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Development of an analytical method for the determination of ciprofloxacin, diclofenac, acetaminophen, and carbamazepine by High-Performance Liquid Chromatography

Desarrollo de un método analítico para la determinación de ciprofloxacino, diclofenaco, paracetamol y carbamazepina por Cromatografía de Líquidos de Alta Resolución

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Technological innovation: Developing a simultaneous chromatographic method capable of quantifying four drugs in an aqueous matrix.

Industrial application area: Monitoring emerging contaminants in water to evaluate the efficiency of wastewater treatment and drinking water plants.

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Resumen

Los medicamentos utilizados en el presente estudio se caracterizan por ser comúnmente usados en México para tratar dolores articulares (diclofenaco), fiebre (paracetamol), infecciones bacterianas (ciprofloxacino) y epilepsia (carbamazepina). La Cromatografía Liquida de Alta Resolución (HPLC, por sus siglas en inglés) ha sido reportados para el análisis de diversos compuestos presentes en fluidos biológicos y ambientales. El objetivo del estudio fue desarrollar un método rápido y sencillo para la determinación de los medicamentos anteriormente mencionados en muestras de agua. Para lo cual se empleó el método de HPLC con detección ultravioleta a 277 nm, fase móvil isocrática y un rango de cuantificación de 1 a 50 $\mu g \cdot mL^{-1}$. Los resultados mostraron tiempos de retención de 6.49, 8.45, 9.56 y 8.56 minutos para el diclofenaco, ciprofloxacino, paracetamol y carbamazepina respectivamente en la misma longitud de onda. La precisión y

exactitud fueron superiores al 95% y el tiempo total de elución fue de 12 minutos por muestra. Se logró desarrollar e implementar un método analítico para la determinación de diclofenaco, ciprofloxacino, paracetamol y carbamazepina en agua, lo suficientemente sensible, exacto y reproductible, con un grado de error menor al 5%, lo cual lo vuelve adecuado para ser usado en posteriores estudios.

Palabras clave: Cromatografía, contaminantes emergentes, fármacos.

Abstract

The drugs used in this study are commonly used in Mexico to treat joint pain (diclofenac), fever (acetaminophen), bacterial infections (ciprofloxacin), and epilepsy (carbamazepine). High-Performance Liquid Chromatography (HPLC) has been reported for the analysis of various compounds present in biological and environmental fluids. The objective of this study was to develop a rapid and simple method for determining the aforementioned medications in water samples. For this purpose, the HPLC method with ultraviolet detection at 277 nm, an isocratic mobile phase, and a quantification range of 1 to 50 $\mu g \cdot mL^{-1}$ was used. The retention times were 6.49, 8.45, 9.56 and 8.56 minutes for diclofenac, ciprofloxacin, acetaminophen, and carbamazepine, respectively, at the same wavelength. Precision and accuracy exceeded 95%, and the total elution time was 12 minutes per sample. An analytical method for the determination of diclofenac, ciprofloxacin, acetaminophen and carbamazepine in water was developed and implemented. This method was sufficiently sensitive, accurate, and reproducible, with a degree of error of less than 5%, making it suitable for use in future studies.

Keywords: Chromatography, emerging pollutants, pharmaceuticals.

1. INTRODUCTION

High-Performance Liquid Chromatography (HPLC) is an analytical method generally used in chemistry, environmental engineering, pharmaceutical applications, and biochemistry, that is based on the separation of semi-volatile and non-volatile compounds from liquid samples [1] by means of a mobile phase that passes under pressure through a column packed with a stationary phase (elution) for its subsequent identification and quantification by translating the signals detected through a chromatography data system (CDS) [2]. Some of the main factors that influence separations by HPLC are: a) the composition of the mobile phase and its interaction with the analyte and the stationary phases, b) the chemical composition of the stationary phase (polarity, charge, and viscosity), c) the temperature, and d) the physicochemical properties of the analyte (size, charge, polarity, and volatility) [3].

Analyte separations by HPLC can be carried out using an isocratic or gradient method. In the isocratic method, separations are carried out using an eluent of uniform composition (A=100%) or a mixture (A:B). In the gradient method, on the other hand, the composition of the eluent changes during the separation process, where the cycle begins with a certain percentage of A versus B, however, these will change over time.

There are multiple HPLC techniques depending on the structure of the analytes as the relative polarity of mobile phase and stationary phase (normal phase chromatography (NP-HPLC) and reverse phase liquid chromatography (RP-HPLC)); the operational pressure (high-performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UH-HPLC)); the type and concentration of the compound to be analyzed (liquid chromatography and mass spectrometry (HPLC-MS), two-dimensional liquid chromatography, dual liquid chromatography, tandem liquid chromatography; among others [4].

For this type of technique, it is essential to consider the retention time, which corresponds to the time elapsed from when the sample is injected into the chromatograph, until the detection of the maximum of the peak [5] and is unique for each compound [6] [7].

The compounds chosen (diclofenac, ciprofloxacin, acetaminophen and carbamazepine) to implement their quantification by HPLC were selected due to their high consumption in the human population, which consequently results in their presence in surface water and wastewater. For instance, some analgesics such as diclofenac can cause environmental issues when they are released in water bodies, generating toxicity in algae and phytoplankton, in addition to harmful effects on the organs of freshwater fish, while carbamazepine (psychiatric medication) is considered recalcitrant in wastewater and can bioaccumulate in aquatic environments and cause neurological, endocrine, and reproductive disorders if it remains in drinking water [8]. On the other hand, exposure of fish embryos to paracetamol can cause alterations in their development, with malformations, changes in swimming behavior and decreased heart rate observed in exposed larvae [9]. Furthermore, antibiotics from the fluoroquinone family, such as ciprofloxacin, can reach terrestrial environments through the excretion of conjugated forms in urine, feces, or through the application of manure to crop fields. In soil, ciprofloxacin can have a negative impact on the health of humans, animals, and terrestrial organisms. Quantification of ciprofloxacin by HPLC may be a useful alternative for determining antibiotics in soil matrices [10] [11]. Since wastewater treatment plants cannot eliminate them completely, and in addition to the practical implications mentioned above, it is relevant to have a simple and reliable simultaneous HPLC quantification method [12].

Therefore, the objective of this work was to develop and validate an HPLC method that allows the simultaneous determination of various drugs in water samples, evaluating validation parameters such as linearity, limits of detection and quantification, precision and accuracy to apply it to the analysis of emerging contaminants in aqueous matrices.

2. MATERIALS AND METHODS

2.1 Chromatographic system

To detect emerging contaminants by qualitative analysis using HPLC, it is common to mix a weak solvent and a strong solvent. The mobile phases were chosen based on their viscosity and boiling point, since lower solvent viscosity requires higher pressure to obtain the desired flow, and lower boiling points will facilitate the removal of the desired compounds from the sample [13].

The proportions of the mobile phases are shown in **Table 1**, while the two mixtures evaluated are mentioned below:

a) Mobile phase (Phase A) HPLC grade water, J.T. Baker brand, and as

organic phase (Phase B) HPLC grade methanol, J.T. Baker brand, was used.

b) Mobile phase (Phase A) HPLC grade water, J.T. Baker brand, acidified with phosphoric acid, J.T. Baker brand, until obtaining a pH=2.4, 3, 4 and 5, and as organic phase (Phase B) HPLC grade acetonitrile, Honeywell brand, was used.

Water was filtered through a 0.45 µm Millipore FHLD filter, while organic solvents were filtered through a 0.45 µm Phenex nylon membrane [14] with a GAST vacuum pump model 3KYY6. The equipment used, as well as the operating conditions, are shown in **Table 1**, while the software was TotalChrom Workstation version 6.3.2.

Table 1. *Chromatographic conditions.*

Instrumental conditions	Specifications
Chromatograph	Perkin Elmer Series 200 UV/VIS Detector, Model 2008/250
Elution	Gradient
Mobile pase	Isocratic
Phase A (%)	60
Phase B (%)	40
Flow rate ($mL \cdot min^{-1}$)	0.3, 0.6 and 0.8
Injection volume (µL)	30
Temperature (°C)	25
Wavelength (nm)	277

2.2 Column

The column used was the ACE-5-C18 with dimensions of 250x4.66 mm and its characteristics are 15.5% carbon content, particle size of 5 µm, pore size of 100 Å, pore volume of 1 $mL \cdot g^{-1}$, surface area equal to 300 $m^2 \cdot g^{-1}$, trace metal content less than 10 ppm, working pH of the mobile phase between 2 and 8 units, pressures less than 4,000 psi (275 bar) and maximum temperatures of 60 °C.

Once the column was finished being used, it was washed with HPLC grade water in the mobile phase A and with methanol in the organic phase in a 90:10 ratio, to later change it to 50:50 and finally to 15:85 to be stored. While, for the regeneration of this, the procedure was in reverse phase with 50 mL of water at 50°C, 50 mL of methanol, 50 mL of acetonitrile and 25 mL of methanol [13].

2.3 Reagents and working solutions

The concentration of the stock solution was 100 $\mu g \cdot mL^{-1}$, for which it was prepared by weighing each drug on an OHAUS analytical balance, model EX324, and then adding HPLC grade water, brand J.T. Baker, and dissolving them in a Cole Parmer ultrasonic bath, model CP505, for 20 minutes according to the methodology cited by Sibinovi et al. (2005) [15]. The specifications of each reagent used are shown in **Table 2**.

The stock solution was refrigerated at 4°C in amber bottles for no more than 15 days to ensure the prevalence of the compounds.

***Table 2**. Reagents used in calibration curves*

Drug	Formula	Brand	Molecular weight ($g\cdot mol^{-1}$)
Acetaminophen	$C_8H_9NO_2$	Sigma-aldrich	151.16
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	Sigma-aldrich	331.34
Carbamazepine	$C_{15}H_{12}N_2O$	Sigma-aldrich	236.27
Diclofenac	$C_{14}H_{10}Cl_2NNaO_2$	Sigma-aldrich	318.13

3 EXPERIMENTAL METHODS

The implementation of the analytical method was experimental; the retention times were given by the particular conditions of each of the compounds (analytes) to be separated. The analysis consisted of detecting the presence of the drugs based on the retention time and comparing their UV-Vis absorption spectra with those of the standards.

3.1 Analytical method

The method was tested to evaluate linearity, accuracy and precision for the quantification of emerging contaminants: acetaminophen, ciprofloxacin, diclofenac and carbamazepine. The concentrations of the method were 1, 5, 10, 20, 25 and 50 $\mu g\cdot mL^{-1}$.

To determine the analytical method's analytical capacity, the detection (LOD) and quantification (LOQ) limits were used. These are parameters that allow determination of the minimum concentration of analyte in a sample that can be detected in an analytical process with an acceptable level of confidence, but not necessarily quantified (LOD) and the minimum concentration of analyte that the equipment can determine (LOQ) [6]. To calculate the LOD and LOQ we use Eq. 1 and Eq. 2 respectively.

$$LoD = 3.3\frac{Sy}{S} \qquad \textbf{\textit{Eq. 1}}$$

$$LoQ = 10\frac{Sy}{S} \qquad \textbf{\textit{Eq. 2}}$$

Where:

Sy, standard deviations of the response at the lower end of the curve

S, slope of the calibration curve

3.2 Sample preparation and calibration curves

From the stock solution, different dilutions were prepared with concentrations of 1, 5, 10, 20, 25 and 50 $\mu g\cdot mL^{-1}$ and dissolved with HPLC grade water, brand J.T. Baker, were subsequently filtered through Corning Incorporated brand syringe filters with a pore size of 0.20 µm. These were prepared daily before use.

3.3 Statistical analysis

For each of the variables to be analyzed, triplicate measurements were performed and the mean ($\bar{x}$) (Eq. 3) was calculated from the values obtained in the measurements, which is the average of the data, and the standard deviation (S) (Eq. 4) which is a measure of dispersion that indicates how far the individual data deviate from the mean, the coefficient of variation (CV) (Eq. 5) which allows us to evaluate the relative dispersion of a set of data in relation to its mean and the percentage of error (Eq. 6).

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \quad \textbf{Eq. 3}$$

$$S = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \quad \textbf{Eq. 4}$$

$$CV = \left(\frac{S}{\bar{x}}\right) * 100 \quad \textbf{Eq. 5}$$

$$\%error = \left(\frac{AV - EV}{AV}\right) * 100 \quad \textbf{Eq. 6}$$

Where:

n, is the total number of data
Σ, is the summation
X_i, is the set of observations
AV, is actual value
EV, is experimental value

To interpret the coefficient of variation, we considered the following ranges:

- Less than 10%: indicates low variability and high homogeneity between the data.
- Between 10% and 20%: represents moderate variability, the sample can be considered relatively homogeneous.
- Between 20% and 30%: shows medium variability, but with some heterogeneity.
- More than 30%: indicates high variability and heterogeneity in the data

The coefficient of variation helps us understand the homogeneity or heterogeneity of the sample. It is a useful tool for comparing the variability between different sets of data, if they have the same unit of measurement [16] [17] [18].

4 RESULTS AND DISCUSSION

Figure 1 you can see some of the materials removed during the filtration process of the dissolved mobile phases. It is possible to see crystals and fibers [19]. Filtration of the mobile phases is considered a fundamental and key step to removing particles that can clog the column and thus increase system pressure. It also helps to reduce background noise and the loss of chromatographic efficiency [20].

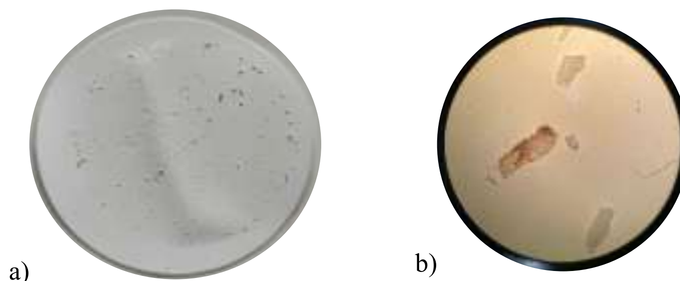

Figure 1. *Particles removed by filtering the mobile phases with a 0.45 µm pore size filter a) with the naked eye and b) with a magnification of 40X.*

The C-18 column is ideal for capturing hydrophobic peptides. Peptides are bound to the reverse phase columns in a high-water mobile phase, salts and buffers are washed away, and peptides are eluted using a high organic mobile phase. Hydrophobic interactions must be overcome to retain the desired compound on the column, so the main solvent options for this are methanol, acetonitrile, and tetrahydrofuran. Strength is transferred to how quickly the analytes will elute with each solvent [21] [22].

4.1 Chromatographic system

Figure 2 shows the chromatogram for each of the drugs analyzed when using water and

methanol (polar organic solvent) as mobile phases with a run time of eight minutes. It can be seen that the appearance times were 1.79, 1.98, 2.31 and 3.53 min for ciprofloxacin, carbamazepine, diclofenac and acetaminophen respectively, while ciprofloxacin and carbamazepine appeared with less than a second of phase lag, which is not desired since the ideal is to correctly show the separation between the compounds to be studied, in addition to the fact that all of these do not show a good definition, especially acetaminophen, whose tail tends to be wider than the others.

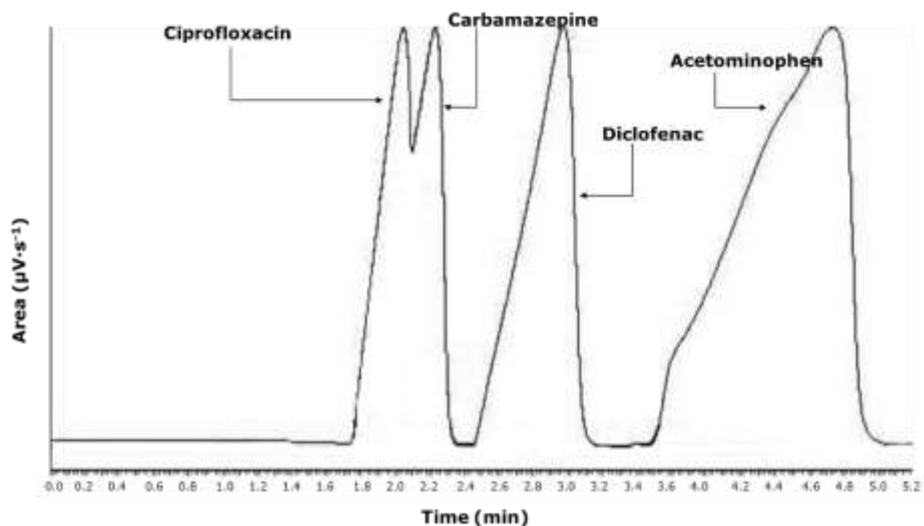

Figure 2. Chromatogram using water and methanol as mobile phase to detect ciprofloxacin, carbamazepine, diclofenac and acetaminophen in water.

To eliminate the low specificity of the method in the detection of contaminants of interest, especially diclofenac and acetaminophen, the mobile phases were changed to acidic water (2.7, 3, 4 and 5 units) and acetonitrile with different run times. Some other mobile phases that have been studied are isopropanol, acetate, chloroform and formic acid (slightly polar solvents).

Table 3 shows the time at peaks detected under different HPLC operating conditions.

Table 3*. Retention times for ciprofloxacin, diclofenac, carbamazepine and acetaminophen under different detection conditions by varying pH and flow rate.*

Compound/ Operating conditions	**Retention times**			
	pH=3 units Flow rate=0.6 $mL{\cdot}min^{-1}$	pH=4 units Flow rate =0.6 $mL{\cdot}min^{-1}$	pH=5 units Flow rate =0.8 $mL{\cdot}min^{-1}$	pH=2.7 units Flow rate =0.8 $mL{\cdot}min^{-1}$
Ciprofloxacin	3.32	3.47	3.55	3.21
Diclofenac	3.00	3.23	3.70	3.25
Carbamazepine	3.50	3.63	3.75	2.85
Acetaminophen	3.75	3.90	4.02	3.90

In **Figure 3** it is possible to observe that all the peaks appeared together in a reasonable time, therefore, to carry out their separation the isocratic elution process was used, which occurs when the composition of the eluent remains constant over time, compared to the

gradient elution method, which is used when the sample peaks are very separated and consists of varying the composition of the eluent over time [23] [24]. In all analyses, the peaks of the compounds were absent when the blanks (HPLC grade water) were injected.

The different appearance times of the four compounds are due to the pKa that each of them has, since when low values are present it is possible that they dissolve better in acidic water and have a more soluble and polar structure, allowing a better interaction with the aqueous medium. The pKa for each of the drugs used are 6.09 (pKa_1) and 8.62 (pKa_2) for ciprofloxacin [25], 0.82-1.43 (pKa_1) and 13.9 (pKa_2) for carbamazepine [26], 9.5 for acetaminophen [25] [27] [28].

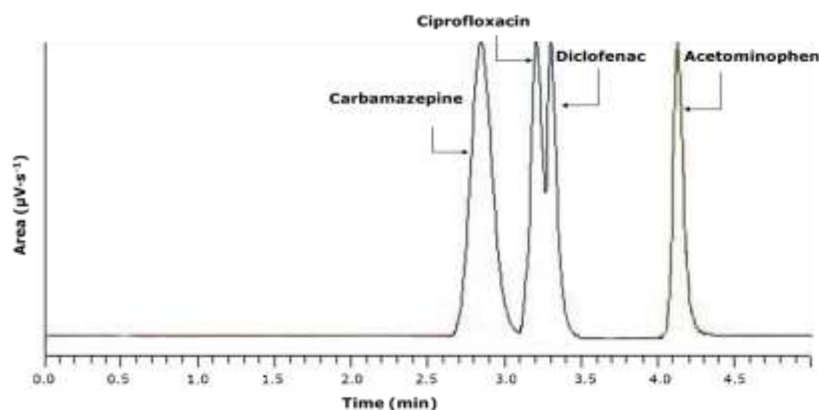

Figure 3. *Chromatogram using acidic water (2.7 units) and acetonitrile as mobile phases to detect ciprofloxacin, carbamazepine, diclofenac and acetaminophen in water with a flow rate of 0.8 mL·min^{-1}.*

To increase the resolution of the chromatogram it is recommended to increase running time. Subsequently, the flow was reduced from 8 mL·min^{-1} to 0.3 mL·min^{-1}, with a run time of 12 min and using acidic water at 2.7 units and acetonitrile (60:40) as mobile phases, during which the appearance of the peaks of the analytes of interest, carbamazepine, ciprofloxacin, diclofenac and acetaminophen, at 8.09, 8.55, 9.86 and 10.59 min respectively, and therefore a good specificity was achieved with the method, which is the ability to differentiate the analyte in the presence of other components of the sample (**Figure 4**).

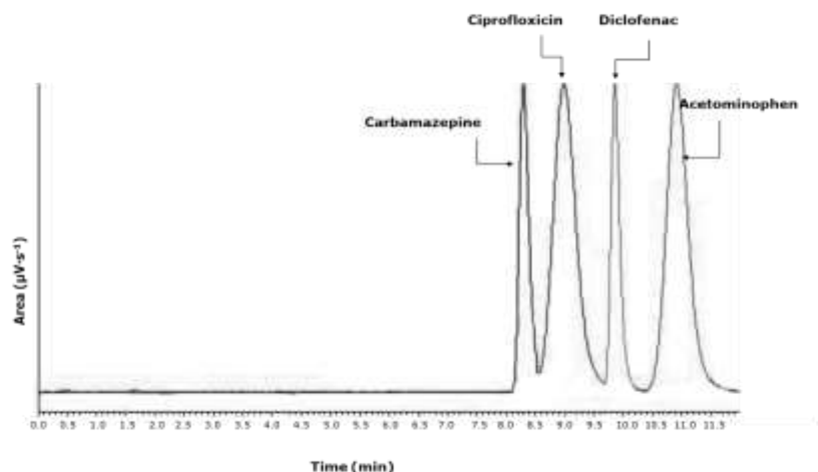

Figure 4. Chromatogram using acidic water (2.7 units) and acetonitrile as mobile phases to detect ciprofloxacin, carbamazepine, diclofenac and acetaminophen in water with a flow rate of 0.3 mL·min^{-1}.

4.2 Validation of methodologies

The parameters for validation are:

Linearity. To establish linearity, at least five points must be considered when constructing the curve [29] [30] [31] [32] [33]. In all analyses, the method was linear in the concentration range between 1 and 50 µg·mL^{-1}, with a correlation coefficient of 0.999. The regression data showed a good linear relationship over the range of concentrations studied, demonstrating the suitability of the method for the analysis (**Figure 5**).

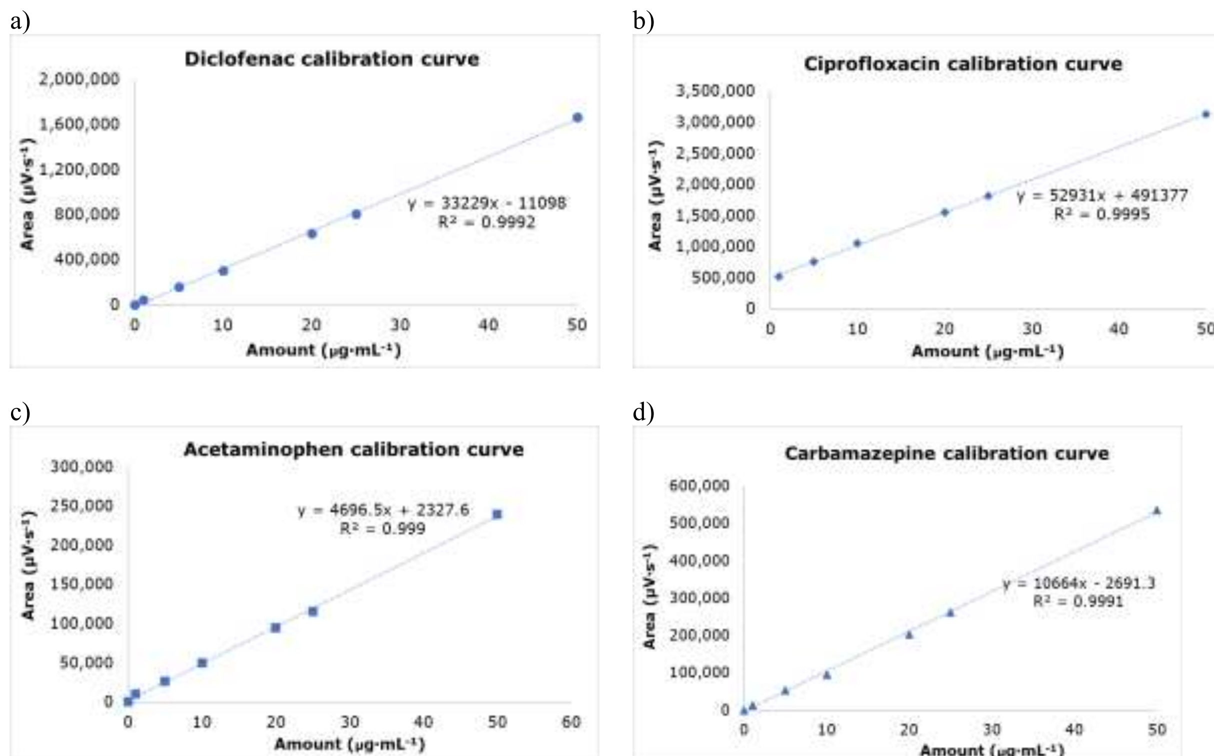

Figure 5. Calibration curve of a) diclofenac, b) ciprofloxacin, c) acetaminophen and d) carbamazepine with a quantification range between 1 and 50 µg·mL^{-1}, all of them in triplicate.

As can be seen, the results of the linear model indicate the correlation between the concentration and the measurement of the area of the peaks obtained from the dilutions with the analytes, in the calibration curve (**Figure 5**), obtaining a statistical R^2 greater than 0.99 in the four drugs.

Precision. Repeatability was carried out at all concentrations; in the statistical analysis, the average values, standard deviation and coefficient of variation between the readings obtained were determined [34] [35].

The precision of the measurements in the analyzed concentrations always had a coefficient of variation less than 5%, while, with respect to accuracy, the percentage of error of the measurements was never greater than 5% (**Table 4**, **Table 5**, **Table 6** and **Table 7**) which indicates that there is very little dispersion between the measurements of the same concentration [36] [37].

Table 4. *Results of the linearity study of the values obtained for Ciprofloxacin in a range between 1 and 50 μg·mL^{-1}*

Ciprofloxacin (μg·mL^{-1})	Peak area (μV·s^{-1})	Average	Standard deviation	Coefficient of variation (%)	% error
1	509,947.65 512,085.00 525,767.88	515,933.51	8,583.60	1.66	4.36
5	780,256.53 725,591.32 760,796.36	755,548.07	27,707.94	3.67	0.02
10	1,065,112.62 1,047,491.97 1,056,520.98	1,056,375.19	8,811.23	0.83	0.67
20	1,544,292.28 1,504,288.94 1,607,024.45	1,551,868.56	51,785.10	3.34	0.02
25	1,724,342.84 1,876,247.60 1,834,704.74	1,811,765.06	78,507.55	4.33	0.02
50	3,278,958.10 3,014,065.08 3,103,206.20	3,132,076.46	134,785.74	4.30	0.02

Table 5. *Results of the linearity study of the values obtained for acetaminophen in a range between 1 and 50 μg·mL^{-1}.*

Acetaminophen (μg·mL^{-1})	Peak area (μV·s^{-1})	Average	Standard deviation	Coefficient of variation (%)	% error
1	9,980.54 10,954.74 10,567.88	10,501.05	440.53	4.67	7.40
5	27,951.22 25,342.73 26,990.77	26,761.57	1,319.26	4.93	0.41
10	50,209.99 49,325.08 50,753.99	50,096.35	721.20	1.44	0.17
20	96,918.39 94,678.09 92,972.06	94,856.18	1,979.18	2.09	0.15

Acetaminophen ($\mu g{\cdot}mL^{-1}$)	**Peak area ($\mu V{\cdot}s^{-1}$)**	**Average**	**Standard deviation**	**Coefficient of variation (%)**	**% error**
25	112,941.53 116,983.26 117,238.09	115,720.96	2,410.43	2.08	0.34
50	233,906.99 239,317.67 244,972.08	239,398.91	5,532.99	2.31	0.10

Table 6. *Results of the linearity study of the values obtained for carbamazepine in a range between 1 and 50 $\mu g{\cdot}mL^{-1}$.*

Carbamazepine ($\mu g{\cdot}mL^{-1}$)	**Peak area ($\mu V{\cdot}s^{-1}$)**	**Average**	**Standard deviation**	**Coefficient of variation (%)**	**% error**
1	13,361.86 12,944.11 13,455.67	13,253.88	272.34	2.05	4.95
5	53,667.36 53,305.48 55,901.00	54,291.28	1,405.75	2.59	0.69
10	92,836.80 98,955.40 96,135.64	95,975.95	3,062.42	3.19	0.75
20	198,782.80 208,867.04 202,734.80	203,461.55	5,081.25	2.50	0.33
25	264,996.41 259,226.03 262,747.78	262,323.41	2,908.50	1.11	0.06
50	522,276.41 555,224.28 528,254.33	535,251.67	17,553.14	3.28	0.09

Table 7. *Results of the linearity study of the values obtained for diclofenac in a range between 1 and 50 $\mu g{\cdot}mL^{-1}$.*

Diclofenac ($\mu g{\cdot}mL^{-1}$)	**Peak area ($\mu V{\cdot}s^{-1}$)**	**Average**	**Standard deviation**	**Coefficient of variation (%)**	**% error**
1	44,166.37 41,552.32 45,979.72	43,899.47	2,225.73	5.07	3.54
5	158,798.53 149,641.34 156,539.13	154,993.00	4,770.37	3.08	0.60
10	317,574.46 287,398.15 301,401.72	302,124.78	15,101.14	5.00	0.87
20	632,748.83 628,446.50 642,398.36	634,531.23	7,144.67	1.13	0.44
25	816,444.96 805,943.51 800,279.33	807,555.93	8,202.55	1.02	0.27
50	1,607,224.98 1,656,730.91 1,738,078.21	1,667,344.70	66,069.14	3.96	0.04

The values obtained for LOD and LOQ were 0.34 $\mu g{\cdot}mL^{-1}$ and 0.94 $\mu g{\cdot}mL^{-1}$ for acetaminophen, 0.54 $\mu g{\cdot}mL^{-1}$ and 1.62 $\mu g{\cdot}mL^{-1}$ for ciprofloxacin, 0.22 $\mu g{\cdot}mL^{-1}$ and 0.67 $\mu g{\cdot}mL^{-1}$ for diclofenac, and 0.08 $\mu g{\cdot}mL^{-1}$ and 0.26 $\mu g{\cdot}mL^{-1}$ for carbamazepine respectively.

The LOQ obtained for ciprofloxacin was higher than the lowest concentration level of the calibration curve, although experimentally no significant dispersion was observed at the lowest level, it is important to consider the statistical approach, since this guarantees compliance with the precision and accuracy criteria required for quantification. The HPLC method can be considered acceptable because it meets the validation parameters (linearity, precision, accuracy) established in the methodology.

5 CONCLUSIONS

An analytical method was implemented to analyze four drugs in water simultaneously; these were ciprofloxacin, diclofenac, acetaminophen and carbamazepine, giving coefficients of variation and percentages of error less than 5%, which indicates that there are a low variability and high homogeneity between the data. In addition, determination coefficients (R2) close to 1 (>0.99) were obtained.

Although the method produced good results for the determination of four emerging contaminants in a synthetic water sample, however, working with wastewater involves a more complex matrix, so the results can vary and be affected by interferences present in it, such as organic matter, nutrients, metals, among others.

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