Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 435–439

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of an inductively coupled plasma mass spectrometry method with optimized microwave-assisted sample digestion for the determination of platinum at ultratrace levels in plasma and ultrafiltrate plasma

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ARTICLE INFO

Article history:
Received 31 October 2007
Received in revised form 2 April 2008
Accepted 3 April 2008
Available online 20 April 2008

Keywords:
Oxaliplatin
Inductively coupled plasma mass spectrometry
Platinum
Validation
Ultrafiltrate plasma
Plasma

ABSTRACT

A highly sensitive inductively coupled plasma mass spectrometry (ICP-MS) method with microwave-assisted sample digestion for the determination of total platinum in rat whole and ultrafiltrate plasma was developed and validated. A first step of this study concerned the optimization of the mineralization procedure, in order to obtain good extraction recovery (higher than 90%) and repeatability (less than 6%) and the absence of matrix effect. ICP-MS analysis was then performed using the "hot plasma/protective ion extraction" mode, achieving high sensitivity and very high signal/noise ratio. Iridium was added as internal standard. The method was then submitted to validation, performed according to the FDA Bioanalytical Validation Methods guidelines and to the Eurachem guide. Validation was carried out in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy and stability. An instrumental LOQ of 1.9 ng L^{-1}, corresponding to a concentration of 955 ng L^{-1} in matrix under the adopted conditions, was obtained, allowing the quantitative analysis of Pt ultratraces. Instrumental linearity was verified in the range 1.9–14,000 ng L^{-1}, corresponding to a concentration range from 955 ng L^{-1} to 6825 μg L^{-1} in matrix. Accuracy was evaluated by analyzing control samples for both matrices at different concentration levels; a good agreement (<15%) was obtained. Sample stability was tested by analyzing control samples maintained for 4 h at room temperature or submitted to three freezing–thawing cycles. Finally, the developed method was applied to the analysis of plasma and ultrafiltrate plasma of rats treated with oxaliplatin-base drug, thus demonstrating its reliability in pharmacokinetic studies.

1. Introduction

Platinum coordination complexes, such as cisplatin, carboplatin and oxaliplatin, are widely used in the treatment of a variety of tumors. In particular, oxaliplatin, a 1,2-diaminocyclohexane (DACH)–Pt complex, is used in the treatment of testicular, colorectal and ovarian cancer [1]. In order to better understand the action of these drugs, the knowledge of the concentration of the active principle and related metabolites even at trace levels as well as the way of its elimination plays a key role (basic pharmacokinetic) [2]. To achieve this goal, the development and validation of accurate and sensitive analytical methods is required, allowing to follow the last part (low concentration range) of the kinetic curve and the long-term retention of the drug that can cause unexpected adverse effects. Moreover a sensitive method could dramatically reduce the quantity of biological fluid required, allowing to perform pharmacokinetic studies in small animals.

The analytical techniques employed for the determination of Pt in biological fluids have been well documented and reviewed since the early 1990 [3–5]. In the past, atomic absorption spectrometry (AAS) had been the method of choice for platinum analysis, due to its great reliability. However, the major limitation of this method is a lack of the adequate sensitivity required for pharmacokinetic studies, as reported by a previous study [6]. Presently, inductively coupled plasma mass spectrometry (ICP-MS) is the method of choice for the determination of Pt in biological fluids since it has been demonstrated to be a valuable tool for the detection of drug containing metals in metabolism studies, due to its excellent sensitivity and selectivity [7–12]. Platinum has six isotopes (^{190}Pt, ^{192}Pt, ^{194}Pt, ^{195}Pt, ^{196}Pt, ^{198}Pt). Two of these, ^{194}Pt and ^{195}Pt, with relative abundances of 32.9% and 33.8%, respectively, are free from the most common interferences and could be useful for the determination of Pt at low concentration levels. The main drawbacks in ICP-MS determination of metals, in particular

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doi:10.1016/j.jpba.2008.04.013

when biological matrices are involved, are non-spectroscopic interferences from the matrix and the memory effect in the introduction system that can cause a carryover and virtually hamper the determination of the analyte at very low concentration levels. In order to minimize these effects usually a high dilution (100- or 1000-fold) is performed, with a substantial degradation of the method performance in terms of detection limit and precision.

Moreover, when ultratrace levels of metals should be determined in complex matrices, a sample treatment method showing high recovery and repeatability is requested.

Microwave-assisted mineralization represents a suitable alternative between the time consuming conventional open-vessel digestion and the dilution methods, enabling relatively fast, complete and reproducible digestions [13]. Most of the microwave equipments available on the market being designed to treat relatively large sample aliquots, lack of reproducibility when used with few microliter samples as the biological ones, due to the inhomogeneity of the radiofrequency field. For this reason special vessels and a further optimization of the suggested time/power cycle are required.

The aim of this work was the development and the validation of an ICP-MS method with optimized microwave-assisted sample digestion for the determination of total Pt in plasma and free Pt (the drug fraction unbound to plasma proteins) in ultrafiltrate plasma. The reliability of the developed method in pharmacokinetic studies was evaluated by analyzing plasma and ultrafiltrate plasma of rats treated with an IV dose (10 mg/kg) of oxaliplatin.

2. Materials and methods

2.1. Chemicals

Pt stock solution (1000 mg L^{-1}) was purchased from Carlo Erba (Milan, Italy), Ir stock solution (1000 mg L^{-1}) were from Sigma Aldrich (St. Louis, MO, USA). Nitric acid 65.5% (w/w), HCl 37% (w/w) and H_2O_2 30% (w/w) Ultrex II grade from J.T. Baker (Deventer, The Netherlands) were used without further purification. Oxaliplatin (*R*, *R*) was kindly provided by Nerviano Medical Science. Deionised water was produced with an Element A10 equipment (Millipore, Bedford, MA, USA). Intermediate standard solutions were prepared weekly from the stock solution with diluted hydrochloric acid (10%, w/v) while the working standards were diluted daily.

2.2. Samples

Plasma samples were obtained by centrifuging whole blood for 5 min (3000 rpm, 4 °C). Ultrafiltrate plasma samples were obtained by centrifuging the plasma fraction through a 30-kDa cut-off ultrafiltrate filter (Centriplus YM-30, Millipore, Bedford, MA, USA) for 15 min (3000 rpm, 20 °C). Plasma and ultrafiltrate plasma samples were transferred in polyethylene vials and stored at −18 °C until analysis.

Method development and validation study were performed on plasma and ultrafiltrate plasma of drug-untreated rats spiked with appropriate amount of Pt standard solutions.

In addition, for each matrix, quality control samples at two different concentration levels, namely Low Quality Control (LQC; 1970 ng L^{-1} for ultrafiltrate plasma and 19,700 ng L^{-1} for plasma) and High Quality Control (HQC; 392 mg L^{-1} for ultrafiltrate plasma and 3920 mg L^{-1} for plasma), were prepared and independently certified by Nerviano Medical Science with a internal protocol.

Finally, the reliability of the method was assessed by analyzing different samples of plasma and ultrafiltrate plasma obtained from rats treated with an IV dose (10 mg/kg) of oxaliplatin. The in vivo part of this study was performed in Nerviano Medical Sciences laboratories.

2.3. ICP-MS conditions

Experimental measurements were made on the ICP-MS X SeriesII (ThermoFisher Corporation, Waltham, MA, USA) equipped with a AS-500 autosampler (CETAC, Omaha, NE, USA) operating under X_i interface standard conditions. The operating parameters of ICP-MS instrument were as follows: RF power 1400 W; coolant gas flow 15.5 L min^{-1}; auxiliary gas flow 0.98 L min^{-1}; nebulizer gas flow 0.87 L min^{-1}; nickel standard X_i cones; peak jumping data acquisition mode: dwell time 100 ms; duration time 60 s; standard resolution. The instrument optimization in hot plasma/protective ion extraction mode was performed daily with the autotune procedure to assure a response of at least 80,000 cps/μg L^{-1} for indium and 100,000 cps/μg L^{-1} for uranium in the high mass range. Data acquisition and all the calculations for internal standard correction were performed with the software Plasmalab 2.02 (ThermoFisher Corporation, Waltham, MA, USA).

All the platinum and iridium isotopes were acquired, but only ^{195}Pt and ^{193}Ir were used for calculations.

2.4. Microwave-assisted mineralization

In order to avoid contamination due to the environmental presence of ultratrace levels of platinum, all glassware, quartzware and polyethylene vials were carefully cleaned with deionised water, then treated with aqua regia for 10 min and finally rinsed with high-purity deionised water.

Mineralization of plasma and ultrafiltrate plasma samples was performed in a MLS-1200 MEGA (Milestone, Sorisole, Italia) apparatus equipped with a MDR-1000-6 rotor.

Each digestion vessel consisted of a 20 mL quartz sample holder inserted in a hollow Teflon® container. 0.9 mL of HCl 37% (w/w) and 0.3 mL of HNO_3 65% (w/w) were added in the quartz vial to 20 μL of appropriately Pt-spiked plasma and ultrafiltrate plasma samples. Then, 0.2 mL of Ir 5 μg L^{-1} solution was added as internal standard. The Teflon® container was then filled with 8 ml of H_2O_2 5% (w/v) to counterbalance pressure via re-oxidation of nitrogen oxides produced during the mineralization process and sealed.

Several time/power cycles of mineralization were tested in order to optimize extraction recovery and repeatability, starting from conditions suggested by the manufacturer:

- Initial program (irradiation time @ power): 2 min @ 250 W; 2 min @ 0 W; 6 min @ 250 W and 5 min @ 650 W.
- Final (optimized) program: 1 min @ 250 W; 3 min @ 0 W; 6 min @ 250 W; 2 min @ 400 W and 1 min @ 600 W.

After cooling, the solution was transferred in a volumetric flask and diluted with deionised water to the final volume of 10 mL.

2.5. Validation

Method validation was carried out according to the FDA Bioanalytical Method Validation guidelines [14] and to Eurachem recommendations [15].

Detection limit (y_D) and quantitation limit (y_Q) expressed as signals were calculated as follows:

$$y_D = \mu_B + 2t_{(\alpha=0.05;\upsilon=n-1)}\sigma_B$$

$$y_Q = \mu_B + 10\sigma_B$$

where μ_B is the mean signal of blank analysis ($n = 10$), σ_B is the standard deviation of blank analysis ($n = 10$), $t_{(\alpha=0.05;\ \upsilon=n-1)}$ is the Student's t-value at α significance level and υ degree of freedom.

The blanks were obtained by applying the full microwave procedure.

A calibration line in the range 1.5–6 ng L^{-1} was used to calculate the corresponding concentration values of limit of detection (LOD) and limit of quantitation (LOQ).

Instrumental linearity was evaluated in the range 1.9–14,000 ng L^{-1}, corresponding to a concentration range from 955 ng L^{-1} to 6825 μg L^{-1} in matrix, considering 13 concentration levels and performing 10 replicated analysis for each concentration level.

Homoschedasticity was preliminarily verified by applying the Cochran test [16].

Repeatability and inter-day precision were calculated in terms of R.S.D.% by analyzing for each matrix the LQC and the HQC in three different days and with three replicated analysis per day.

The same quality control samples were used to assess accuracy, which was calculated in terms of percentage recovery. Sample stability was tested by analyzing quality control samples maintained for 4 h at room temperature or submitted to three freezing–thawing cycles.

All statistical analyses were performed by using the SPSS v. 9 software package (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. ICP-MS conditions

As preliminary work, the relative sensitivity of the instrument versus the different species of Pt was calculated by comparing the slopes of the calibration curves obtained from mineralized solutions of Pt standards (platinum chloride complex) and oxaliplatin; no significant differences were found as shown in Table 1.

In addition, the use of the "hot plasma/protective ion extraction" mode, with a positive voltage on the frontal extraction lens, allows the achievement of very good detection limits since sputtered and matrix-related ions, that are the main responsible of unspecific noise, could not reach the quadrupole analyzer, due to kinetic discrimination: as a result very low blank counts (few cps) were measured regardless of the species used. Finally, the absence of relevant spectroscopic interferences was verified by comparing the various isotope ratios for platinum and iridium both in the standard solutions and in spiked matrices.

3.2. Microwave-assisted mineralization

The ICP-MS determination of metals at very low concentration levels is seriously hampered by the sticking of the biological matrix, namely proteins and non-polar compounds, on the system introduction walls. This could cause in turn, long-term unpredictable effects on the resulting concentration values, due to the selective getter of the analytes on the contaminated walls and their subsequent release, for instance when the pH of the delivered solution is changed. This behavior could be not easily corrected by the use of internal standard since it is strictly dependent on the nature of the element determined. For this reason the simple dilution of the samples was not performed and the mineralization process was selected.

Table 1
Comparison of sensitivity vs. different forms of platinum

Pt sdt regression	$y = (99 \pm 3) + (28.7 \pm 0.5)x$, $R^2 = 0.996$ ($n = 18$)
OxaliPt regression	$y = (107 \pm 7) + (26 \pm 1)x$, $R^2 = 0.970$ ($n = 18$)
t-Value	−1.83
t-Critical ($\alpha = 0.05$; $\upsilon = 32$)	2.03

Table 2
Calibration curves obtained by using the different mineralization programs

Standard solution initial	$y = (20.6 \pm 1.5) + (25.71 \pm 0.24)x$, $R^2 = 0.995$ ($n = 54$)
Ultrafiltrate plasma initial	$y = (8.2 \pm 1.2) + (24.31 \pm 0.19)x$, $R^2 = 0.998$ ($n = 54$)
Plasma initial	$y = (11.5 \pm 1.3) + (16.82 \pm 0.21)x$, $R^2 = 0.992$ ($n = 54$)
Standard solution final	$y = (85.91 \pm 2.69) + (56.75 \pm 0.42)x$, $R^2 = 0.997$ ($n = 54$)
Ultrafiltrate plasma final	$y = (81.5 \pm 3.0) + (57.84 \pm 0.46)x$, $R^2 = 0.997$ ($n = 54$)
Plasma final	$y = (84.0 \pm 2.8) + (57.67 \pm 0.44)x$, $R^2 = 0.997$ ($n = 54$)

The fit-for-purpose of the mineralization process was evaluated by comparing the results obtained from the treatment of simple standard solutions and spiked plasma and plasma ultrafiltrate matrices.

Three concentration levels (1, 5, 10 ng L^{-1}) were considered; each solution was mineralized in triplicate and 6 instrumental replicated analyses were performed for each extract, giving a total of 18 replicated for each concentration level.

Results obtained by using the initial microwave power program are shown in the first part of Table 2.

A more than 100% scattering of the replicate data was observed in both the data from standard solution samples and from the spiked-matrix ones when the above time/power cycle was used.

A mean recovery of 86% was obtained for ultrafiltrate plasma and of 62% for plasma.

In order to asses the matrix effect presence, the slopes of the regression curves were compared by means of a t-test. For both matrices, the calculated t-value, respectively $t = 27.87$ for plasma and $t = 4.57$ for ultrafiltrate plasma, resulted higher than the critical t-value ($t_{(\alpha=0.05;\ \upsilon=104)} = 1.98$), meaning that an effect due to the matrix is present.

The ratio between the acid mixture and the sample volumes suggests that a classical matrix effect, i.e. due to incomplete mineralization, is hard to believe, while the data scattering even in the standard solution suggested that the mineralization process could be too strong causing the analyte loss by dissolution in the surrounding hydrogen peroxide solution.

All the subsequent steps were aimed at trying more mild mineralization conditions.

Final results obtained by using the optimized mineralization program are shown in the last part of Table 2.

By using the optimized program a very good reproducibility was obtained (R.S.D.% <5) and the mean recovery rose up to 100% for ultrafiltrate plasma and to 102% for plasma.

Using the final mineralization program, no effect due to the different matrices was detected; in fact the calculated t-values, respectively $t = 1.51$ for plasma and $t = 1.75$ for ultrafiltrate plasma, resulted lower than the critical t-value ($t_{(\alpha=0.05;\ \upsilon=104)} = 1.98$).

Since the plasma matrix is considered much heavier than the ultrafiltrate one, these results indirectly demonstrate also the absence of non-spectroscopic interferences, typical of the dilution-based methods [12], making unnecessary the verification of the effectiveness of the internal standard and probing the absence of sticking in the sample introduction system.

On the basis of these findings, also considering the low volumes of plasma and ultrafiltrate plasma available, further validation experiments were performed by using mineralized standard solution for calibration purposes.

3.3. Validation

Very low LOD and LOQ values were calculated, being LOD equal to 0.9 ng L^{-1} and LOQ equal to 1.9 ng L^{-1}, corresponding, respec-

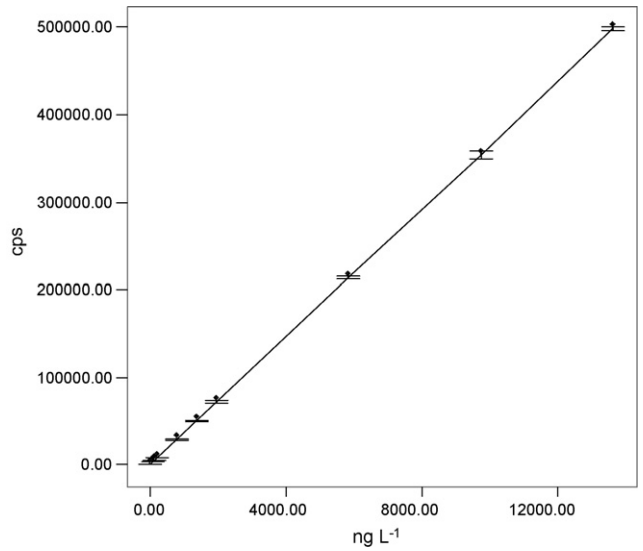

Fig. 1. Calibration curve obtained from mineralized standard solutions in the range 1.9–14,000 ng L^{-1}, R^2 = 0.999 (n = 130).

tively to a concentration of 452 ng L^{-1} and of 955 ng L^{-1} in matrix under the adopted conditions.

These findings demonstrate the capability of the developed method of quantifying platinum at ultratrace levels and the benefits of the "hot plasma/protective ion extraction" mode configuration, since the blank counts were very low.

Linearity was evaluated in the range 1.9–14,000 ng L^{-1} in the mineralized solution considering 13 concentration levels and performing 10 replicated analysis for each concentration level.

Homoscedasticity was preliminarily verified by applying the Cochran test.

The results of Cochran test showed that the variance significantly differs among concentration levels at 0.05 significance level; in particular, it can be observed that variance increases with the concentration level, while the relative standard deviation is quite constant (about 5%).

When homoscedasticity is not verified, the simple least-squares procedure cannot be used without reducing the reliability of the estimation [16]; nevertheless the problem of the non-constant variance can be overcome by using the weighted least-squares regression [17].

The regression model calculated by mean of the weighted least-squares procedure was described by the following equation:

$$y = (28.04 \pm 2.2) + (36.9 \pm 0.1)x, \quad R^2 = 0.999 \ (n = 130)$$

Calibration curve is shown in Fig. 1.

The wide linearity range allows the analysis of plasma and ultrafiltrate plasma samples with very different platinum concentration levels, as requested in pharmacokinetic studies.

Quality control samples were used to verify intra-day precision (repeatability), inter-day precision (intermediate precision) and accuracy. The results, reported in Table 3, confirm the suitability of the developed method for the intended application.

Table 3
Precision, recovery and accuracy on quality control samples

Quality control sample	CV% intra-day	CV% inter-day	Recovery (%)	Bias (%)
Ultrafiltrate-LQC	4	8	98 ± 8	−2
Ultrafiltrate-HQC	2	3	96 ± 3	−3
Plasma-LQC	6	4	89 ± 3	−11
Plasma-HQC	3	2	87 ± 1	−13

Table 4
Stability of quality control samples under different storage conditions

Quality control sample	Treatment	cps (mean ± S.D.)
Ultrafiltrate-LQC	None	10.81 ± 0.35
Ultrafiltrate-LQC	4 h RT	11.08 ± 0.65
Ultrafiltrate-LQC	Three freeze–thaw cycles	10.93 ± 0.41
Ultrafiltrate-HQC	None	1,950 ± 34
Ultrafiltrate-HQC	4 h RT	1,979 ± 20
Ultrafiltrate-HQC	Three freeze–thaw cycles	1,975 ± 55
Plasma-LQC	None	87 ± 4
Plasma-LQC	4 h RT	90 ± 4
Plasma-LQC	Three freeze–thaw cycles	92 ± 2
Plasma-HQC	None	17,085 ± 371
Plasma-HQC	4 h RT	17,134 ± 786
Plasma-HQC	Three freeze–thaw cycles	17,163 ± 421

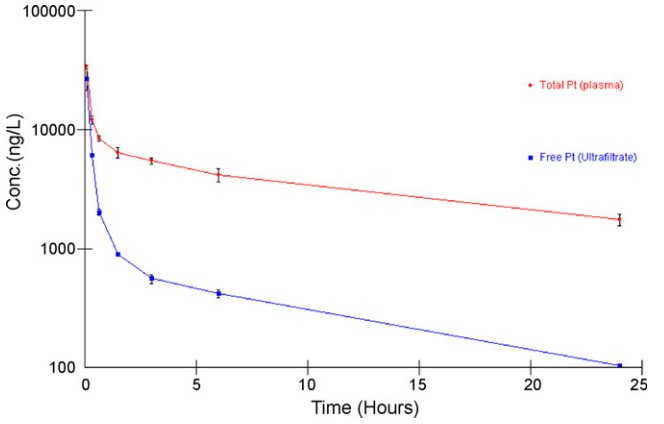

Fig. 2. Profile of ultrafiltrate plasma and plasma concentrations vs. time in treated rats (dose 10 mg kg^{-1}).

Finally, the stability under different storage conditions of the quality control samples was tested, verifying the presence of significant differences by means of a t-test; not significant differences were observed, meaning that the considered samples are stable under the adopted storage conditions (Table 4).

As a last step, the reliability of the developed method in pharmacokinetic studies was evaluated by analyzing plasma and ultrafiltrate plasma of rats treated with an IV dose (10 mg kg^{-1}) of oxaliplatin collected at different time after the drug administration and obtained from Nerviano Medical Sciences.

As example, Fig. 2 shows the profile of the Pt concentration versus time in plasma and ultrafiltrate plasma samples.

4. Conclusions

The optimized sample treatment method based on microwave mineralization showed a very good reproducibility and absence of non-spectroscopic interferences. The more labor-intense sample preparation is well counterbalanced by the subsequent very easy ICP-MS determination, due to the absence of sticking on the sample introduction system. The detection limits are comparable to those reported in the latest papers [10] but with very good long-term stability. This method is therefore suitable when very large batch of samples should be determined.

The suitability of the developed method for the intended purpose was demonstrated, taking also into account the very low volumes of plasma and ultrafiltrate plasma availables.

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