ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 1097–1103

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Validation of a LC method for the analysis of oxaliplatin in a pharmaceutical formulation using an experimental design☆

R. Ficarra [b], M.L. Calabrò [b], P. Cutroneo [a], S. Tommasini [a], S. Melardi [a], M. Semreen [a], S. Furlanetto [c], P. Ficarra [a,*], G. Altavilla [d]

[a] *Dipartimento Farmaco-Chimico, Università di Messina, Viale Annunziata, 98168 Messina, Italy*
[b] *Dipartimento di Scienze Farmaco-Biologiche, Università di Catanzaro 'Magna Græcia', Complesso 'Ninì Barbieri', 88021 Roccelletta di Borgia, Catanzaro, Italy*
[c] *Dipartimento di Scienze Farmaceutiche, Università di Firenze, Firenze, Italy*
[d] *Dipartimento di Patologia Umana-Oncologia Medica, Università di Messina, Messina, Italy*

Received 25 July 2001; received in revised form 20 October 2001; accepted 27 October 2001

Abstract

A rapid and sensitive RP-HPLC method with UV detection for routine control of oxaliplatin in a pharmaceutical formulation (Eloxatin®) was developed. Quantitation was accomplished with the internal standard method. The procedure was validated by linearity (correlation coefficient = 0.999948), accuracy, robustness and intermediate precision. Experimental design was used during validation to calculate method robustness and intermediate precision. For robustness test three factors were considered: percentage v/v of acetonitrile, flow rate and temperature; an increase in the flow rate results in a decrease of the drug found concentration, while the percentage of organic modifier and temperature have no important effect on the response. For intermediate precision measure the considered variables were: analyst, equipment and days. The RSD value (2.27%, $n = 24$) indicated a good precision of the analytical method. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Oxaliplatin; RP-HPLC; Validation; Robustness testing; Intermediate precision; Experimental design

1. Introduction

Oxaliplatin is a new derivative in which the central platin atom is surrounded by an oxalate and a 1,2 diaminocyclohexane (DACH) in the *trans* position (Fig. 1). As with other platin derivatives, oxaliplatin acts on DNA by producing alkyl bonds resulting in formation of intrastrand and interstrand cross-links and by inhibiting DNA synthesis and subsequent replication.

The pharmacokinetic of binding to DNA is a rapid event and occurs within a maximum of 15 min, while that of cisplatin is a two-phase event with a late phase lasting 4–8 h. In humans, measurement of adducts in white blood cells has

☆ Presented at the 9th International Meeting on Recent Developments in Pharmaceutical Analysis, Lipari, 5–8 June, 2001.

* Corresponding author. Fax: + 39-90-6766-407
E-mail address: pficarra@pharma.unime.it (P. Ficarra).

0731-7085/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(02)00151-6

shown their presence 1 h after treatment. Synthesis of DNA by replication and subsequent separation thus are inhibited as well as subsequent RNA and cell protein synthesis [1].

The rapid pharmacokinetic and its ability of killing some cell lines resistant to cisplatin, make the oxaliplatin a very promising anticancer drug.

HPLC methods have been developed for determining cisplatin and some of the second-generation agents in body fluids. However, these methods have not been widely used for pharmacokinetic studies because of the obstacles to detection. In those methods, various detection systems have been adopted: off-line atomic absorption spectrophotometry [2], on-line inductively coupled plasma atomic emission spectrophotometry [3], quenched phosphorescence detection [4], reductive electrochemical detection with mercury drop electrode [5], the direct UV detection [6] and UV detection coupled with post column derivatization by potassium dichromate and sodium bisulfite [7,8].

This paper reports a rapid and sensitive HPLC determination method with UV detection, useful for routine control of oxaliplatin in pharmaceutical formulations and in pharmacokinetic studies. The method was validated by linearity, accuracy, precision and robustness. Experimental design was used during validation to evaluate method robustness and for the determination of intermediate precision.

2. Experimental

2.1. Apparatus

Different HPLC systems were used at the two laboratories involved in the studies. The specifies are provided below.

Lab. A: the HPLC 1 apparatus was a Perkin Elmer chromatographic system (series 410 liquid chromatograph) equipped with a septumless injector (Rheodyne 7125-075) and a column heather (Perkin Elmer TC 931). A variable wavelength diode array detector (Perkin Elmer LC 235) was used. Peak area integration was performed using a chromatographic data system (Perkin Elmer LCI 100 laboratory computing integrator). A Vydac reversed-phase C_{18} column (25 cm × 4.6 mm i.d., particle size 10 μm), thermostated at 22, 24, 26 °C, was used as the stationary phase.

Lab. B: the HPLC 2 apparatus was a Merck Hitachi chromatographic system pump (LaChrom L-7100) equipped with a septumless injector (Rheodyne 7125-075) and a column oven (LaChrom L-7300). An UV detector (LaChrom L-7400) was used. Peak area integrations were performed using a D-7000 HPLC system manager program. A Vydac reversed-phase C_{18} column (25 cm × 4.6 mm i.d., particle size 10 μm), thermostated at 24 °C, was used as the stationary phase.

The experimental design and statistical analysis of the data were performed, by Nemrod software [9] (LPRAI, Marseille, France).

2.2. Reagents

Lichrosolv® acetonitrile was purchased from Merck (Darmstadt, Germany). Water used in the mobile phase was deionized, distilled and filtered through a 0.22 μm Millipore (Bedford, USA) before use.

The determination of oxaliplatin in commercial formulation was carried out on Eloxatin® vials kindly obtained from Sanofi Winthrop (France). The composition of a vial is: oxaliplatin mg 50, lactose monohydrate mg 450.

2.3. Standard solutions

Two methanolic working stock solutions at a concentration of 0.7 mg ml^{-1} of oxaliplatin (solution A) and 0.8 mg ml^{-1} of internal standard flavone (solution B) were prepared in volumetric flasks.

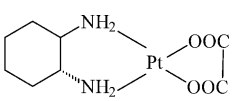


Fig. 1. Structure of oxaliplatin.

2.4. Calibration procedure

In order to study the linearity of the response, aliquots of sol. A equal to 0.7, 0.49, 0.42, 0.21, 0.1 and 0.07 mg ml^{-1} were accurately withdrawn and added with 0.5 ml (0.4 mg) of sol. B. Before injecting solutions, the column was equilibrated for at least 30 min. with the mobile phase flowing through the system. Quantitation was accomplished using an internal standard method. Five determinations were carried out for each solution. Peak areas were recorded for all the solutions. The correlation graph was constructed by plotting the peak areas obtained at the optimum wavelength of detection versus the injected amounts.

2.5. Chromatographic conditions

The mobile phase was a mixture of acetonitrile/water (80/20, v/v).

The flow rate was 0.8 mL min^{-1}. The UV detector wavelength was set at 255 nm and was used an attenuation of 0.05 a.u.f.s.. The temperature was set at 24 °C.

3. Results and discussion

The applied chromatographic conditions permitted to obtain a good separation of oxaliplatin and internal standard in a short time (Fig. 2), meanwhile no drug decomposition was observed. The slight tailing of the oxaliplatin peak is an effect of the underlying integration line, altering the gaussian curve. The LC method was validated for the parameters reported below.

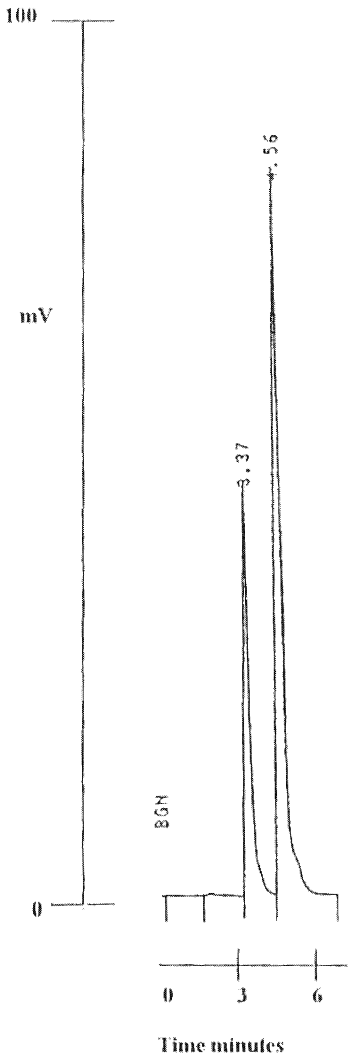


Fig. 2. Chromatogram of a solution containing oxaliplatin (tr. 3.37) at a concentration of 0.49 mg ml^{-1} and internal standard (tr. 4.56) at a concentration of 0.08 mg ml^{-1} at the described chromatographic conditions.

3.1. Linearity

Oxaliplatin and internal standard were chromatographed using a mixture of acetonitrile and water (80/20, v/v). The flow-rate was 0.8 mL min^{-1}.

The linearity of peak area responses versus concentrations was studied from 0.7 to 0.07 mg ml^{-1} for oxaliplatin. A linear response was observed over the examined concentration range, with a R^2 of 0.999948 and a cross-validated R^2 of 0.99989 [10]; an intercept of 4.73×10^{-3} (RSD = 1.70×10^{-4}) and a slope of 0.9449 (RSD = 3.90×10^{-4}).

3.2. Accuracy and repeatability

Accuracy was studied using three different solutions, containing 0.7, 0.49 and 0.07 mg ml^{-1} of oxaliplatin. Recovery data are reported in Table 1. The obtained values were within the range of

99.99 and 98.86%, RSD was 0.56%, satisfying the acceptance criteria for the study.

The system repeatability was calculated from ten replicate injections of oxaliplatin solutions at the analytical concentration of about 0.7 mg ml^{-1}; the RSD% found was 0.15.

3.3. Robustness testing

As defined by the ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters [11]. In order to study the simultaneous variation of the factors on the considered responses, a multivariate approach using design of experiments is recommended in robustness testing.

A response surface method was carried out to obtain more information and to investigate the behaviour of the response around the nominal values of the factors. Response surface methodology (RSM) has the following advantages: (a) to allow a complete study where all interaction effects are estimated; (b) to give an accurate description of an experimental region around a center of interest with validity of interpolation [12–15]. Generally the large number of experiments required by standard designs applied in RSM discourage their use on the validation procedure. However, if an analytical method is fast and requires the testing of few factors (three or less), a good choice for robustness testing may be the central composite design (CCD), widely employed because of its high efficiency with respect to the number of runs required. A CCD in k factors requires 2^k factorial runs, $2k$ axial experiments, symmetrically spaced at $\pm\alpha$ along each variable axis, and at least one center point. Three to five center repetitions are generally carried out in order to know the experimental error variance and to test the predictive validity of the model [16].

Table 1
Accuracy for oxaliplatin

Concentration (mg ml^{-1})	n	Recovery (%)	RSD (%)
0.70	4	99.48	1.36
0.49	4	99.99	1.46
0.07	4	98.86	1.62
Mean	4	99.44	0.56

Table 2
Chromatographic conditions and range investigated during robustness testing

Variable	Optimized value	Range investigated
Mobile phase (CH_3CN/H_2O)	80/20	75/25–85/15
Flow rate (ml min^{-1})	0.8	0.6–1.0
Temperature (°C)	24	22–26

In order to study the variables at no more than three levels (-1, 0, $+1$), the design used in robustness testing of oxaliplatin is a face-centered design (FCD) with $\alpha = \pm 1$ [13,17].

Three factors were considered: percentage v/v of acetonitrile (x_1); flow rate ml min^{-1} (x_2); temperature °C (x_3). The experimental domain of the selected variables is reported in Table 2. The ranges examined were small deviations from the method settings and the considered response was the found drug concentration mg ml^{-1} (Y).

A three-factor FCD requires 18 experiments, including four replicates of the center point. The experimental plan and the corresponding responses are reported in Table 3. All experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the response.

A classical second-degree model with a cubic experimental domain was postulated.

Experimental results were computed by Nemrod software [9]. The coefficients of the second-order polynomial model were estimated by the least squares regression. The equation model for Y (found concentration) was as follows:

$$Y = 0.47097 + 0.0003x_1 - 0.1173x_2 - 0.0041x_3 + 0.02267x_1^2 - 0.02433x_2^2 + 0.03367x_3^2 - 0.01225x_1x_2 - 0.0060x_1x_3 - 0.0125x_2x_3$$

Only the factor flow rate (x_2) was significant for the regression model assumed.

The model was validated by the analysis of variance (ANOVA). The statistical analysis showed that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors, showing a good agreement between experimental and predicted values.

The interpretation of the results has to start from the analysis of the whole model equation rather than from the analysis of the single coefficients; it is important, for the response surface study, to consider also the factors whose coefficients are statistically non-significant. For this reason the analysis of the response surface plot is necessary.

As shown in Fig. 3(a–c), the analysis produces three-dimensional graphs by plotting the response model against two of the factors, while the third is held constant at a specified level, usually the proposed optimum. Fig. 3a shows a graphical representation of the isoresponse surface for variation of percentage of ACN (x_1) and flow rate (x_2), while the temperature (x_3) is maintained constant at its optimum of 24 °C. An increase in the flow rate results in a decrease of the found concentration (Y), while the percentage of organic modifier had no important effect on the response. Analogous interpretation may be derived by examining Fig. 3b that plots the factors flow rate (x_2) versus temperature (x_3). In Fig. 3c, where the factor flow rate is maintained constant, the method can be considered robust for the studied experimental response.

In conclusion, the analysis of response surface confirms that Y is not robust for factor x_2, thus a precautionary statement should be included in the analytical procedure for this factor.

3.4. Intermediate precision

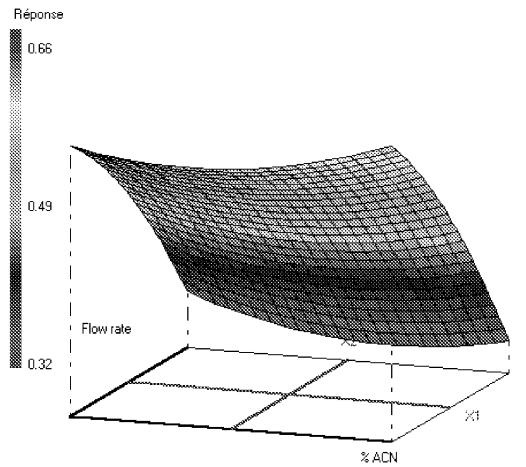
The intermediate precision is a measure of precision between repeatability and reproducibility and it should be established according to the circumstances under which the procedure is intended to be used. The analyst should establish the effects of random events on the precision of the analytical procedure.

The intermediate precision is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory [11].

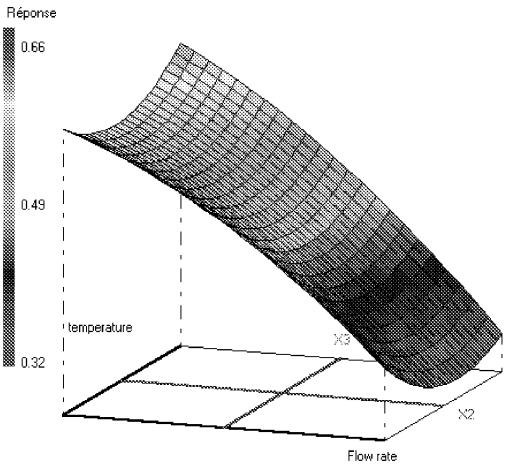
In order to study these effects simultaneously, a multivariate approach was used.

Table 3
Experimental plan for robustness testing and obtained responses

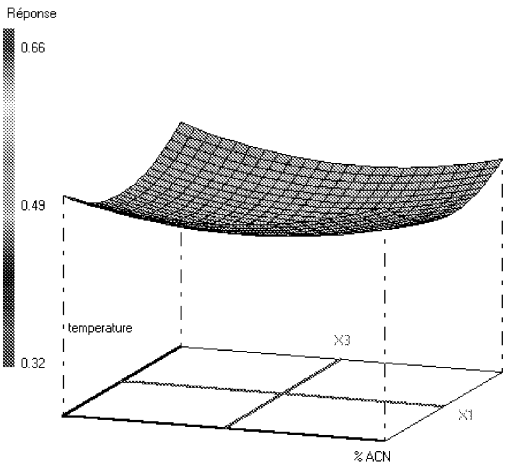
No. exp.	Random	Acetonitrile (%)	Flow (ml min^{-1})	Temperature (°C)	Found concentration (mg ml^{-1})
1	15	75	0.6	22	0.600
2	1	85	0.6	22	0.658
3	8	75	1.0	22	0.405
4	3	85	1.0	22	0.381
5	9	75	0.6	26	0.643
6	4	85	0.6	26	0.644
7	12	75	1.0	26	0.365
8	13	85	1.0	26	0.350
9	14	75	0.8	24	0.491
10	18	85	0.8	24	0.474
11	7	80	0.6	24	0.500
12	16	80	1.0	24	0.371
13	5	80	0.8	22	0.493
14	2	80	0.8	26	0.494
15	17	80	0.8	24	0.499
16	6	80	0.8	24	0.511
17	10	80	0.8	24	0.457
18	11	80	0.8	24	0.461



3a



3b



3c

Fig. 3. Three-dimensional plot of the response surface for Y (found drug concentration). (a) Variation of the response Y as a function of x_1 (% acetonitrile) and x_2 (flow rate); fixed factor: x_3 (temperature) = 24 °C. (b) Variation of the response Y as a function of x_2 (flow rate) and x_3 (temperature); fixed factor: x_1 (% acetonitrile) = 80% v/v. (c) Variation of the response Y as a function of x_1 (% acetonitrile) and x_3 (temperature); fixed factor: x_2 (flow rate) = 0.8 ml min^{-1}.

The considered variables included analysts (1 and 2), equipment (HPLC 1 and 2) and days (1 and 2). The considered response was the found drug amount (mg ml^{-1}). A linear model ($y = b_0 + b_1x_1 + b_2x_2 + b_3x_3$) was postulated and a 2^3 full factorial design was employed to estimate the model coefficients. Each experiment was repeated three times in order to evaluate the experimental error variance. The analyses were carried out in a randomized order according to the experimental plan reported in Table 4. The level of oxaliplatin was about 0.49 mg ml^{-1}. No considered factor was found significant for the regression model assumed. The RSD found (2.27%, $n = 24$) was acceptable, indicating a good precision of the analytical procedure.

Table 4
Experimental plan for intermediate precision testing and obtained responses

No. exp.	Random	Analyst	Instrument	Day	Found concentration (mg ml^{-1})
1	12	Analyst 1	HPLC 1	Day 1	0.498
2	9	Analyst 1	HPLC 1	Day 1	0.510
3	20	Analyst 1	HPLC 1	Day 1	0.509
4	23	Analyst 2	HPLC 1	Day 1	0.480
5	1	Analyst 2	HPLC 1	Day 1	0.481
6	21	Analyst 2	HPLC 1	Day 1	0.503
7	11	Analyst 1	HPLC 2	Day 1	0.481
8	18	Analyst 1	HPLC 2	Day 1	0.499
9	8	Analyst 1	HPLC 2	Day 1	0.482
10	3	Analyst 2	HPLC 2	Day 1	0.509
11	13	Analyst 2	HPLC 2	Day 1	0.488
12	22	Analyst 2	HPLC 2	Day 1	0.486
13	6	Analyst 1	HPLC 1	Day 2	0.479
14	16	Analyst 1	HPLC 1	Day 2	0.501
15	7	Analyst 1	HPLC 1	Day 2	0.481
16	24	Analyst 2	HPLC 1	Day 2	0.480
17	5	Analyst 2	HPLC 1	Day 2	0.501
18	2	Analyst 2	HPLC 1	Day 2	0.496
19	17	Analyst 1	HPLC 2	Day 2	0.510
20	10	Analyst 1	HPLC 2	Day 2	0.498
21	4	Analyst 1	HPLC 2	Day 2	0.482
22	19	Analyst 2	HPLC 2	Day 2	0.497
23	14	Analyst 2	HPLC 2	Day 2	0.481
24	15	Analyst 2	HPLC 2	Day 2	0.486

References

[1] E. Raymond, S.G. Chaney, A. Taamma, E. Cvitkovic, Ann. Oncol. 9 (1998) 1053–1071.
[2] C.M. Riley, L.A. Sternson, A.J. Repta, R.W. Siegler, J. Chromatogr. 229 (1982) 373–386.
[3] F.J. De Waal, M.J. Maessen, J.C. Kraak, J. Chromatogr. 407 (1987) 253–272.
[4] R.A. Baumann, C. Gooijer, N.H. Velthorst, R.W. Frei, I. Klein, W.J.F. van der Vijgh, J. Pharm. Biomed. Anal. 5 (1987) 165–170.
[5] M. Treskes, J. De Jong, O.R. Leewenkamp, W.J.F. van der Vijgh, J. Liq. Chromatogr. 13 (1990) 1321–1338.
[6] R. Kizu, K. Hayakawa, M. Miyazaki, Biomed. Chromatogr. 3 (1989) 14–19.
[7] K.C. Marsch, L.A. Sternson, A.J. Repta, Anal. Chem. 56 (1984) 491–497.
[8] R. Kizu, T. Yamamoto, T. Yokoyama, M. Tanaka, M. Miyazaki, Chem. Pharm. Bull. 43 (1995) 108–114.
[9] D. Mathieu, R. Phan-Tan-Luu, Nemrod software, LPRAI, Marseille, F-13331, France.
[10] I.E. Frank, R. Todeschini (Eds.), The Data Analysis Handbook, Elsevier, Amsterdam, 1994.
[11] International Conference on Harmonisation, Topic Q2B, Validation of Analytical Methods: Methodology. The Third International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, (ICH) Yokohama, Japan, 29 November–1 December, 1996.
[12] D.K. Lin, J. Quality Technol. 31 (1999) 61–66.
[13] K.K. Hockman, D. Berengut, Chem. Eng. 102 (1995) 142–8.
[14] H. Fabre, J. Pharm. Biomed. Anal. 14 (1996) 1125–1132.
[15] R. Ragonese, M. Mulholland, J. Kalman, J. Chromatogr. A 870 (2000) 45–51.
[16] G.A. Lewis, D. Mathieu, R. Phan-Tan-Luu, Pharmaceutical Experimental Design, Marcel Dekker, New York, 1999.
[17] S. Pinzauti, P. Gratteri, S. Furlanetto, P. Mura, E. Dreassi, R. Phan-Tan-Luu, J. Pharm. Biomed. Anal. 14 (1996) 881–889.