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A Validated HPLC Multichannel DAD Method for the Simultaneous Determination of Amoxicillin and Doxycycline in Pharmaceutical Formulations and Wastewater Samples

Anca Becze [1], Maria-Alexandra Resz [1], Aranka Ilea [2] and Oana Cadar [1,*]

[1] INCDO-INOE 2000, Research Institute for Analytical Instrumentation, 67 Donath Street, 400293 Cluj-Napoca, Romania
[2] Department of Oral Rehabilitation, Faculty of Dentistry, Iuliu Haţieganu University of Medicine and Pharmacy, 400012 Cluj-Napoca, Romania
* Correspondence: oana.cadar@icia.ro

Abstract: The quality of marketed pharmaceutical formulations must be guaranteed to attain better remedial effects and lower toxicity. The wide exploitation of antibiotics may lead to their presence as residues in body fluids and wastewaters, potentially toxic to human health. Consequently, determining antibiotics in pharmaceutical formulations and water samples is of significant importance. This paper aims to explore the possibilities of a high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) method to obtain a simple, fast, and efficient analytical tool for the simultaneous determination of antibiotics in pharmaceutical formulations and environmental samples. The method was completely validated with regard to specificity, linearity, detection and quantification limits, precision, accuracy, and robustness according to the requirements of existing guidelines, and was proven to be reliable and suitable for the envisioned application. The linearity study was conducted for the calibration curves in the range of 10–100 µg/mL. The limits of detection and quantification were found to be 0.2 and 0.7 µg/mL for amoxicillin and 0.3 and 1.0 µg/mL for doxycycline, respectively. The high recovery of drugs from their commercial pharmaceutical formulations (93%) and from wastewater samples (98%) indicated good accuracy and precision. The method is robust for small or deliberate changes to the chromatographic parameters, and it was successfully applied for the quantitative determination of amoxicillin and doxycycline in wastewater and commercial tablets. The obtained results proved that the validated method is appropriate for its intended use in the routine quality control and assay of both antibiotics studied.

Keywords: amoxicillin; doxycycline; HPLC; DAD; validation; tablets; wastewater

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1. Introduction

Antibiotics used in human and animal therapy has been one of the leading scientific achievements of the last century that revolutionized the treatment of infectious diseases worldwide [1]. These substances are known to kill or inhibit the growth of microorganisms at very low concentrations. In this context, quantifying the antibiotics in pharmaceutical formulations is extremely important for quality control and quality assurance [2].

In recent years, many studies have reported the existence of antibiotics in the environment, these drugs being released through diverse paths, such as discharges from wastewater treatment plant effluents to surface water, where hospitals and private households have significant contributions [3]. Consequently, their occurrence and persistence in the environment, and harmful and deleterious impact, has attracted significant attention [1,4]. Therefore, monitoring antibiotics that reach the environment, even at very low concentrations, is necessary.

Amoxicillin (Amox) and doxycycline (Dox) (Figure 1) are some of the most prevalent antibiotics prescribed in dental practice due to their broad range of activity and low

cost [1,4,5]. Amox, which belongs to the β-lactam antibiotic class of high pharmacological, clinical and economical status, is one of the most commonly used to treat various conditions and patient groups against Gram-positive and Gram-negative bacteria [5,6]. Amox has better absorption from the intestinal tract, higher capacity for achieving effective concentrations at the receptors, and an enhanced ability to penetrate Gram-negative microorganisms' cellular wall compared to other β-lactam antibiotics [7].

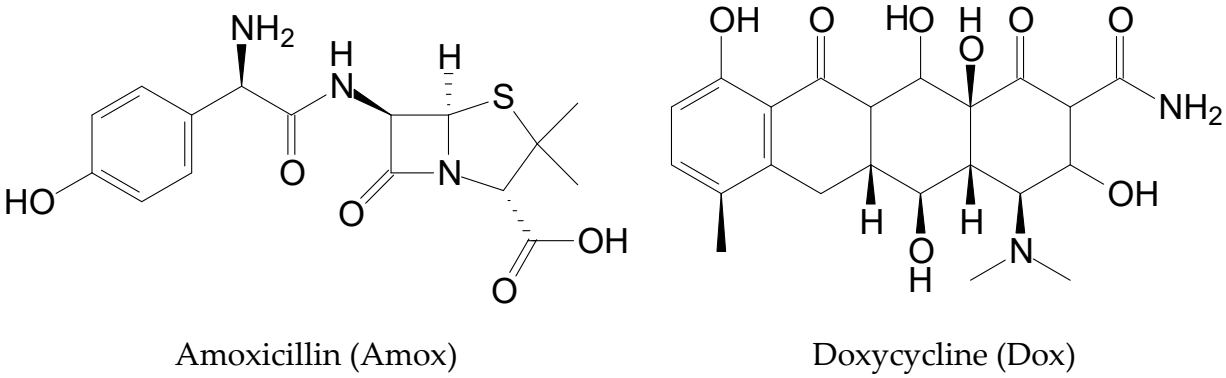


Figure 1. Chemical structure of amoxicillin (Amox) and doxycycline (Dox).

The literature depicts various analytical approaches for the quantitative determination of Amox in commercial drugs and wastewater samples, namely spectrophotometry [8,9], voltammetry [10,11], fluorimetry [12,13], microbiological methods [14], capillary electrophoresis [15,16], and high-performance liquid chromatography (HPLC) [2,6,17–22].

Dox is a broad-spectrum antibiotic belonging to the tetracycline class, usually used in the management and treatment of various infections caused by Gram-positive and Gram-negative bacteria, rickettsiae, chlamydiae, spirochetes, mycoplasmas, and protozoans [23,24]. Compared with other tetracyclines, doxycycline has a better absorption profile, longer half-life, safety profile, and excellent efficacity for susceptible microorganisms, even at lower concentrations [25,26]. Doxycycline monohydrate and doxycycline hyclate are equally effective, but doxycycline hyclate is more frequently uwatersed in pharmaceutical samples due to its good water solubility [24].

Many efforts have been dedicated to the quantitative determination of Dox in both commercial pharmaceutical forms and wastewater samples, such as spectrophotometry [27–29], capillary electrophoresis [30], micellar electrokinetic chromatography [31], and HPLC [32–37].

HPLC is a valuable tool in the quantification of antibiotics in various types of samples due to its simplicity, speed, low cost, and applicability to various analyte types. Moreover, HPLC has been renowned as a robust separation technique with high selectivity [20]. Unfortunately, several HPLC methods validated for the simultaneous determination of different antibiotics in the same sample suffer from several drawbacks, such as the need for extensive runtime or large sample volumes. Moreover, in some of these methods, the mobile phases following the antibiotic need to be analyzed [38].

In recent years, HPLC-DAD (Diode Array Detector) has been demonstrated to be a fast, reliable, and highly reproducible method for a drug screen for clinical research and forensic toxicological applications, where the ruggedness and the fairly simple sample preparation are impetuously required [39]. Therefore, as an option to the existing methods, this work is focused on the optimization and validation of an inexpensive, useful, and simple HPLC coupled with DAD method for the simultaneous determination of Amox and Dox in pharmaceutical formulations and wastewater with reliable accuracy, linearity, precision, and reproducibility. The method is customized by a simple sample preparation protocol and identical chromatographic conditions, namely stationary and mobile phases, applied to both of the investigated compounds. These characteristics make the designed method particularly appropriate for performing several daily measurements. Moreover, this method is applicable to small sample volumes. Consequently, this paper is essential

for quality control analytical laboratories dealing with determining Amox and Dox in pharmaceutical formulations and wastewater using HPLC since it presents a simultaneous and fully validated method for this purpose. This method can easily and conveniently be implemented for the routine quality control analysis of Amox and Dox in pharmaceutical forms and water samples.

2. Results and Discussion

The multichannel DAD HL uses provides (*i*) an ultra-wide dynamic range, ideal for the simultaneous detection of major compounds and trace level impurities; (*ii*) data acquisition up to 200 Hz using up to 10 absorption channels; (*iii*) one spectral field delivers the best support of ultrafast separations; and (*iv*) multichannel data acquisition that permits the simultaneous determination of compounds with different optimal wavelengths of detection [40]. In this context, one run of DAD analysis guarantees, besides the analysis efficiency, a complex evaluation of low amounts of sample and a low cost and rapid analysis.

2.1. Method Validation

In order to ensure the best chromatographic parameters and low detection and quantification limits, different wavelengths were chosen for each antibiotic, considering the full advantage of the multichannel capacity of the DAD detector. The multichannel DAD detector allows for the optimal detection and quantification of compounds that would be impossible to be analyzed in the same run over single wavelength detection. The optimal wavelength for Amox is 350 nm, ensuring a LOQ of 0.7 µg/mL. The peak corresponding to Dox is visible at this wavelength, but the LOQ is more than 20 times higher than that obtained at the optimal wavelength of Dox (230 nm). Therefore, an intermediate wavelength should have been chosen for an even response from bought compounds in the same run on a single wavelength, resulting in a method with lower chromatographic parameters, and a higher limit of detection (LOD) and limit of quantification (LOQ). Consequently, two different wavelengths (230 and 350 nm) were selected to monitor Amox (retention time at 1.70 min) and Dox (retention time at 5.23 min) (Figure 2).

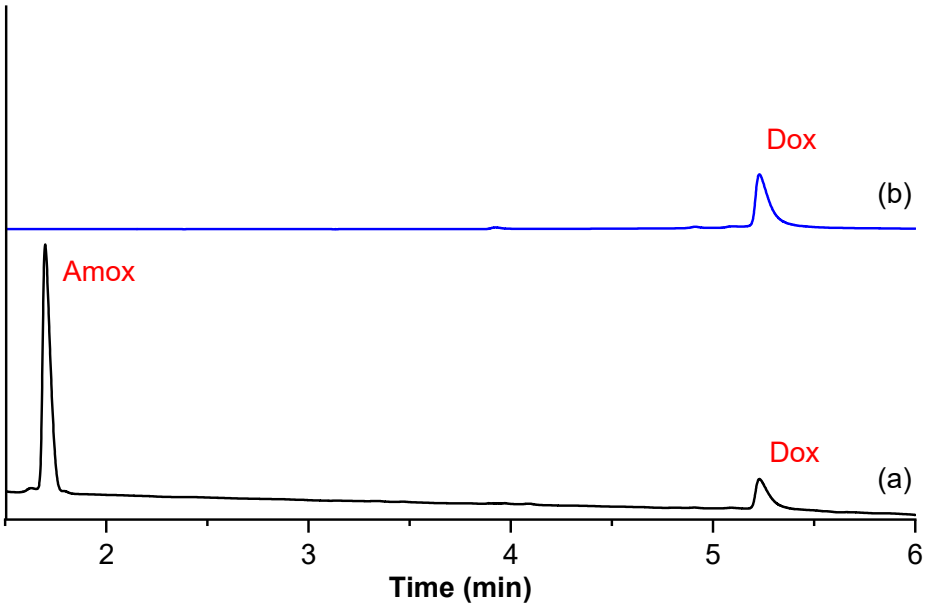


Figure 2. HPLC chromatograms of Amox and Dox recorded at two different wavelengths: (**a**) 350 nm and (**b**) 230 nm, at 100 µg/mL.

The proposed method for the simultaneous determination of Amox and Dox in pharmaceutical formulations and wastewater samples was validated relating to the conformity

of the chromatographic parameters (tailing factor, resolution, peak symmetry, theoretical plates), LOD, LOQ, working and linear ranges, precision, and recovery, according to current guidelines and previously published papers [41–47].

2.1.1. Chromatographic Parameters

To evaluate the suitability of the HPLC-DAD system according to European Pharmacopeia [45], the retention factor, peak asymmetry, resolution, specificity, and number of theoretical plates were calculated using the Chromeleon 7 Chromatography Data System (Thermo Fisher Scientific, Waltham, MA, USA) Software Version 7.3 (Table 1). The resolution should be >2 between the peak of interest and the closest eluting possible interference, such as impurity, related substance, degradation product, excipient, and internal standard, while the theoretical plates should be >2000 [48]. The high number of theoretical plates (N > 11,500) confirmed the high column efficiency [48]. The asymmetry factors close to 1 indicated a small tailing but precise quantification measurements, according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) ($T \leq 2$) [45,48].

Table 1. Parameters of the HPLC-DAD Method for the Simultaneous Determination of Amox and Dox.

Parameter	Amox	Dox
retention time (min)	1.72	5.23
RSD of retention time (%)	0.65	0.58
asymmetry factor	1.31	1.52
resolution	1.92	6.43
theoretical plates	11,558	26,328

2.1.2. Specificity

Specificity is defined as the capability of the method to differentiate between the analyte and other components present in the sample matrix [49]. It allows for the unequivocal assessment of the analyte in a sample. Specificity evaluation was carried out by injecting blank matrix samples into the HPLC system [46,49,50]. No interfering matrix peaks were encountered in the blank samples, demonstrating that the excipients used did not interfere, indicating specificity of the method. The excipients used for the preparation of the physical mixture used as a blank sample in the case capsules were corn starch, monohydrated lactose, microcrystalline cellulose, polyvinyl-pyrrolidone and talcum (Merck, Darmstadt, Germany) and magnesium stearate, titanium dioxide (Sigma Aldrich, Taufkirchen, Germany).

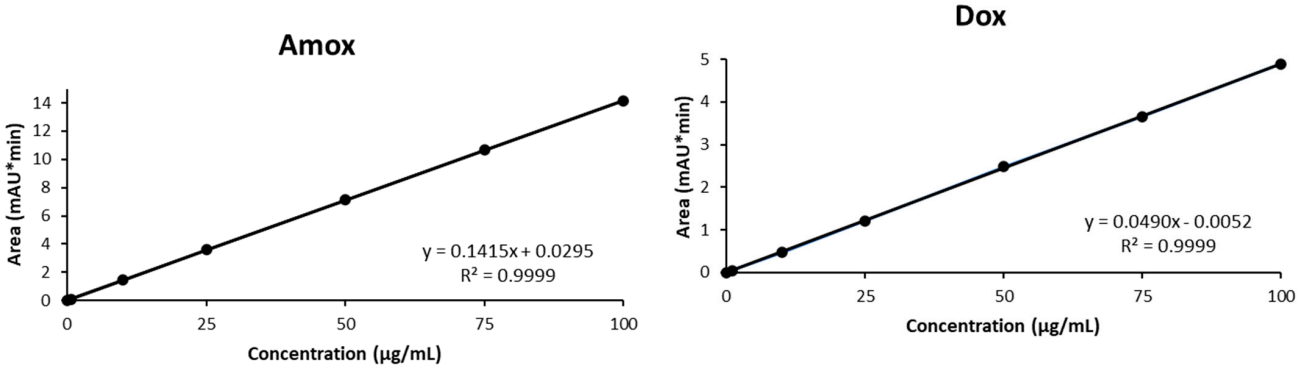
2.1.3. Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Calibration is a common and important step in analytical methods. Preferably, the calibration range should be set so that most analyte concentrations fall near the range's center. Since we intended to develop and validate an analytical method to quantify the amoxicillin and doxycycline in various matrices (wastewater, tab), based on the previous studies [6,41–44], we established an optimal, large range of calibration from the LOQ to 100 ug/mL to be relevant for each investigated matrix. Moreover, the calibration curves were forced to go through the origin resulting in high accuracy at low concentrations, which is proven by the repeatability of the LOQ (Table 2).

Table 2. Repeatability and Recovery for the LOQ of the HPLC-DAD Method for the Simultaneous Determination of Amox and Dox in Capsules and Wastewater.

Analyte	Capsules		Wastewater	
	Amox (μg/mL)	Dox (μg/mL)	Amox (μg/mL)	Dox (μg/mL)
1	0.754	1.12	0.711	1.064
2	0.712	1.05	0.728	0.981
3	0.673	0.947	0.694	0.976
4	0.684	0.935	0.683	0.937
5	0.746	0.935	0.648	1.037
6	0.709	1.10	0.624	0.907
RSD (%)	4.54	8.61	5.73	6.00
Recovery (%)	102	101	97.3	98.4

The linearity is defined as the ability of the method to attain test results that are directly proportional to the analyte concentration in samples within a specific range [47]. The linearity study was conducted for the calibration curves in the range of 10–100 μg/mL (Figure 3). Each concentration was injected three times and evaluated under identical conditions. For both analytes, the correlation coefficient $R^2 > 0.996$ indicates a linear relationship between the concentration of the analyte and the peak area. ANOVA analysis for both analytes also evidenced that the regression model was statistically significant and envisaged the outcome variable ($p < 0.05$). Calibration curves were prepared in solvent and by spiking both matrices (capsules and wastewater). No significant difference was observed, permitting the use of the same calibration curve (in solvent) for the Amox and Dox analysis in both matrices. The different matrices used to prepare the calibration curves were analyzed previously with the HPLC–DAD method, and no Amox and Dox levels were detected. High values of the correlation coefficients ($R^2 = 0.9999$) were also obtained for the determination of Amox in tablets [6,41] and water [42], and for Dox in tablets [33] and water [44] by HPLC.



(**a**) (**b**)

Figure 3. HPLC calibration curves of (**a**) Amox and (**b**) Dox standards.

This study attended to decrease the detection (LOD) and quantification (LOQ) limits to as low as practically attainable while still achieving good accuracy and precision. The LOQ is expressed as the lowest concentration of an analyte that can be quantitatively measured with acceptable accuracy and precision, while the LOD is the concentration that can be detected, but not certainly quantified [47]. The noise level of the developed method was too small to calculate the LOQ and LOD based on the signal-to-noise ratio. Therefore, the LOQ (0.7 μg/mL for Amox and 1.0 μg/mL for Dox) and LOD (0.2 μg/mL for Amox and 0.3 μg/mL for Dox) were calculated as 10 times and 3.3 times, respectively; the standard deviation of the response for the lowest feasible analyte concentration in the range and the calibration curve's slope [46].

The determined LOQs (0.7 µg/mL for Amox and 1 µg/mL for Dox (equivalent to 14 µg/g for Amox and 20 µg/g for Dox in tablets)) were verified using six spiked solutions at LOQ level for both targeted matrices (capsules and wastewater) (Table 2). In both cases, the RSD values were lower than 10%, and the recovery values were included in the range of 95–105%. The LOQs were accepted as valid [51–53]. Moreover, the validated method supplied recoveries in the range of 97.3 to 102% (Table 2), which supported the suitability of the HPLC technique for the envisioned application, namely the simultaneous determination of Amox and Dox in the targeted matrices. The LOQs of the optimized method were low enough to perform the quantitative determination of Amox and Dox in a variety of matrices. Our study's LOD and LOQ values are much lower than those of 0.436 and 1.32 µg/mL [20], 2.14 and 7.15 µg/mL [54], 1.579 and µg/mL, 0.1 and 0.3 mg/mL [41], and 4.785 µg/mL [17] reported for the quantitative determination of Amox, and 1.08 and 3.27 µg/mL [55], and 16 and 54 µg/L [6] stated for Dox in pharmaceutical formulations and wastewater samples using the HPLC method.

2.1.4. Accuracy and Precision

Accuracy conveys the closeness of the test results generated by the method to the true value [47]. The accuracy was determined by six measurements of spiked samples, using magnesium stearate and wastewater as the matrix, at different concentration levels (10 µg/mL for wastewater and 20 µg/g for commercial tablets) (Table 3). Acceptable accuracy was within the range of 80% to 120%, and the precision of the %RSD was not more than 2.0%.

Table 3. Results of Accuracy.

Analyte	Capsules				Wastewater			
	Fortified (µg/g)	Found (µg/g)	Recovery (%)	RSD (%)	Fortified (µg/mL)	Found (µg/mL)	Recovery (%)	RSD (%)
Amox	20.1	18.6 ± 0.21	92.8	1.11	10.1	9.92	98.6	1.03
Dox	20.1	18.6 ± 0.29	92.5	1.54	10.1	9.95	98.4	1.37

Average of six determinations.

The method's precision (repeatability) is defined as the closeness of the agreement among a series of measurements attained from several samples of the same homogeneous sample under the established conditions, and it is usually stated as the relative standard deviation [47]. Our system's precision was determined by analyzing six independent replicates at two concentration levels, for each matrix (magnesium stearate and wastewater), over the working range of the method on the same day (intraday). Amox and Dox concentration levels were 60.0 and 30.0 µg/g for magnesium stearate and 50 and 20 µg/mL for wastewater (Table 4). In order to evaluate the interday precision, two lots of spiked samples containing a placebo matrix and a standard of Amox and Dox at different concentrations (20.0 and 35.0 µg/g, and 10.0 and 25.0 µg/mL, respectively) were determined over five consecutive days. In terms of method precision, the %RSD of the assay results for both studied antibiotics (Amox and Dox) in the calculation of repeatability and intermediate precision were less than 2% [45,47].

Table 4. Intraday and Interday Precision and Accuracy for Amox and Dox in Commercial Tablets and Wastewater Samples.

			Intraday (n = 5)		RSD (%)	Interday (n = 6)		RSD (%)
Analyte	**Matrix**	**Fortified (µg/g)**	**Measured (µg/g)**	**RSD (%)**		**Measured (µg/g))**	**RSD (%)**	
Amox	Tablet	30.0	30.3 ± 0.34	1.12	101	-	-	-
		60.0	60.4 ± 0.72	1.19	101	-	-	-
Dox		30.0	29.9 ± 0.55	1.12	99.8	-	-	-
		60.0	60.4 ± 0.80	1.32	101	-	-	-
Amox		20.0	-	-	-	20.1 ± 0.65	3.23	100
		35.0	-	-	-	35.3 ± 0.86	2.44	101
Dox		20.0	-	-	-	20.1 ± 0.87	4.34	101
		35.0	-	-	-	34.8 ± 0.84	2.40	99.3
Analyte	**Matrix**	**Fortified (µg/mL)**	**Measured (µg/mL)**	**RSD (%)**	**Accuracy (%)**	**Measured (µg/mL)**	**RSD (%)**	**Recovery (%)**
Amox	Wastewater	20.0	20.2 ± 0.37	1.82	100	-	-	-
		50.0	49.9 ± 0.60	1.20	101	-	-	-
Dox		20.0	20.1 ± 0.29	1.73	101	-	-	-
		50.0	50.1 ± 0.87	1.46	101	-	-	-
Amox		10.0	-	-	-	10.1 ± 0.17	1.72	101
		25.0	-	-	-	24.3 ± 0.71	2.93	97
Dox		10.0	-	-	-	10.1 ± 0.13	1.29	101
		25.0	-	-	-	24.3 ± 0.27	1.10	97

2.1.5. Robustness

The robustness of an analytical method embodies its ability to remain unaffected by the minor, deliberate variations of one or more parameters, demonstrating its reliability during normal usage [43]. In this case, the robustness was evaluated in terms of small but thoughtful changes in the chromatographic parameters, i.e., column temperature (±5 °C), flow rate (±0.2 mL/min), mobile phase ratio (±2%) and wavelength (±2 nm) (Table 5). The separation between the two peaks is of no interest since their time gap is very large (≥3.6 min). Thus, the separation is no issue regardless of the changes made to the chromatographic parameters. The peak areas of both compounds remained constant. The most significant difference was noticed in the asymmetry factor, especially for Dox, when lowering the column oven temperature, the flow, and the percentage of acetonitrile (ACN) in the mobile phase. These factors directly influence the column and compound interaction. No significant changes were observed in the Amox and Dox retention time in different conditions tested (RSD < 2%). Therefore, the obtained results indicated that the effects of the considered, deliberate variations on the chromatographic parameters were insignificant, and the proposed method was robust for its envisioned applications.

Table 5. Method Robustness.

Analyte	**Chromatographic Parameters**	**Column Temperature (°C)**		**Flow Rate (mL/min)**		**Mobile Phase Ratio (%)**		**Wavelength (nm)**	
		27.5	**32.5**	**1.55**	**1.45**	**11 ACN**	**9 ACN**	**232 * 348 ****	**228 * 352 ****
Amox	Assay (%)	99.6	98.9	100	99.1	100	99.7	99.9	98.7
	Retention time (min)	1.72	1.72	1.72	1.71	1.71	1.72	1.73	1.72
	Asymmetry factor	1.16	1.11	1.15	1.57	1.12	1.67	1.12	1.14
	Resolution	28.2	27.8	25.3	26.8	20.3	24.1	10.2	11.4
Dox	Assay (%)	98.7	98.6	99.1	99.2	98.6	98.4	99.3	99.4
	Retention time (min)	5.33	5.30	5.27	5.25	5.23	5.27	5.23	5.30
	Asymmetry factor	2.07	1.44	1.45	2.45	1.45	2.18	1.13	1.31
	Resolution	23.4	3.8	27.6	31.0	11.4	17.4	11.4	18.1

*—Amox; **—Dox.

Although several analytical techniques are available for the individual quantification of these antibiotics, their analysis is challenging due to their low levels and complex sample matrices. Liquid chromatography is one of the most used analytical techniques used in pharmaceutical analysis and drug control [56]. The validated HPLC-DAD method for

the simultaneous determination of Amox and Dox is simple, rapid, reliable and low cost considering its main advantages; namely, it requires no sample preparation and, thus, no expensive equipment, no expensive solvents (especially, it uses only ultrapure water), time-consuming steps; requires no buffers in the mobile phase; the mobile phase is easy to prepare; it uses only a binary pump and a DAD detector, which are, besides the most common type of pump and detector, the ones with the lower prices. Moreover, the method allows the simultaneous analysis of two antibiotics belonging to different classes with dissimilar chemical and physical properties: Dox (hyclate form) being highly soluble in water (50 mg/mL) [25], while Amox is less soluble in water (54.6 µg/mL) [2].

To our knowledge, no analytical methods were reported for the simultaneous determination of Amox and Dox in the investigated matrices (pharmaceutical formulations and wastewater samples). Fourier transform infrared (FT-IR) spectroscopy [57], UV–VIS spectrophotometry [58–60], and near-infrared (NIR) spectroscopy [61] have the advantages of a reduced volume of chemical waste generated and low initial investment for the equipment. However, their disadvantages are higher LOQ (~5 mg/L), low specificity, strictly controlled laboratory room temperature conditions and they require a chemometric method for quantification of more the one compound [57–61]. The capillary electrophoresis for the individually determination of Amox and Dox showed similar LOD (0.4 µg/mL), but higher relative standard deviation and a longer analysis time (30-40 min) [16,62]. Voltametric methods are frequently used for the individual, quantitative analysis of Amox and Dox. However, their applications focus on solid samples containing a high concentration of antibiotics, such as in capsules and tablets. Consequently, the concentration range and LOD are much higher than for liquid chromatographic methods [10,11,63].

2.2. Determination of Amox and Dox in Commercial Drugs

Quantitative determination plays a crucial role in the pharmaceutical industry due to the direct effect of active pharmaceutical ingredients on human health. A precise and accurate analytical method permits quantitative determination at trace levels without any interference effects. The high extraction efficiency of Amox and Dox from pharmaceutical formulations to extraction solutions is also a key factor.

Three different brands of Amox (250, 500 and 1000 mg/capsule) and Dox (100, 250 and 500 mg/capsule) tablets were extracted and analyzed using the validated HPLC-DAD method. The removal of excipients using an extraction step before the HPLC-DAD analysis was appraised as unneeded. When comparing the experimental data with the label information, both of the antibiotics found in all of the commercial formulations matched with the label information, with recoveries in the range of 98.2–101% for Amox and 98.5–98.9% for Dox. Moreover, the obtained results indicated that using about 50 mg of each sample or tablet was sufficient for precisely quantifying both antibiotics (Amox and Dox). Consequently, the proposed HPLC-DAD method requires no sophisticated software and is applicable for the quality control and routine analysis of the studied antibiotics. A higher recovery (110.07%) was stated by Kogawa et al. for the determination of Dox in pharmaceutical formulations (tablets) [33].

2.3. Occurrence of Amox and Dox in Wastewater Samples

The effluent of wastewater treatment plants is an important route by which antibiotics may enter the aquatic environment. The continuous monitoring of antibiotics in wastewater or treated wastewater effluent is of growing concern worldwide due to: (*i*) the development of a generation of antibiotic-resistance bacteria and other microorganisms; (*ii*) the effect of antibiotics on animal life in surface water; and (*iii*) the consequences to human health via their uptake via crops irrigated with treated wastewater [64].

The validated method was used to quantitatively determine Amox and Dox in wastewater. The recovery tests (Section 2.1.4) performed on wastewater samples with Amox and Dox spiked at a concentration of 10 µg/mL resulted in good recovery (~98.0%). No antibiotics (Amox and Dox) were detected in the wastewater collected from the sewage system of

the two stations from Cluj-Napoca. Lower recovery (97.0 ± 1.6%) for the determination of Amox in spiked wastewater at 10 mg/L was found by Unutkan et al. [6], while Almaki et al. reported 100.99 ± 1.632% for the assay of Amox in capsules [20].

3. Materials and Methods

3.1. Materials

All chemicals were of analytical grade (Merck Millipore, Darmstadt, Germany) except for the porcine mucin, which was purchased from Sigma–Aldrich (Steinheim, Germany), and were used as received without purification. The reference standard compounds were HPLC grade: Amox, amoxicillin trihydrate, 99.2%, CPAchem, Bogomilovo, Bulgaria and Dox, doxycycline hyclate, 99.3%, CPAchem, Bogomilovo, Bulgaria. The HPLC grade solvents were supplied by VWR (Radnor, PA, USA) and used as received. Ultrapure water (18.2 MΩ cm at 25 °C) was prepared from an ELGA Purelab water purification system (Veolia Water Technologies, High Wycombe, UK).

3.2. Preparation of Standards and Quality Controls

The standard (individual and mixed) stock solutions of 1 mg/mL of each compound were prepared in the mobile phase with various concentrations and deposited in amber-colored vials at 5 °C prior to use. The working standard solutions of Amox (0–10–25–50–75–100 µg/mL) and Dox (0–10–25–50–75–100 µg/mL) were prepared by proper dilution of the stock solution with the mobile phase.

3.3. Sample Preparation

3.3.1. Commercial Tablets

Three different brands of Amox (1000 mg/capsule, randomly named Amox-1, Amox-2, and Amox-3) or Dox (100 mg/capsule, randomly named Dox-1, Dox-2, and Dox-3) tablets were purchased from local pharmacies. All antibiotic tablets tested were in their original sealed containers and were within their expiration dates.

The 500 mg homogenized capsules were added into a centrifuge tube with 5 mL ACN and vortexed for 1 min, at room temperature. Afterward, the mixed samples were separated by centrifugation at 11,000 rpm using a Hettich D-78532 (Kirchlengern, Germany) and the obtained supernatant was filtered through a 0.45 µm filter (Chromafil Xtra RC, Macherey, Nagel, France). The extraction was repeated from the residual mixture with 5 mL ACN. The clean supernatant was collected and analyzed using an HPLC-DAD. For each antibiotic, three replicate samples were prepared.

3.3.2. Wastewater

The developed analytical method was also applied to the analysis of real wastewater for the quantitative determination of Amox and Dox. The wastewater sample was collected in 1 L sterile glasses in April 2022, from the sewage system of two different stations from Cluj-Napoca, mixed, and stored at 4 °C until use. The wastewater sample (pH = 7.2) was centrifuged at 11,000 rpm for 15 min and the supernatant was filtered through a 0.45 µm filter to eliminate contamination and to protect the column from undesirable particles [65].

3.4. Chromatographic Conditions

The analysis was performed using a Vanquish ultra-high-pressure liquid chromatography (HPLC) system (Thermo Scientific, Germering, Germany) with a diode array detector (DAD HL, Dionex, Germering, Germany). The chromatographic separation was carried out on a Zorbax SB column, 150 × 46 µm, 5 µm (Agilent, Santa Clara, CA, USA). The mobile phase comprised 0.1% formic acid in ultrapure water (A) and ACN (B) (Table 6). The flow rate of the mobile phase was 1.5 mL, the injection volume was 10 µL and the column temperature was 30 °C. The detection UV wavelength was set at 350 nm and 230 nm due to the multichannel option that concomitantly allows up to 10 wavelength settings.

Table 6. HPLC Gradient Program.

		Composition (%)	
Running Time (min)	**Flow (mL/min)**	**Mobile Phase-A**	**Mobile Phase-B**
0.00	1.5	90	10
10.00	1.5	40	60
10.10	1.5	90	10
15.00	1.5	90	10

3.5. Method Validation

Validation of the proposed method in respect of LOD and LOQ, specificity, linearity, working range, trueness, and precision was performed in correspondence with the European Pharmacopeia [45] for commercial tablets and the International Conference on Harmonization (ICH) guidelines [46] for wastewater samples.

4. Conclusions

The validated HPLC-DAD method is simple, selective, rapid, sensitive, repeatable, precise, and accurate for the estimation of Amox and Dox in pharmaceutical formulations and wastewaters. The chromatographic determination of Amox and Dox was completed in 15 min, the peak was close to the signal, and the resolution was high. The LOD and LOQ values were calculated as 0.7 μg/mL for Amox and 1.0 μg/mL for Dox, and 0.2 μg/mL for Amox and 0.3 μg/mL for Dox, respectively, which allow us to perform sensitive measurements of the analyte using a simple HPLC–DAD system. The method was validated according to the existing guidelines (Pharmacopeia and ICH) and proved suitable for the envisioned application; providing high recoveries, and accurate and precise quantitative results under minor variations of the chromatographic parameters. Moreover, a qualitative aspect was the peak identification on the chromatogram of the Amox and Dox samples, which was at the same retention time as for the Amox standard. In view of the foregoing, the proposed method can be easily and conveniently implemented for the routine quality control analysis of Amox and Dox in bulk and pharmaceutical formulations and at the low analyte levels usually found in real water samples. The validated HPLC–DAD method would be used with diverse extraction and preconcentration methods to attain lower detection limits.

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