

## Research Article

# Development and Validation of a Simple and Selective Analytical HPLC Method for the Quantification of Oxaliplatin

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Considering that currently published oxaliplatin (OXPt) analytical methods require complex procedures and equipment, the objective of this paper was to present the validation of a simple, rapid, precise, and specific isocratic HPLC method for quantification of OXPt. Such method will be essential during the future development of innovative, topical drug formulations for the treatment of mucosal and skin cancers. Validated method demonstrated OXPt separation without interference from the solvents or polymers with the most relevance for developing of bioadhesive pharmaceutical drug carries (chitosan and poloxamer). Method was linear ( $r > 0.999$ ) over studied OXPt concentration range (0.5–15.0  $\mu\text{g/mL}$ ) with acceptable precision and accuracy. Limit of detection (LOD) and limit of quantification (LOQ) were 0.099  $\mu\text{g/mL}$  and 0.331  $\mu\text{g/mL}$ , respectively.

## 1. Introduction

Platinum compounds (cisplatin, carboplatin, nedaplatin, tetraplatin, and oxaliplatin) constitute an important class of anticancer drugs [1], which act by formation of DNA adducts, resulting in damage and apoptosis of tumoral cells [2, 3]. Oxaliplatin (OXPt, Figure 1) has currently been used as first-line antitumor compound in adjuvant and palliative chemotherapy regimens [4, 5] and has proven to be effective in digestive cancers [2] and in some solid tumors [4]. As a third-generation drug of platinum group, OXPt has showed, at least in part, overcoming clinical problems related to the first-generation drug cisplatin, for example, the occurrence of important toxic effects and tumoral resistance along the treatment [1, 6, 7].

Given OXPt therapeutic relevance, our research group intends to develop topical, mucoadhesive formulations to make possible targeted therapy of mucosal and skin cancers. This approach could reduce toxic effects related with systemic therapy [8, 9], improving tolerability, effectiveness, and patients compliance. However, a reliable quantification method is required to give support to formulations development.

Considering that currently published OXPt quantitative methods require complex procedures and equipment, as mass detection chromatographers [10, 11], the objective of this paper was to develop and validate a simple, rapid, precise, and specific isocratic HPLC method with UV detection for quantification of OXPt with future topical formulations developments purposes.

## 2. Material and Methods

**2.1. Chemical and Reagents.** OXPt (99%) was purchased from Simagchem Co. (Xiamen, China). Chitosan of low molecular weight, poloxamer 407, and phosphoric acid were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol of HPLC grade were purchased from J. T. Baker (Phillipsburg, USA). The water used in all preparations was of Milli-Q grade (Millipore, Illkirch-Graffenstaden, France).

**2.2. Standard Solution Preparation.** Working solutions of OXPt (100.0  $\mu\text{g/mL}$ ) were prepared by dissolving 10.0 mg of the drug in 100 mL of purified water. These standard solutions were used to prepare the calibration curves samples.

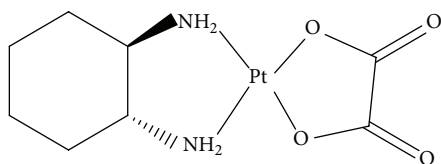


FIGURE 1: Molecular structure of OXPt (MW = 397.29 Da.).

**2.3. Method Development.** Development of the method involved the selection of wavelength of OXPt maximum absorption based on spectrophotometric scans (Lambda Bio model, PerkinElmer, United Kingdom). Different chromatographic conditions were tested, such as composition of mobile phase, flow rate, and injection volume, aiming to obtain OXPt chromatographic peaks with acceptable analytical performance.

**2.4. Apparatus and Chromatographic Conditions.** The HPLC system (LC-20AD model, Shimadzu, Kyoto, Japan) consisted of two pumps (LC 20-AT model), an automatic injector (9SIL-20AD model), an oven (CTO-20AS model), a spectrophotometric detector (SPD-M20A model), and a computer equipped with the analysis software Shimadzu LC. A reverse-phase  $C_{18}$  column (150 mm  $\times$  4.6 mm, 5  $\mu$ m) from Dionex Co. (Salt Lake City, UT, USA) was used as stationary phase. The mobile phase consisted of aqueous acid solution (0.01 M phosphoric acid): acetonitrile (95:5 v/v) mixture, which flowed at a rate of 1.0 mL/min. Oven was set at room temperature (25  $\pm$  2  $^{\circ}$ C), and UV detection was performed at 255 nm. Samples volumes of 20  $\mu$ L were injected in each analysis.

**2.5. Method Validation.** The method was validated according to ICH guideline Q2 (R1) [12] with respect to specificity, linearity, accuracy and precision, limit of detection (LOD), and limit of quantification (LOQ).

**2.5.1. Specificity.** The specificity was investigated by injecting OXPt samples contaminated or not with chitosan and poloxamer, which are the most relevant polymer excipients for preparation of bioadhesive drug carriers.

Six independent OXPt working solutions were prepared as previously described and diluted at the concentration of 5.0  $\mu$ g/mL for the HPLC analysis. Then, the same six OXPt solutions contaminated with different polymers (chitosan or poloxamer at 5.0  $\mu$ g/mL) were reanalyzed. The results of area and retention time were analyzed by ANOVA one-way with significance level of 0.05.

**2.5.2. Linearity.** Three calibration curves at the range of 0.5 to 15.0  $\mu$ g/mL were prepared from different OXPt working solutions. Linear fit was carried out using linear least square regression. Angular coefficient significance and proportionality tests were evaluated by student *t*-test. Variance homogeneity and residues normality were checked by ANOVA one-way with significance level of 0.05. Linearity response factors were calculated from the ratio between the peak

area and OXPt theoretical concentration. The analysis of this parameter was carried out by the average response factors and its variation coefficient.

**2.5.3. Limit of Detection and Limit of Quantification.** Limit of detection (LOD) and limit of quantification (LOQ) were defined by theoretical equations, respectively, described below:

$$\text{LOD} = \frac{3 \times s}{S}, \quad (1)$$

$$\text{LOQ} = \frac{10 \times s}{S},$$

where *s* is the standard deviation of *y*-axis interception values of the calibration curves and *S* is the angular coefficient of the calibration curve.

**2.5.4. Precision and Accuracy.** Precision and accuracy were determined in terms of recovery of known concentrations of OXPt. Authentic samples were prepared in triplicate at three levels of OXPt concentration (0.5, 3.0, and 15.0  $\mu$ g/mL). The analyses were performed on the same day to determine intraday variability and on different days to establish the variability interday. Precision was evaluated by calculating the CV.

The appropriate number of replicates for an analytical experiment containing OXPt was estimated from the repeatability of the method, based on a confidence interval of 98–102%, using the following equation:

$$\text{CV} = \frac{(|100 - AV| * \sqrt{n})}{Z}, \quad (2)$$

where CV is the coefficient of variation of repeatability of the method (intraday analysis); AV is the accepted value; *n* is the number of replicates; and *Z* ( $\alpha = 0,01$ ) is 2.58 [13].

### 3. Results and Discussion

**3.1. Method Development.** Maximum UV absorption of OXPt occurs at 255 nm; hence, this wavelength was fixed in all drug analysis. Main variations in the chromatographic conditions tested are described in Table 1. The mobile phase compositions containing methanol and water provided low OXPt interaction with the column, resulting in rapid drug elution. Similarly, mobile phases containing acetonitrile and water mixtures resulted in early OXPt elution (before 2 min), showing peaks with inadequate symmetry factors.

OXPt is a water-soluble compound, which presents a pKa equal to 6.1 [14]. At pH below this value, molecule polarity is decreased, providing better drug interaction with  $C_{18}$  column. In this way, a phosphoric acid solution, pH 3.5, was used as the polar solvent, yielding better results. The phosphoric acid: acetonitrile mixture at 95:5 (v/v) provided the most adequate OXPt peak separation with appropriate retention time (3.7 min, Figure 2).

TABLE 1: Variation in analytical method conditions. OXPt samples ( $15.0 \mu\text{g/mL}$ ) were analyzed in HPLC equipment using a  $C_{18}$  column, with UV detection fixed at  $255 \text{ nm}$ , at  $25^\circ\text{C}$ .

Mobile phase (volume : volume)	Injection volume ( $\mu\text{L}$ )	Flow rate ( $\text{mL/min}$ )	Result
Acetonitrile : water (90 : 10)	50	1	Peak tailing
Acetonitrile : water (80 : 20)	50	1	Peak tailing
Acetonitrile : water (70 : 30)	50	1	Peak tailing
Acetonitrile : water (50 : 50)	50	1	Peak tailing
Acetonitrile : water (20 : 80)	50	1	Peak tailing
Acetonitrile : water : Methanol (60 : 30 : 10)	50	1	Peak spike
Methanol : water (85 : 15)	50	1	Early peak
Methanol : water (65 : 35)	50	1	Early peak
Methanol : water (70 : 30)	10	0.5	Peak tailing
Methanol : water (70 : 30)	30	0.3	Broad peak
Methanol : water (30 : 70)	50	1	Broad peak
Acid solution : acetonitrile (80 : 20)	20	1	Early peak
Acid solution : acetonitrile (90 : 10)	20	1	Early peak
<b>Acid solution : acetonitrile (95 : 5)</b>	<b>20</b>	<b>1</b>	<b>Suitable peak</b>

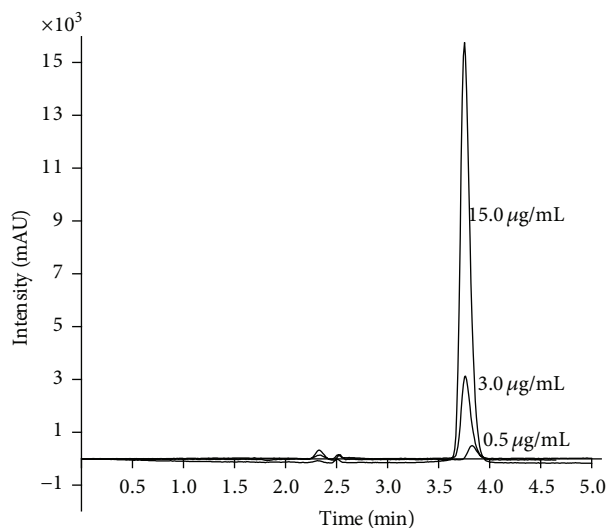


FIGURE 2: Overlaid HPLC chromatograms of OXPt aqueous solutions analyzed at different concentrations ( $0.5$ ,  $3.0$ , and  $15.0 \mu\text{g/mL}$ ).

### 3.2. Validation

**3.2.1. Specificity.** Specificity is a defining parameter, which is analyzed considering the use of the analytical method. Here, the developed method is intended to assay OXPt from bioadhesive formulations further prepared with the selected polymers poloxamer and chitosan [15].

Previous experiments conducted using UV spectrophotometer, as a simpler and lower cost analytical method, have shown polymeric solutions, analyzed as pharmaceutical contaminants, absorbed UV light at the same wavelength of OXPt (data not shown). Hence, more selective equipment as HPLC would be necessary.

Figure 3 shows the overlapped chromatograms of OXPt solutions contaminated or not with the above-mentioned polymers. Peaks of the drug were clearly separated from

the solvents (mobile phase) and the polymers in all situations. In the set condition analysis, polymeric pharmaceutical contaminants showed no detectable response in the OXPt retention time, allowing the unequivocal identification of the drug in formulations containing such materials.

Peak areas and retention time of OXPt from samples containing the pharmaceutical matrix or not were compared to evaluate whether the method is affected by the presence of contaminants (Figure 4).

In the presence of chitosan, the peak area response has no difference from that without chitosan (Figure 4(a)). In the case of poloxamer, a response difference of 2% was obtained when the polymer concentration was as high as  $5 \mu\text{g/mL}$  (Figure 4(a);  $p = 0.008$ ,  $\alpha = 0.05$ ). Nevertheless, this difference is still within the tolerance of the ICH standard.

ANOVA statistical analysis has also pointed no difference between retention times of OXPt in the presence or absence of the evaluated polymers (Figure 4(b)), with all variations observed being attributed to random errors ( $p = 0.1005$ ,  $\alpha = 0.05$ ).

**3.2.2. Linearity.** The analytical curve is shown in Figure 5. The variance analysis, ANOVA, confirms the linearity significance of the curve, homogeneity of variances, and normality of residues, which were randomly distributed without tendency.

Resulting calibration curve follows the following equation:  $y = 7159.7x - 140.9$ , where  $y$  is the peak area and  $x$  is the concentration of OXPt ( $\mu\text{g/mL}$ ). The correlation coefficient is close to the unit ( $r = 0.9998$ ), which shows a high probability of correlation between variables (area and concentration), following the minimum recommendation value of 0.99 [12].

The proportionality test shows the ordinate (140.9) is significantly equal to zero, proving that the curve passes through the origin. This value is less than 1% of the analyte response based on nominal concentration, fulfilling another analytic condition requirement.

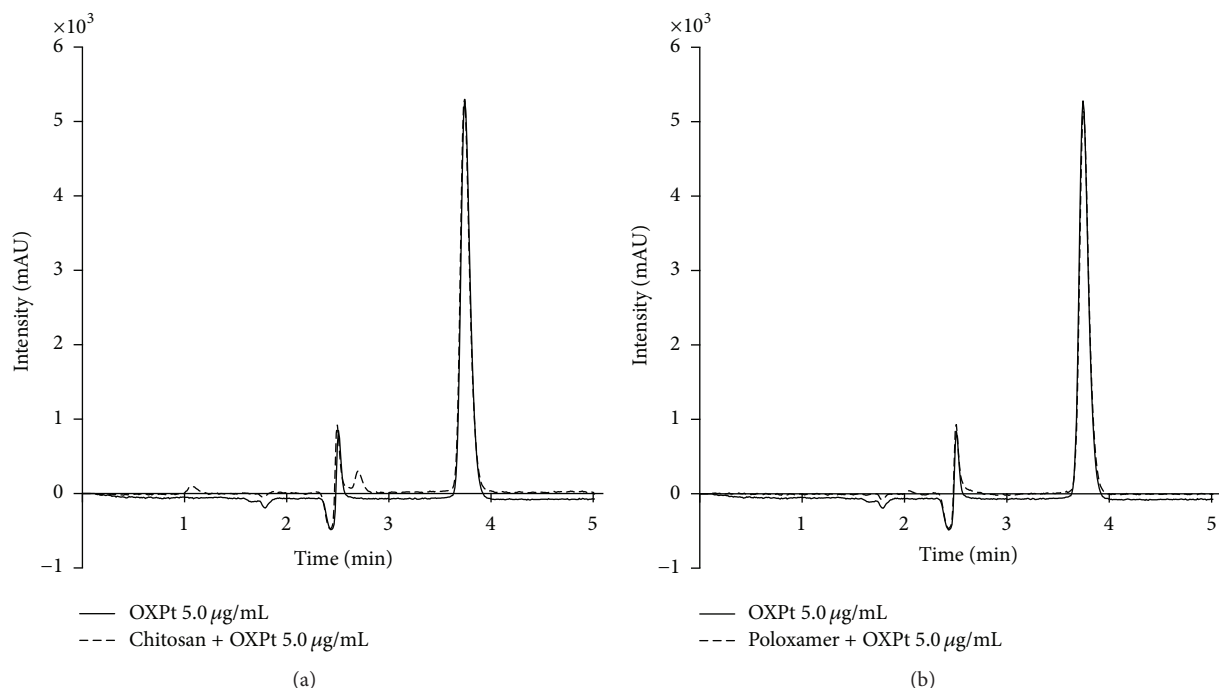


FIGURE 3: Overlaid HPLC chromatograms of a simple OXPt solution ( $5.0 \mu\text{g/mL}$ ) and OXPt solutions contaminated with  $5.0 \mu\text{g/mL}$  of (a) chitosan or (b) poloxamer.

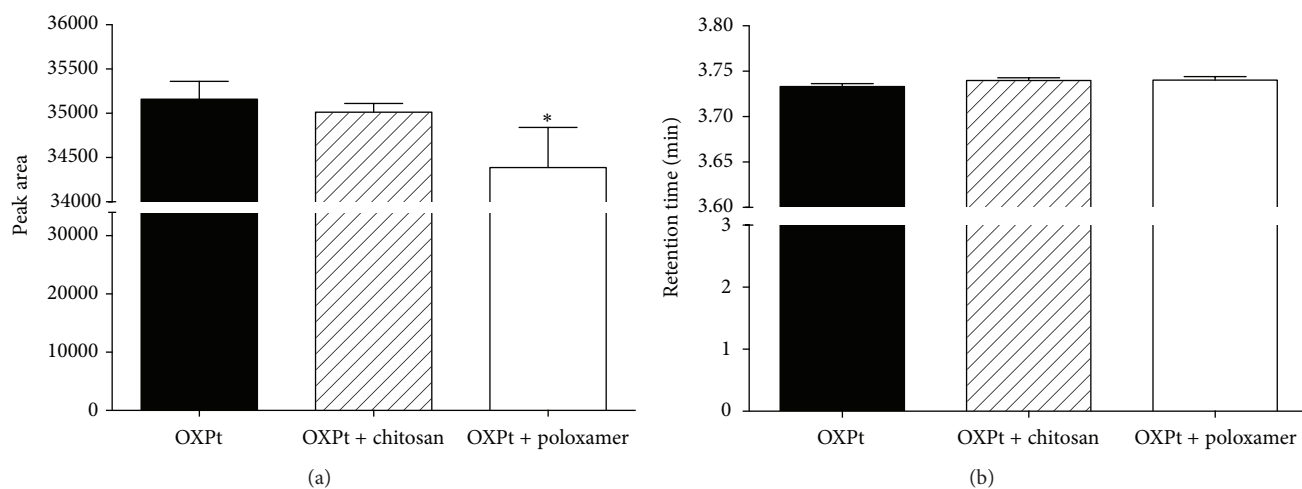


FIGURE 4: Selectivity of the method in quantification of OXPt ( $5.0 \mu\text{g/mL}$ ), alone, with poloxamer ( $5.0 \mu\text{g/mL}$ ), or with chitosan ( $5.0 \mu\text{g/mL}$ ), analyzed in terms of differences in (a) peak areas and (b) retention times. \* $p < 0.05$ .

The slope of analytical curve was different from zero ( $t = 217.9$ ;  $\alpha = 0.05$ ) and its high value (7159.7) indicates good methodological sensitivity. The average value of the response factors (7151.4) was very close to the slope (7159.7), indicating a suitable linear data calibration. Response factors coefficient of variation was 2.5%, below recommended limits by the ICH (5%) [12].

Based on these results HPLC method proved to be linear, following all requirements of international protocols for pharmaceutical analytical methods [12].

**3.2.3. Limit of Detection (LOD) and Limit of Quantification (LOQ).** The limits of detection and quantification obtained were calculated as  $0.099 \mu\text{g/mL}$  and  $0.331 \mu\text{g/mL}$ , respectively. Hence, proposed method is sensible for OXPt analysis in pharmaceutical formulations and might be useful to determine kinetics of drug release from the pharmaceutical matrix.

**3.2.4. Precision and Accuracy.** The results for precision and accuracy (intra- and interday) of OXPt quantification are presented in Table 2.

TABLE 2: Precision (CV) and accuracy of the HPLC method for OXPt quantification.

Theoretical concentration ( $\mu\text{g/mL}$ )	Experimental concentration (mean $\pm$ SD; $\mu\text{g/mL}$ )	CV (%) <sup>a</sup>	Accuracy <sup>b</sup>
Intraday ( $n = 9$ )			
0.5	$0.54 \pm 0.01$	1.75	107.33
3.0	$3.00 \pm 0.07$	2.32	100.07
15.0	$15.04 \pm 0.22$	1.45	100.26
Interday ( $n = 9$ )			
0.5	$0.52 \pm 0.01$	1.66	104.79
3.0	$2.86 \pm 0.12$	4.17	95.28
15.0	$14.82 \pm 0.08$	0.57	98.77

<sup>a</sup>CV = (SD/mean); <sup>b</sup>accuracy = (experimental concentration/theoretical concentration)  $\times$  100.

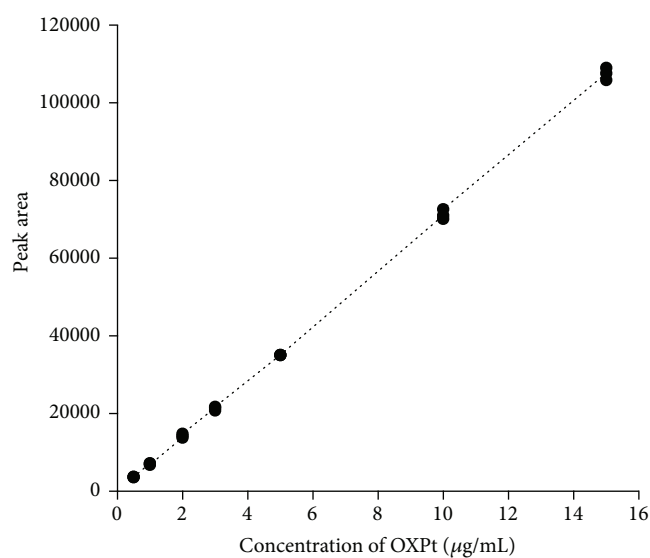


FIGURE 5: Calibration plot of peak area against concentration of OXPt ( $y = 7159.7x - 140.9$ ;  $r = 0.9998$ ;  $n = 3$ ).

Precision was evaluated to identify variability due to random errors that cannot be controlled, as those related to the analysis time, reagents, glassware, and sample preparation [16]. For intra- and interday analysis, coefficients of variation (CV) ranged from 0.6% to 4.2%. These values were in accordance with acceptable limits determined by the international requirements that establish a maximum CV of 5% [14]. CV obtained from method repeatability (intraday analysis) also allowed estimating (2) that a minimum of 5 replicates is recommended during a routine assay, considering a mean CV of 1.8% (Table 2).

Drug recovery (accuracy) ranged from 95.3% to 107.3% (Table 2). These values were in accordance with acceptable limits for a pharmaceutical matrix assay method determined by the ICH [12] and indicate that there is proximity between experimental and theoretical concentration values of OXPt.

#### 4. Conclusion

This work showed the validation of a simple and selective HPLC method for OXPt quantification in pharmaceutical

delivery systems. The method was capable of selectively quantifying OXPt from any interference, that is, solvents or tested polymers (chitosan and poloxamer). Accuracy, precision, and low LOD and LOQ were demonstrated during all analysis.

#### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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