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Voltammetric glassy carbon sensor approach for the extended stability studies of doxorubicin in lyophilized dosage form

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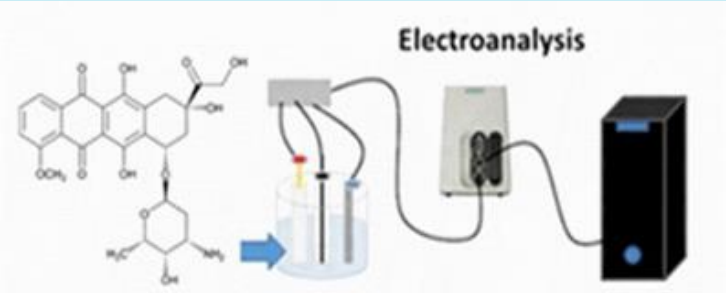
ABSTRACT: Doxorubicin (DOX) is an anthracycline antibiotic that is widely used in the clinical treatment of cancer patients. DOX has a high market value. Electroanalytical methods for DOX analysis are an alternative and promising approach compared to chromatographic techniques. In this context, electroanalysis provides a low-cost method for determining drugs such as DOX lyophilized powder for the injection. Differential pulse voltammetry with a glassy carbon electrode was used. DOX stability after reconstitution was performed, and the correct time for safe administration to patients in hospitals was determined. The electroanalytical method showed a limit of detection of 0.54 µmol L^{-1} and limit of quantification of 1.83 µmol L^{-1}, which is enough for the application in quality control of DOX. The high-performance liquid chromatography analysis was also applied in pharmaceutical samples containing DOX to compare with the proposed method, showing that the obtained results are relatively similar for both methods. Therefore, the electroanalytical approach shows the viability of an attractive alternative technique for applying this sensor for drug quality control.



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1. Introduction

Doxorubicin hydrochloride (DOX) (Fig. 1) is an anthracycline antibiotic which starting material is *Streptomyces percetius* var. *cesium*, which is widely used in the clinical treatment of patients with leukemias and tumors in the lung or breast. Doxorubicin hydrochloride is composed of an amino sugar linked to anthraquinone aglycone, as shown in in its chemical structure (Fig. 1). Its mechanism of action is elucidated in four different ways, i.e., a) inhibition of deoxyribonucleic acid (DNA) synthesis in tumor cells; b) creation of free radicals, which can damage DNA; c) induction of DNA damage due to DOX interference with topoisomerase II; and d) induction of apoptosis (Alhareth *et al.*, 2012; Li *et al.*, 2020; Skalová *et al.*, 2020).

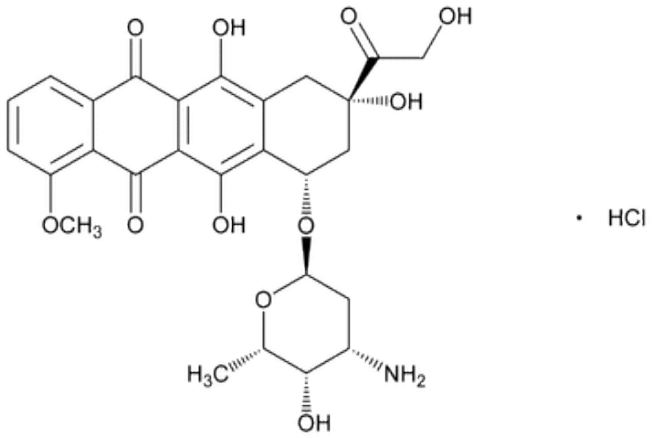

Figure 1. Doxorubicin chemical structure.

Doxorubicin hydrochloride is highly prescribed for therapeutic use in the form of a lyophilized powder for injection, so it is necessary to ensure the quality of the drug. Doxorubicin hydrochloride has a high market value, so it is a drug with less accessibility to patients with less purchasing power However, hospitals allow the use of the DOX injection form to grant access to more patients. Nonetheless, it is necessary to carry out stability control after reconstitution of the lyophilized powder for DOX injection to ensure the drug's efficacy and safety (Navas *et al.*, 2013; Radi, 2003; Rodrigues *et al.*, 2018).

Quality control tests involve checking the content and possible impurities and stability of the drug. Techniques with good sensitivity are needed to perform the quality control tests for DOX, with the official methods used to determine the content consisting of high-performance liquid chromatography (HPLC) tests with UV detection and UV-VIS spectrometry (Felix and Angnes, 2018; Navas *et al.*, 2013). These techniques require expensive instrumentation, centralized laboratory and well-qualified personnel, time-consuming sample pretreatment steps in addition to making use of many organic reagents, also not environment-friendly (ACS, 2019; Hahn and Lee, 2004; Shellaiah and Sun, 2020).

As an alternative to those above conventional analytical methods, electrochemical sensors offer several remarkable attributes, such as low-cost instrumentation, elimination or reduction of sample pretreatment steps, fast response, and the possibility of online and *in situ* detection. Furthermore, electroanalytical methods provide good sensitivity, versatility, and cleaner and more sustainable analysis. The glassy carbon electrode (GCE) and the carbon paste electrode (CPE) have been used frequently among many electrodes employed in electroanalysis. Modifications of these electrodes, mostly with catalysts and/or nanoparticles, have displayed a significant increase in sensitivity for electrochemical detection (Hajian *et al.*, 2017; Shah *et al.*, 2018; Skalová *et al.*, 2020).

This work aims to perform the electroanalytical determination of DOX in lyophilized powder form, by differential pulse voltammetry (DPV) with a GCE sensor. We also aimed to verify the stability of DOX after reconstitution, establishing the correct time for safe administration to patients in hospitals. For comparison, DOX determination was also performed by HPLC-UV.

2. Materials and Methods

Electrolyte solutions were prepared using high analytical grade salts, which were solved in Milli-Q water (conductivity $\leq$ 0.1 µS cm^{-1}) (Millipore S. A., Molsheim, France).

Analytical grade DOX was obtained from the United States Pharmacopeia (USP) ($\geq$ 99%). Pharmaceutical formulations of DOX (10 mg mL^{-1}) were kindly provided by a private oncology health unit (Rio de Janeiro/RJ – Brazil). The stock standard solution was prepared from a test dose of 27.18 mg in a 50 mL volumetric flask, then 25 mL of Milli-Q water was added and taken to ultrasound until complete solubilization, then the volume of the flask obtaining a concentration of 1.0 mmol L^{-1} DOX, the solution was prepared immediately before the experiments.

The samples of the reconstituted drug destined for the evaluation of the stability against the ambient temperatures and at 2 to 8 °C by voltammetric tests were prepared from an initial solution of DOX reconstituted in water for injection in its original packaging and divided into four 10-mL amber glass volumetric flasks. In contrast, those for monitoring by

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chromatographic tests were packaged in four 100-mL amber glass volumetric flasks. The tests were performed at zero time and repeated in 2, 4, 6, 12, 24, 48, and 96 h in the samples exposed to the environmental conditions proposed by the study.

2.1. Electroanalytical tests

Voltammetric measurements were performed using a potentiostat/galvanostat PGSTAT model 204 with a FRA32M module (Metrohm Autolab, Eco Chemie, Netherlands) integrated with NOVA 2.1 software. All measurements were carried out in a 1-mL one-compartment electrochemical cell coupled to a three-electrode system consisting of a GCE, a Pt wire counter electrode and an Ag/AgCl/KClsat reference electrode (both purchased from Lab Solutions, São Paulo, Brazil).

The experimental conditions used for the DPV were pulse amplitude of 50 mV, pulse width of 0.5 s, and scan rate of 10 mV s^{-1}. All voltammetric assays were performed in 0.1 mol L^{-1} phosphate buffer solution (PBS), pH 7.0. The DP voltammograms were background-subtracted and baseline-corrected. All experiments were conducted in triplicate, and data were analyzed using Origin Pro 9 software (Northampton, MA, USA). Between each voltammogram reading, the GCE was sanded with 0.3 µm alumina suspension and subsequently rinsed with Milli-Q water, in order to renew the electrode surface, thus ensuring the reproducibility of the tests.

2.2 Chromatography tests

For the chromatographic assay, a stainless-steel column (250 × 4.6 mm) was used, packed with octadecylsilyl silica gel for chromatography (5 µm) (Hypersil C18). The elution was isocratic with the mobile comprised of acetonitrile and acid surfactant solution (containing 0.288% w/v dodecyl sulfate and 0.225% w/v orthophosphoric acid) in a 50:50 (v/v) proportion. The analyses were performed at room temperature (25 °C). The flow was 1 mL min^{-1}, the wavelength was 254 nm, and the injection volume was10 µL. The samples were prepared with 0.01% w/v of the reconstituted solution for injection diluted in the mobile phase, compared to the USP standard's declared value (USP, 2020; Zhao and Dash, 1999).

2.3 Method validation

The method was developed and validated for linearity, accuracy, precision, quantification limit, detection limit, and selectivity in accordance with ICH Q2 (R1). The linearity of the method was performed through three analytical curves using DOX standard solutions. The results were statistically analyzed by linear regression analysis using the least squares method (ICH, 2014).

3. Results and discussion

3.1 Electroanalytical tests

The DP voltammogram obtained with GCE showed one main oxidation peak for DOX at E_{pa} = 0.33 V corresponding to oxidation in the quinonic portion of the anthracyclines drug class, followed by a second and third oxidation peaks, at E_{pa} = 0.60 V and E_{pa} = 0.75 V that are explained by the adsorption of DOX, attributed to the formation of a hydrogen bond between the hydroxyl groups of the phenolic compound and the carbonyl group (Piovesan and Spinelli, 2014).

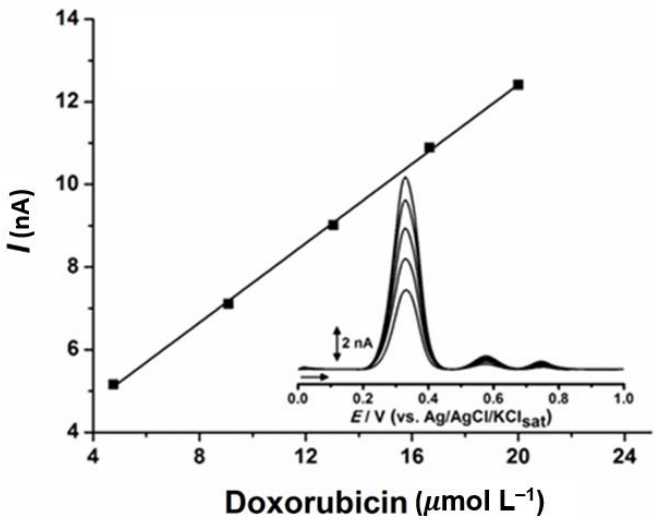

Figure 2. Calibration curve of DOX with DPV with GCE in PBS pH 7.0.

A calibration curve (Fig. 2) was constructed to determine the ideal concentration for DOX recovery in the stability study. Linearity was found on the calibration curve for the anodic peak E_{pa1} a.a. 0.33 V. As E_{pa1} presented a good linearity coefficient (r^2 = 0.9993), it was used to calculate the regression equation: y = (2.81± 0.09) + (0.48 ± 0.01 × [DOX] (µmol L^{-1}). As the p-value (0) found in the ANOVA F test is less than 0.05, it was rejected the null hypothesis (zero slope) at the significance level of 5%, whereas the P-value of 2755.7954 of the t-test is greater than 0.05, therefore, it was not rejected the null hypothesis (intercept equal to zero) at the significance level of 5%.

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The correlation coefficient found of 0.9993 is greater than 0.9900, so it was concluded that there is an adequate linear relationship, showing residual sum of squares of 3.94×10^{-9}.

A comparison of the analytical parameters obtained by the proposed method and HPLC for the determination of DOX in pharmaceutical samples is described in Tab. 1.

Table 1. Precision and Repeatability assays values for voltammetric and chromatographic assays.

Samples	Sample amount (%)	Methods	
		DPV – GCE (% ± SD) (n = 6)	HPLC (% ± SD) (n = 6)
DOX lyophilized powder for injection Repeatability (intraday)	100.0	101.4 ± 0.8	100.9 ± 0.2
DOX lyophilized powder for injection tablets Intermediary precision (interday)	100.0	101.0 ± 0.5	100.6 ± 0.1

SD: standard deviation.

For comparison reasons, HPLC recovery assays were performed. Repeatability assays were performed within a single day with intervals of 2 h. Intermediary precision assays were performed on three different days with three different analysts with intervals of 4 h for each day. It can be seen from Tab. 1 that both methods displayed acceptable precision.

The voltammetric method displayed slightly higher deviations of precision in both assays in comparison with HPLC, as expected. However, due to the great difference between equipment and preprocessing of both approaches, the precision difference shows that the method developed in this work is within an appropriate range of precision in comparison with pharmacopoeia methods. The results are further in agreement with the study conducted by Macêdo *et al.* (2020) and Cunha *et al.* (2019). Accuracy assays were also performed for both methods and the results are shown in Tab. 2.

Table 2. Accuracy assays validation data for voltammetric and chromatographic assays for DOX lyophilized powder for injection.

Samples	Sample amount (%)	Methods	
		DPV – GCE (% ± RSD)	HPLC (% ± RSD)
DOX lyophilized powder for injection	**80.0**	81.4 ± 0.7	80.7 ± 0.2
	100.0	101.1 ± 0.5	101.5 ± 0.2
	120.0	120.7 ± 0.8	120.1 ± 0.4

RSD: Relative standard deviation.

As observed in Tab. 2, the accuracy of both methods was satisfactory, with results within 5% deviation range. The chromatography approach showed only slightly lesser deviation than the voltammetric method with the GCE, corroborating the considerations made previously. Table 3 presents the limit of detection (LOD) and limit of quantification (LOQ) values of this study compared to the results obtained from the literature data used to determine DOX.

Table 3. Comparisons of the limits of detection and the limits of quantification in the determination of DOX with other methods.

Method	LOD	LOQ	Reference
DPV	0.54 µmol L^{-1}	1.83 µmol L^{-1}	This work
HPLC	0.5 ng mL^{-1}	5.0 ng mL^{-1}	Skalová *et al.*, 2020
DPV-PGE	9.9 µmol L^{-1}	33.31 µmol L^{-1}	Cunha *et al.*, 2019
DPCSV	0.44 µmol L^{-1}	0.6 µmol L^{-1}	Deepa *et al.*, 2020
HPLC	0.2 ng mL^{-1}	0.6 ng mL^{-1}	Thomaz *et al.*, 2018

Next, a summary and a comparison of our method depicted in Tab. 3 were provided, which shows the current analytical approaches for the detection of DOX, from which it is possible to infer that an adequate sensitivity was obtained for the simplified electroanalytical approach used. The results further demonstrate the availability of a faster analytical method and at a lower cost compared to other methods.

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The electroanalytical method proposed in this work showed a LOD of 0.54 µmol L^{-1} and LOQ of 1.83 µmol L^{-1}, which is sensitive enough for the application in quality control of DOX in lyophilized powder for injection.

3.2 Extended stability study test on a DOX pharmaceutical sample

The GCE sensor was also used to monitor the stability of DOX lyophilized powder for injection applications. In this context, injectable DOX solutions were evaluated after reconstitution at room temperature and at temperatures from 2 to 8 °C. Analytical assays were performed at different times monitoring the decay of the DOX content after reconstitution, where the recovery in percentage after each period was evaluated, determining the stability after its reconstitution through the assay found (Tab. 4). As expected, the recovery values for voltammetric and chromatographic determinations showed DOX degradation after reconstitution. However, at room temperature, it shows DOX degradation after 48 h, while from 2 to 8 °C it remained stable until 96 h. (Tab. 4). All concentrations found were less than 5% of the relative standard deviation. Both methods showed effective approaches to assess the stability of DOX lyophilized powder for injection after reconstitution.

Table 4. Results obtained for the recovery of DOX in the evaluation of the extended stability study at different times.

Time (h)	% DOX (at room temperature)		% DOX (2–8 °C)	
	DPV	HPLC	DPV	HPLC
0	92.74	92.33	92.74	92.33
2	92.18	93.36	92.22	93.34
4	91.13	92.92	93.62	93.84
6	92.04	92.61	91.61	91.50
12	91.38	90.20	92.55	92.33
24	92.07	88.47	91.12	90.74
48	83.36	84.23	91.78	90.89
96	79.51	81.52	91.06	91.41

4. Conclusions

The GCE sensor, when compared to other sensors and evaluation methods, offers efficiency in its analytical performance for the determination of lyophilized powder for injection. This characteristic, when associated with low cost, easy access, quick and efficient cleaning of the electrode surface area, indicates that the GCE sensor can be a useful tool for DOX analysis. Also, the GCE sensor exhibited satisfactory detection and recovery, although the standard deviation values were slightly higher than most of the sensors and methods applied. However, the results are following the specifications for such an analysis. The general analytical performance and the low cost of the material associated with the immediate analysis provided by both electrodes, consistently justify the choice of these analytical devices as alternative approaches to quality control and extended drug stability studies.

Authors' contribution

Conceptualization: Cunha, C. E. P.; Gil, E. S.; Rodrigues, E. S. B; Sgobbi, L. F.; Taveira, S. F
Data curation: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R; Sgobbi, L. F.; Taveira, S. F
Formal Analysis: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R; Sgobbi, L. F.; Taveira, S. F
Funding acquisition: Not applicable.
Investigation: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R; Sgobbi, L. F.; Taveira, S. F
Methodology: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R; Sgobbi, L. F.; Taveira, S. F
Project administration: Gil, E. S.
Resources: Gil, E. S.
Software: Not applicable.
Supervision: Gil, E. S.
Validation: Somerset, V.
Visualization: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R; Sgobbi, L. F.; Taveira, S. F
Writing – original draft: Cunha, C. E. P.; Rodrigues, E. S. B.; Oliveira Neto, J. R
Writing – review & editing: Gil, E. S.; Somerset, V.

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Data availability statement

All dataset were generated or analyzed in the current study.

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