—Rivotril® 2 mg (Roche Farma, Madrid, Spain).

CLO reduction solution (500 μg/mL). Reduction solution of CLO was prepared as previously reported [11] by dissolving 0.05 g of CLO in 50 mL of ethanol. This solution was transferred into a 125 mL beaker to which 20 mL of distilled water, 20 mL of concentrated HCl (11.64 M), and 3 g of zinc powder were added. In order to complete the reduction process, the beaker was allowed to stand for 15 min at room temperature (25°C). Then the solution was filtered into a 100 mL volumetric flask and the residue was washed with distilled water. Finally, the volume was diluted to the

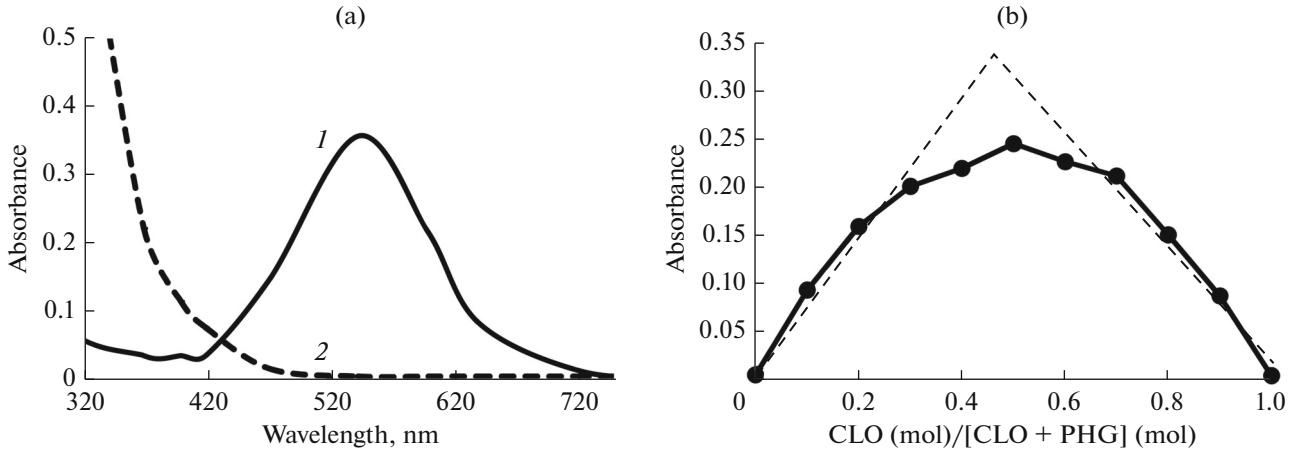


Fig. 2. (a) The absorption spectra of 40 μg/mL of CLO measured against the reagent blank (*1*) and the reagent blank measured against distilled water (*2*); (b) study of the mole ratio of the reaction between CLO and PHG (0.001 M each).

mark with distilled water to obtain a concentration of 500 μg/mL of CLO. More dilute solution was prepared daily using distilled water.

PHG solutions (0.005 and 0.1 M). These solutions were freshly prepared by dissolving 0.0631 and 1.2611 g of PHG (British Drug Houses, UK) and diluting to 100 mL with distilled water in volumetric flasks and then transferred into brown bottles.

Solutions of pharmaceutical tablets. Twenty five tablets of commercial CLO (Rivotril® 2 mg) were accurately weighted and finely powdered. Then an amount of the powder equivalent to 50 mg of CLO was dissolved in 30 mL of ethanol. The solution was filtered into a 50 mL volumetric flask, the residue was washed with ethanol and finally the volume was diluted to the mark with the same solvent to obtain a concentration of 1000 μg/mL of CLO. This solution was transferred into a 125 mL beaker and was subjected to a reduction reaction as described above. Further appropriate solutions of pharmaceutical tablets were made using distilled water.

General procedures. *Batch procedure.* An increasing amount of the sample (100–1125 μg of reduced CLO) was transferred into a series of 25 mL standard flasks. 3 mL of PHG solution (5 mM) and 0.1 mL of $NaIO_4$ solution (100 mM) were added. The contents of the flasks were diluted to the 25 mL mark with distilled water, mixed well and left for 20 min. The absorbance was measured at 543 nm at room temperature (25°C) against reagent blank containing all compounds except reduced CLO. A calibration graph and a regression equation were obtained. A solution of 750 μg of CLO in a 25 mL final volume (i.e. 30 μg/mL) was used for the optimization of all conditions in subsequent experiments.

nFIA procedure. A series of reducing solutions of CLO in a concentration ranging from 50 to 400 μg/mL were prepared from the stock solution. A volume of 200 μL of CLO was injected into the stream of a mixture solution of $NaIO_4$ (50 mM) and PHG (100 mM) at a flow rate of 1.25 mL/min in each channel (Fig. 1a). The result was a pink dye product, the absorbance of which was measured at 543 nm. The optimization of the conditions for this reaction was carried out using 200 μg/mL of CLO.

rFIA procedure. A 200 μL portion of PHG (30 mM) was injected into the stream of $NaIO_4$ (70 mM). This mixture was then combined with a stream of reduced CLO solution prepared at a range of 30 to 400 μg/mL at a flow rate of 0.38 mL/min (Fig. 1b). After mixing in the reaction coil, a pink dye product was formed and the absorbance was measured at 534 nm. Similar to what is mentioned above, the optimization of the conditions of this reaction was carried out using 200 μg/mL of CLO.

RESULTS AND DISCUSSION

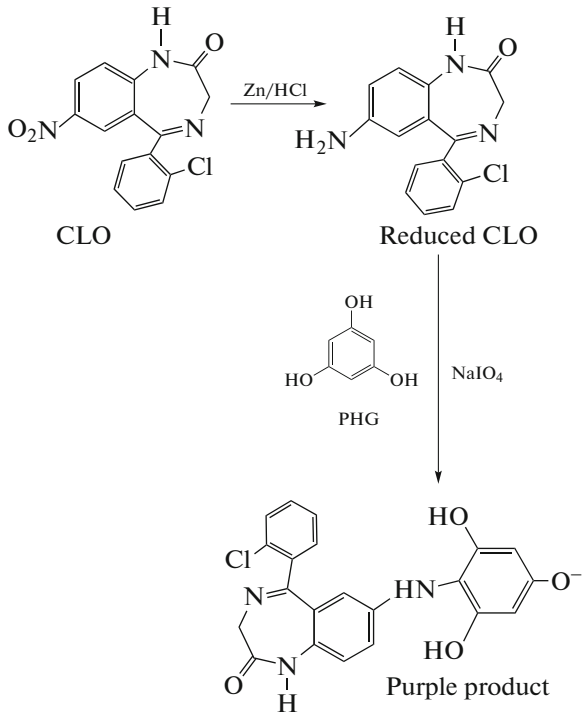
Batch spectrophotometric method. All the variables that may affect sensitivity and stability of the color product were studied and optimized. Optimum conditions were obtained by changing one parameter at a time and keeping the others fixed with observing the effect produced on the absorbance of the colored species. An intensively pink-colored product, with a maximum absorbance at 543 nm with a molar absorption coefficient of 2778 L/mol cm, was formed immediately after adding PHG and the oxidant to CLO solution. The absorption spectra of the colored product and the blank are shown in Fig. 2a.

Different orders of the addition of reagents were studied. One order (CLO + PHG + $NaIO_4$) was used in all following experiments and was found to be efficient in producing the results. In order to understand the mechanism of the reaction, the stoichiometry of the reaction between each CLO and PHG was investi-

Table 1. Analytical features of the procedures developed for the determination of CLO

Parameter	Batch	nFIA	rFIA
Regression equation	$y = 0.0088x - 0.0097$	$y = 0.0048x - 0.2163$	$y = 0.0056x - 0.1585$
Molar absorption coefficient, L/(mol cm)	2778	1515	1768
Linear range, μg/mL	4–45	50–400	30–400
Correlation coefficient	0.9972	0.9994	0.9991
Limit of detection ($S/N = 3$), μg/mL	0.7	11	8
Recovery, %	101.98	100.37	100.30
Reproducibility, %	<3.2	<1.3	<0.6
Throughput , h^{-1}	3	51	28

gated under the recommended optimum conditions by Job's method [15]. The results showed that a one to one (CLO : PHG) ratio product is formed (Fig. 2b). The reduced form of CLO, by virtue of its strong electron donating ability, couples with PHG (oxidized by sodium periodate) and results in the formation of an oxidative coupling product, as shown in Scheme 2.



Scheme 2. Proposed mechanism of the reaction between CLO and PHG.

The conditional stability constant of the pink colored product was calculated from the continuous variation data using the following equation [16]:

$$K_f = \frac{A/A_m}{\left(\frac{1-A}{A_m}\right)^{n+1} c^n n^n},$$

where A and A_m are the maximum absorbance of the continuous variation curve and the absorbance corresponding to junction of the two tangents of the continuous variation curve respectively (Fig. 2a); n is the number of molecules of the reagent in the reaction product (the stoichiometric constant); c is the molar concentration of CLO at the maximum absorbance. The conditional stability constant (K_f) in this study was found to be equal to 7.47×10^2 L/mol which indicates a stable reaction product. The Gibbs free energy of the reaction ΔG was also calculated using the following equation:

$$\Delta G = -2.303RT \log K_f$$

where R is the universal gas constant (8.314 J/mol deg), T is the absolute temperature (273 + 25 K), K_f is the formation constant of the complex.

The value of ΔG in the present study was found to be −16.395 kJ/mol. The pink product appeared directly in neutral medium after direct reaction between reduced CLO and PHG in the presence of sodium periodate. Therefore, there was no need to add any acids or bases. The optimum experimental conditions for the determination of CLO were established for PHG (0.5–3.5 mL of 5 mM) and $NaIO_4$ (0.05–2.5 mL of 0.1 M) by varying one variable at a time and measuring the absorbance at 543 nm. The results indicated that a volume of 3 mL of PHG and 0.1 mL of $NaIO_4$ gave the highest intensity and stability of the dye. The color product becomes stable after 20 min and the absorbance remains constant for more than 120 min. Temperature has a great effect on some spectrophotometric methods, therefore the proposed reaction was studied under different temperatures and the results showed that a high absorbance was obtained when the color is developed at an ambient temperature (25°C) than when exposure to low (0°C) or high (60°C) temperatures. All analytical figures for the batch procedure and the comparison with other methods are summarized in Table 1.

nFIA and rFIA spectrophotometric methods. Two kinds of FIA (nFIA and rFIA) used two different systems for automation of the previously proposed batch method. The manifold is the core of the flow system

Table 2. Optimum conditions for the determination of CLO using two types of FI-systems

Variable	Studied range	Optimum values	
		nFIA	rFIA
Concentration of PHG, mM	10–150	100	30
Concentration of $NaIO_4$, mM	30–200	50	70
Reaction coil, cm	50–250	100	50
Total flow rate, mL/min	0.75–15	2.5	0.75
Sample volume, μL	100–250	200	200

and therefore different reaction manifolds were utilized to conduct different paths of reactions for both types of FIA methods. By using preliminary established conditions, the results obtained showed that the manifolds (a) and (b) in Fig. 1 gave the best absorbance for nFIA and rFIA respectively and were therefore chosen for further use. The influences of different physical or chemical parameters on the intensity of the colored product were also optimized (each sample was injected three times and the average absorbance was calculated).

Optimization of the chemicals variables. In order to compare between the two systems (nFIA and rFIA), the variables of both methods were optimized based on a univariate experimental design. Various concentrations of PHG in the range of 10 to 150 mM were investigated. PHG concentration of 100 mM in nFIA compared to 30 mM in rFIA was found to give the highest absorbance (Fig. 3a). Furthermore, the amount of the oxidant was optimized using different concentrations of $NaIO_4$ (30–200 mM) in both methods. It was found that the greatest absorbance intensity was obtained at 50 and 70 mM of $NaIO_4$ for nFIA and rFIA, respectively (Fig. 3b). The result showed a gradually increase in the absorbance of the blank or the sample in nFIA and rFIA systems which was parallel to the increase in the concentration of PHG and $NaIO_4$. However, the net absorbance (subtracting the absorbance of blank from the sample absorbance) was decreased slightly when the reagent concentration was greater than the optimum value.

Optimization of manifold parameters. The current study showed that a flow rate of 2.5 and 0.75 mL/min gave the highest absorbance for nFIA and rFIA, respectively (Fig. 3c) and were used in all subsequent experiments. The sensitivity and absorbance decreased with increased flow rate because at higher flow rate, the dispersion increases, which means a decreased residence time and increased reagent consumption. To improve the sensitivity and increase mixing of the reactants, different lengths of the reaction coils (50–250 cm) were examined. A coil length of 100 and 50 cm gave the highest absorbance for nFIA and rFIA, respectively (Fig. 3d) and was used in all subsequent experiments. By varying the injection volume from 100 to 250 μL, the highest sensitivity was obtained at 200 μL for both methods (Fig. 3e).

A standard calibration graph, obtained from a series of CLO standards and the main analytical figures of merits of the developed procedures are compared in Table 1. All the optimum values of the studied variables are summarized in Table 2.

Accuracy, reproducibility and interferences. Under the optimum conditions, the accuracy and reproducibility of the proposed methods for the determination of CLO using batch, nFIA and rFIA methods were evaluated. The recovery and replicate analysis of three different concentrations of the standard solution of CLO were determined. All the proposed methods gave acceptable results where by RSD (%) did not exceed 3 (1.4 for batch and 0.6 for nFIA and rFIA) and with good recoveries. Table 3 shows *E*, Recovery, and RSD of the three proposed methods. Recovery testing was checked the freedom of the procedure from interference by the tablet excipients. The common excipients used in the tablets were studied by analyzing synthetic sample solutions containing CLO in the presence of 10-fold concentration excess of each excipient. The recovery values indicated that there were no interferences in the determination of CLO in the presence of the excipients (Table 4).

Pharmaceutical applications. Solutions of pharmaceutical preparations were prepared as mentioned in the previous sections. The proposed methods were applied successfully for the determination of CLO in tablets and by injecting of three different concentrations of each sample in nFIA (or ejection three different concentrations of drug in rFIA) using optimum conditions (Table 2). The results obtained are summa-

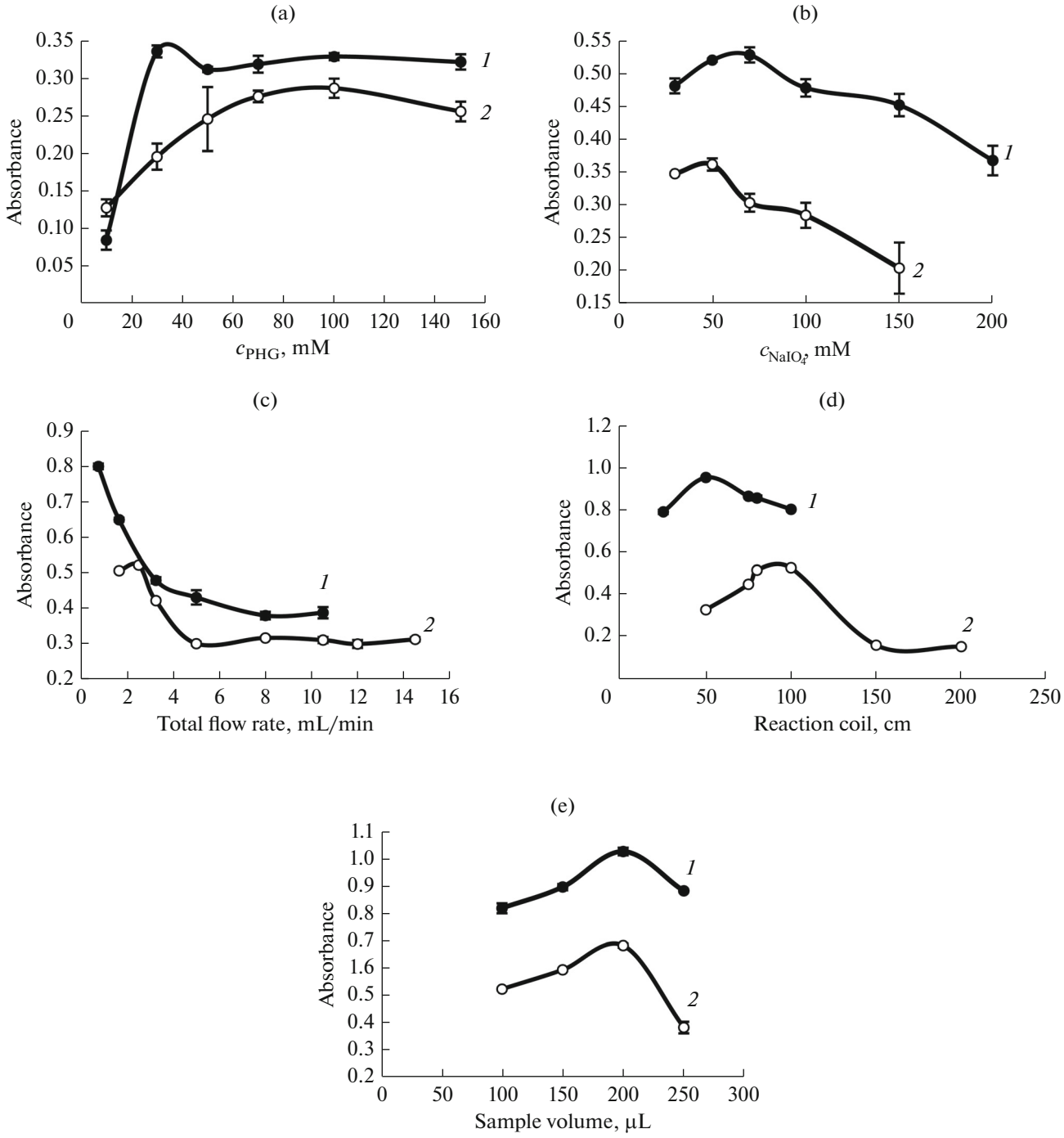


Fig. 3. Effect of chemical and physical parameters on the reaction between CLO and PHG (explanations in the text): *1*—rFIA, *2*—nFIA.

rized in Table 5 and compared with those obtained by standard methods [4].

The results obtained by the three different methods were statistically compared, using Student's *t*-test and variance ratio *F*-test at 95% confidence level. In all cases, the calculated *t*- and *F*-values (Table 5) did not exceed the theoretical values, which indicate that there was no significant difference between either methods in accuracy and precision.

* * *

The current study proved that batch and FIA-spectrophotometric systems (normal and reverse) are sim-

Table 3. Accuracy and precision of the proposed methods

Batch				nFIA				rFIA			
added, μg/mL	found, μg/mL	recovery, %*	RSD, %*	added, μg/mL	found, μg/mL	recovery, %*	RSD, %*	added, μg/mL	found, μg/mL	recovery, %*	RSD, %*
10	10.1	102.3	2.9	100	101.9	101.9	1.4	200	203.5	101.7	0.4
20	20.9	104.5	3.2	300	299.0	99.7	1.3	300	296.3	98.8	0.3
30	30.1	100.2	1.7	400	397.9	99.5	0.1	400	401.5	100.4	0.6

* Average of five determinations

Table 4. Determination of CLO* in presence of common interferences by batch method

Excipent (300 μg/mL)	Found CLO, μg/mL	Recovery ± SD, %**
Poly vinyl pyrrolidone	30.1	100.2 ± 0.5
Lactose	29.7	99.1 ± 0.5
Starch	30.0	100.0 ± 0.6
Magnesium stearate	31.4	104.7 ± 0.4
Total (contains all previous excipents)	29.5	98.3 ± 0.4

* Present 30 μg/mL of CLO.
** Average of five determinations.

Table 5. Application of proposed and official methods to the determination of CLO in different dosage forms ($n = 5$, $P = 0.95$)

Dosage form	Proposed methods									Official method [4]		
	batch			nFIA			rFIA					
	taken, μg/mL	recovery, %	RSD, %	taken, μg/mL	recovery, %	RSD, %	taken, μg/mL	recovery, %	RSD, %	taken, μg/mL	recovery, %	RSD, %
Rivotril® tablet (2 mg) Hoffman La Roche, Switzerland	20	99.4	2.1	100	99.2	0.9	50	98.6	3.0	20	100.1	2.8
	30	98.3	1.2	200	99.9	0.6	75	101.5	0.9	30	98.8	0.2
	40	99.3	2.4	300	99.7	0.3	150	98.6	0.8	40	96.8	0.1
Rivotril® tablet (2 mg) Roche Farma, S.A-Madrid, Spain	20	100.0	0.9	100	103.9	0.7	50	102.7	3.9	20	96.7	0.4
	30	100.9	0.9	200	100.3	0.7	75	99.9	1.2	30	98.9	0.8
	40	98.3	0.5	300	100.2	0.2	150	100.9	0.6	40	100.8	0.5
t (2.776)*	0.831			1.392			1.291					
F (19.000)*	1.967			1.474			1.772					

* Theoretical value.

ple and cost effective methods for the determination of CLO in pharmaceutical preparations. In comparison to the batch method, FIA procedures are faster (sample throughput of 51 and 28 h^{-1} for nFIA and rFIA, respectively) and have a wider linear range (Table 1). rFIA has many advantages over the other listed methods as it is more sensitive (two times more sensitive than nFIA) and suitable in case of using expensive reagents. Taken together, the methods described proved to be simple, sensitive and precise in analyzing pure and pharmaceutical samples of CLO.

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