Application of atomic absorption spectroscopy method for platinum content determination to study functionalization of bone substitute materials with anticancer drug

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Abstract. Methods of quantitative determination of the platinum content in high-salt solutions have been developed using atomic absorption spectroscopy (AAS). Definitions were carried out for small volume bioassays during the research of functionalization of calcium phosphate substitute materials with anticancer drug, cisplatin. The conditions for the determination of Pt in the flame were optimized. The effects of the cisplatin matrix solution s, salt components and concentrations on the Pt absorption value were found out. The relevance of application of the flame version of the AAS method was shown in order to determine the platinum content in high-salt solutions, over 200 g/l, without matrix separation in a wide range of Pt concentrations from 0.1 to 50 µg/ml. This issue was targeted through the estimation of efficiency of Pt-containing drug incorporation and its dynamics release as an application method in the development of drug delivery systems for bone tumors treatment.

1. Introduction

Improvement of approaches to chemotherapeutic treatments of skeletal metastases and fractures after resection has been developed intensively to be used for drug delivery to a local region of the pathology [1-3]. Major complications of the chemotherapy involve the maximum effect of therapeutic agents on the tumor with minimal toxicity. The anticancer agents, based on complexes of divalent platinum (cisplatin, carboplatin, platin), are the most active and popular in the chemotherapeutic treatment. However, these drugs are of high toxicity, have broad side effects and, in addition, have low bioavailability to bone tissue.

After surgically removing of damaged bone area the osteoplastic materials are used to stimulate the processes of new bone formation. Being the carriers for drugs, released directly into the area of tumor lesion, these materials serve as an alternative to the chemotherapeutic treatment [4,5]. The application of bone substitute materials functionalized with drugs provides significant reduction of the systemic toxicity of drugs together with antitumor efficacy comparable to systemic dose [6]. Recent approaches
to the biomaterials functionalization with anticancer drugs have been actively developed for bone reconstruction and treatment [7-9].

In the proposed work octacalcium phosphate (OCP) ceramics were chosen as a platform for drug functionalization. OCP, a new generation material, is considered to be a possible precursor of biological bone apatite and possess enhanced osteoconductive properties [10,11]. Cisplatin (cis-diaminedichloroplatinum) was chosen as a medicinal antitumor drug. The OCP functionalization was obtained by cisplatin incorporation onto ceramics surface by co-precipitation of calcium phosphates and cisplatin from water solutions with different calcium and phosphate ions contents. Evaluation of the cisplatin incorporation efficiency and its dynamics release is an essential issue in the development of an effective method of ceramic functionalization. It requires to identify platinum content of small-volume high-salt bioassays in a wide range of concentrations from 0.05 to 50 µg/ml.

Quantitative determination of platinum content in drugs can be carried out by application of different methods: gravimetry, titrimetry neutron activation, electrochemical method, spectrophotometry, atomic absorption spectrometry — flame and electrothermal versions, atomic emission spectrometry with inductively coupled plasma (AES-ICP), mass spectrometry with inductively coupled plasma (MS-ICP) [12-15]. Comparative study of proposed analytical methods showed the rational application of the flame version of atomic absorption spectroscopy (AAS) in the determination of platinum content in metal-containing drugs. Low detection limits, selectivity, high accuracy, expressiveness and ease of implementation put this method in a number of AES-ICP and MS-ICP methods in terms of competitiveness. Possibility of Pt determination by AAS in drugs and metal-containing compounds has been demonstrated earlier [16,17]. The represents are not complete and also are contradictory; do not reveal several specific for our work subjects and that is the influence of the complex systems composition on parameters of AAC determination of Pt content. The methods of matrix separation and concentration are quite embarrassing in the small-volume bioassays. This work is devoted to the adaptation of AAS parameters and data estimation in order to determine platinum content in high-salt solutions of complex composition without matrix separation in a wide range of concentrations.

2. Materials and methods

2.1. Materials

All reagents used in the work corresponded to the qualification of “analytical grade” and higher. Standard platinum solutions were prepared from high purity metal [18] or from “Merk” fixanal with Pt concentration of 1.00 mg/ml. Cisplatin was of “Sigma-Aldrich” with purity of 99.9% (Cat. No. 2.15663-27-1). Cisplatin is a complex chloride-ammonia of bivalent platinum, cis-[Pt(NH3)2Cl2], an antitumor drug. Aqueous and aqueous-salt solutions of cisplatin with concentration of 1 mg/ml were prepared, in which Pt content was calculated of 0.65 mg/ml. OCP (Ca8H2(PO4)4 ∙ 5H2O) porous ceramic granules of average size 500-1000 µm have been used as biomaterial.

2.2. Analytical equipment and methods

The research was carried out using atomic absorption spectrometer iCE 3000 (Thermo Fisher Scientific, USA). Platinum determination was performed in the flame of acetylene-air with deuterium background corrector. Lamp with a hollow cathode served as the source of resonance radiation. The sensitivity of AAS determination of platinum was assessed by the magnitude of the analyte concentration that provides 1% absorption or absorbance A = 0.0044 [12].

The microwave sample preparation system MARS 5 (CEM Corporation, USA) was used for cisplatin destruction. The procedure was consisted of destruction of 5 ml of cisplatin solution in 5 ml of mixture of the concentrated nitric and hydrochloric acids within 30 minutes at temperature of 210°C and at pressure of 2400 kPa.
2.3. Procedure
Functionalization of OCP ceramics with cisplatin was performed by cisplatin solution infiltration into ceramic granules. Different buffer solutions with pH value 7.4 were prepared as matrix solutions for cisplatin: PB 1M (Phosphate Buffer saline with cation concentration of 1 Mol), PB 1.8 mM (Phosphate Buffer saline with cation concentration of 1.8 mMol), SCS (Supersaturated Calcification Solution), SCSm (SCS modified), TRIS (tris(hydroxymethyl)aminomethane buffer solution) (Table 1). Incorporation solutions were prepared by dissolution of cisplatin in buffer solutions and distilled water to get the cisplatin concentration of 1 mg/ml. PB 1M, PB 1.8 mM, SCS, SCSm were used as supersaturated calcium and/or phosphate ions solutions. During incubation of calcium phosphate ceramics in such buffer solutions at 37°C, the new amorphous calcium phosphate coating had formed onto its surface via biomimetic precipitation [19]. The SCSm was of the same ion and concentration compound as SCS, only had NO3− ions instead of Cl− ions, since it is known that the interaction of cisplatin with calcium phosphates took place in absence of Cl− ions in incorporation solution [20]. The H2O and TRIS were taken for comparison of matrix solution impact on cisplatin incorporation.

The incorporation procedure was carried out by incubation of 50 μg of ceramic granules in 1 ml of incorporation solution at 37°C during 48 h and constant stirring. The solutions were sampled to get the assays after incubation.

The dynamics release of cisplatin from the ceramics was studied for 7 weeks in DPBS (Dulbecco’s Phosphate-Buffer Saline). The functionalized materials were placed in tubes with 1.0 ml of DPBS. The buffer solutions were completely sampled in established terms to determine the Pt content.

Table 1. Compound of the solutions used in experiments

| Solution designation | Solution compound | Salt concentration, g/l |
|---------------------|------------------|------------------------|
| H2O                 | H2O              | 0                      |
| TRIS                | (HOCH2)3CNH2     | 1.21                   |
| PB 1M               | K+; HPO4−2; H2PO4−; pH = 7,4 | 230                   |
| PB 1.8 mM           | K+; HPO4−2; H2PO4−; pH = 7,4 | 0.414                 |
| SCS                 | Na+; K+; Ca2+; Cl−; HPO4−2; pH = 7,4 | 8.72              |
| SCSm                | Na+; K+; Ca2+; NO3−; HPO4−2; pH = 7,4 | 10.8              |
| DPBS                | Na+; K+; Mg2+; Ca2+; Cl−; HPO4−2; | 9.93               |
| NaCl                | Na+, Cl−         | 9.00                   |

2.4. Optimization of analysis conditions for the determination of Pt
The absorbance measuring of platinum was performed at a slit width of 0.2 nm and at the analytical wavelength of 265.9 nm. At other resonance lines the Pt detection limit was reduced in 2-10 times. It was found out that the maximum Pt absorption was executed at atomization in stoichiometric air-acetylene flame (figure 1), in the area of 10 mm above the burner. The enriched air-acetylene flame reduced the sensitivity of platinum determination in 2 times. In the nitrous oxide-acetylene flame the Pt absorption was in several times feebler.

2.5. Influence of acid nature and concentration on Pt signals intensity
The effects of the concentration of acids contained in experimental samples (HCl, HNO3, H2SO4 and H3PO4) on the platinum analytical signals were studied. Figure 2 shows the dependence of the atomic absorption of Pt on the nature and concentration of the acids. Thus, the effect of 2M HCl in the solution resulted in 9% reduction of Pt absorption, the 2M H2SO4 — of 25% reduction, and 2M H3PO4 — in 30% reduction. The platinum analytical signals were suppressed not more than of 5 % with increasing of both HCl and HNO3 concentration in solutions from 0.01 M to 1 M. The presence of phosphoric and sulfuric acids influenced significantly on the Pt absorption through depressing effects on the spray system. Uncontrolled volatilization in the concentration of the acids could lead to
the errors significantly exceeding the instrumental ones. Fortunately, a practical error could be avoided by maintaining an adequate acid content in samples and standard solutions.

![Figure 1](image1.png) **Figure 1.** AAS calibration curves for Pt determination in stoichiometric (1) and enriched (2) air-acetylene flame.

![Figure 2](image2.png) **Figure 2.** The dependence of the atomic absorption of Pt on the nature and concentration of acids: 1, 2 — HNO₃; 3 — H₂SO₄; 4 — H₃PO₄.

2.6. Influence of acid nature and concentration on Pt signals intensity

The effects of the concentration of acids contained in experimental samples (HCl, HNO₃, H₂SO₄ and H₃PO₄) on the platinum analytical signals were studied. Figure 2 shows the dependence of the atomic absorption of Pt on the nature and concentration of the acids. Thus, the effect of 2M HCl in the solution resulted in 9% reduction of Pt absorption, the 2M H₂SO₄ — of 25% reduction, and 2M H₃PO₄ — in 30% reduction. The platinum analytical signals were suppressed not more than of 5% with increasing of both HCl and HNO₃ concentration in solutions from 0.01 M to 1 M. The presence of phosphoric and sulfuric acids influenced significantly on the Pt absorption through depressing effects on the spray system. Uncontrolled vacillations in the concentration of the acids could lead to the errors significantly exceeding the instrumental ones. Fortunately, a practical error could be avoided by maintaining an adequate acid content in samples and standard solutions.

2.7. Influence of cisplatin coordination complex on the Pt determination

Figure 3 demonstrates the calibration curves of AAS Pt determination for pure standards, cisplatin solutions and DPBS solutions contained cisplatin. There were several factors that occurred during the detection. Firstly, in standard solutions there was the tetravalent Pt, and in cisplatin solutions there was the divalent Pt. The oxidation state of the element was influenced slightly on its signals in the flame version of the AAS method [12]. Secondly, the cisplatin possessed the stability of complex compound that reduced significantly the method sensitivity. Figure 3 illustrates the salt background contribution to the Pt analytical signals through interaction between inorganic ions and cisplatin in DPBS solution. The temperature-time conditions of the air-acetylene flame (T = 2000°C) were not rigid enough for its completely destruction and for free platinum atoms production at the same extent as for pure solutions.

The influence of intramolecular bonds of cisplatin as the coordination complex of platinum on the Pt atomization in comparison with its inorganic standard was investigated. To reveal the influence of cisplatin coordination in matrix solution, the Pt determination was carried out both without decomposition and after decomposition of the matrix. Cisplatin destruction was performed in the microwave sample preparation system MARS 5. The platinum calibration graphs obtained after cisplatin degradation were identical to the pure Pt solutions. However, the preliminary acid decomposition of the matrix extended significantly the analysis time, especially for a large number of samples. In order to avoid preliminary mineralization procedure, the calibration curves against the
cisplatin background were plotted (figure 3). In this case the metrological characteristics were fallen down as well as the method sensitivity was decreased almost in 2 times. Despite this, the application of the method without matrix mineralization proved to be more economical and less time-consuming process, because it afforded to reduce significantly the analysis time of a large number of samples.

Figure 3. Calibration curves of AAS Pt determination in (1) – pure Pt solution, (2) – cisplatin solution, (3) – cisplatin in DPBS.

Figure 4. Calibration curves of AAS Pt determination in different salt solutions with cisplatin (Cis): 1 — Cis-H2O; 2 — Cis-TRIS; 3 — Cis-SCSm; 4 — Cis-PB 1,8 mM; 5 — Cis-SCS; 6 — Cis-PB 1M.

2.8. Influence of matrix composition on the Pt determination

It is known, that excess of inorganic salts in the samples influences on Pt analytical signals [13]. On the one hand, the buffering technique using the intentional salts introduction (copper and cadmium sulfates, lanthanum chloride and others) is applied to increase the platinum analytical signals. On the other hand, significant interference can appear due to high salt background (concentration of associative elements from 0.1 to 1 mg/ml) [13].

Influence of cationic and anionic composition of the experimental solutions and its concentrations on the analytical Pt signals were investigated. Figure 3 and 4 display the calibration graphs of AAS platinum determination in cisplatin solutions of different salt background. The sensitivity of Pt determination decreased in 1,5-3 times as a result of solutions matrices involvement. High salt concentrations from 9 (SCSm, table 1) to 200 g/l (PB 1M, Table 1) strongly inhibited the Pt absorption. The inhibitory effect amplified with increase of ions concentration that resulted in rising of slope and curvature of the graphs. As for analytical conditions, the spraying efficiency and the method sensitivity decreased. Presence of ions K⁺, Na⁺, Ca²⁺, Mg²⁺, PO₄³⁻ in the analyzed solution, even in amounts equal to the Pt content, led to changes both in the spray system and in the flame by that impaired Pt absorption [13]. The studied solutions were of high concentrations of these ions and of small concentrations of Pt (from 0.1 to 50 µg Pt/ml, Table1).

To reduce the effect of salt background the solutions with cisplatin had been pre-diluted to the Pt content not less than of 0,5 µg/ml. To improve the metrological characteristics it is preferably to carry out the Pt determination in the optimum range of concentrations (Table 2). To eliminate the negative influence of the matrix platinum standard solutions that modelled the full chemical composition of the analyzed samples (matrix ion composition, the acidity of the solutions) were prepared for the spectrometer calibration. In the sections of the calibration curves with a small violation of the linear dependence, the concentration of platinum in the analyzed solution was calculated by measuring the absorption of the analyte and two nearby standards.

The method of interactive matrix matching was used to eliminate matrix background of the same salt solutions, but of different salt concentrations (PB 1M and PB 1,8 mM) [15]. Such solutions were
diluted to the one background concentration, agreed with the calibration graph. Table 2 presents the metrological characteristics of Pt determination in experimental solutions of various compositions and concentrations. The Pt detection limit depended on the solution composition and varied from 0.05 to 0.50 µg/ml. The developed technique was described by relative standard deviation $S_r$ of the element definition not above 0.15. The results relevance was controlled by the “entered-found” way because of the absence of standard solutions. The convergence of results of Pt determination obtained by different methods was satisfactory (Table 3).

Table 2. Metrological characteristics of Pt determination in cisplatin solutions of different salt composition.

| Solution designation | Detection limit 3σ | Graph linearity | Optimal range | $S_r$ for optimal range |
|----------------------|---------------------|-----------------|---------------|------------------------|
| H$_2$O               | 0.05                | 0.05-100        | 1-20          | 0.014-0.005            |
| TRIS                 | 0.07                | 0.07-60         | 1-20          | 0.019-0.01             |
| PB 1M                | 0.5                 | 0.5-5           | 1-5           | 0.15-0.06              |
| PB 1.8 mM            | 0.1                 | 0.1-20          | 1-20          | 0.09-0.02              |
| SCS                  | 0.1                 | 0.1-20          | 1-20          | 0.08-0.02              |
| SCS$_m$              | 0.1                 | 0.1-25          | 1-20          | 0.10-0.02              |
| DPBS                 | 0.5                 | 0.5-10          | 1-5           | 0.12-0.05              |
| NaCl                 | 0.4                 | 0.4-10          | 1-10          | 0.12-0.04              |

3. Results

A series of experimental samples of various salt compositions were analyzed according to the developed AAS methods. The optimal conditions for the measurement of analytical signals were found out. The spectrometer calibration was performed by measuring of prepared standard cisplatin solutions modelling chemical composition and concentration of the analyzed samples. To eliminate matrix background the method of interactive matrix matching was successfully used. Incorporation effectiveness and dynamics release of cisplatin from the ceramic surface were investigated.

The estimation of amount of the incorporated cisplatin onto OCP ceramics was done by calculation of difference between concentrations values before and after incorporation procedure. Using the developed AAS methods the Pt contents in all experimental solutions were determined.

The results were represented as an average of several values (at least three) of parallel samples.

It was found that PB 1.8 mM-based cisplatin matrix was the most effective incorporation solution compared to others, due to more continuous and less intense release of the drug from OCP incubated in it. Incorporation took place with the equal efficiency when water, TRIS and SCS$_m$ were used as incorporation matrix solution for drug saturation. The most intense release of the drug was observed from OCP functionalized with SCS that was consistent with the literature data, as this solution contained chloride ions [20].

Table 3. Results of verification of correctness of Pt determination in cisplatin solutions of different salt composition ($n = 5$, $P=0.95$).

| Solution for analysis | Pt concentration, µg/ml | $S_r$ | Pt concentration, µg/ml | $S_r$ |
|----------------------|-------------------------|-------|-------------------------|-------|
|                      | Entered | Found | Entered | Found | Entered | Found |
| TRIS                 | 0.10    | 0.11±0.02 | 0.10 | 0.50 | 0.48±0.02 | 0.06 |
| PB 1M                | 1.00    | 1.0±0.09 | 0.08 | 5.00 | 5.10±0.15 | 0.06 |
| PB 1.8 mM            | 0.20    | 0.21±0.02 | 0.07 | 1.00 | 1.11±0.12 | 0.05 |
| SCS                  | 0.30    | 0.29±0.02 | 0.08 | 0.90 | 0.88±0.05 | 0.07 |
| SCS$_m$              | 0.20    | 0.22±0.02 | 0.08 | 1.00 | 1.00±0.09 | 0.08 |
| DPBS                 | 0.50    | 0.52±0.04 | 0.15 | 2.00 | 1.94±0.13 | 0.09 |
| NaCl                 | 0.80    | 0.83±0.04 | 0.06 | 2.00 | 2.12±0.14 | 0.05 |
Figure 5. Dynamics of cisplatin release from OCP ceramics functionalized in different salt solutions: 1 — PB 1.8 mM; 2 — H2O; 3 — TRIS; 4 — SCSm; 4, 5 — PB 1M; 6 — SCS.

4. Conclusion
In the proposal work the methods of the flame version of AAS platinum determination in solutions with different composition and high salts concentration have been developed. The use of atomic absorption spectrometer iCE 3000 Thermo Scientific allowed to carry out the express control of Pt content in solutions, contained complex salts, and to determine platinum in a wide range of concentrations from 0.05 to 50 µg/ml without matrix separation in small bioassays (3 ml) with good metrological characteristics. The relative standard deviation \( S_r \) is 0.06-0.005 when the Pt content is from 1 to 20 µg/ml and \( S_r \) does not exceed 0.15 when the content of the element is from 0.05 to 1 µg/ml. The optimal analytical parameters for the AAS Pt determination were selected. Dependences of Pt analytical signals on various concentrations of hydrochloric, nitric, phosphoric and sulfuric acids were established. It was revealed that the H2SO4 and H3PO4 affect significantly on platinum absorption.

The developed methods of platinum determination were applied for calculation of cisplatin content in solutions with different salt compounds and concentrations. Cisplatin is complex of divalent platinum and possess the stability of complex compound that reduced significantly the AAS sensitivity. To avoid the depressive effect of cisplatin on Pt absorption we prepared platinum standard solutions that modelled the full chemical composition of the analyzed samples. As a result the calibration curves were plotted based on cisplatin salt background.

The influence of concentration and composition of experimental solutions on the values of Pt analytical signals was studied. The Pt detection limit depends on the composition of the solutions and varies from 0.05 to 0.50 µg/ml. The method of interactive matrix matching using standard solutions of identical composition was applied to eliminate the interference caused by concentration effects of the matrix background.

The developed optimal conditions and obtained calibration plots were used during the studies of calcium phosphate ceramic biomaterial (OCP) functionalization with cisplatin. Functionalization of OCP ceramics with cisplatin was performed by cisplatin solution infiltration into ceramic granules. Different supersaturated buffer solutions were prepared as matrix solutions for cisplatin to use as incorporation solutions. Co-precipitation of new-formed calcium phosphates and drug incorporation occur simultaneously during infiltration. It was found that from 32 to 50% of cisplatin were incorporated onto the OCP ceramics surface when the different incorporation solutions were used. It was shown that the solution PB 1.8 mM has the greatest efficiency for incorporation; biomaterial functionalized with PB 1.8 mM possessed more continuous and less intense release of the drug in the model medium.
Therefore, the developed AAS methods demonstrate the relevance application in determination and evaluation of platinum content in Pt-containing medications, such as cisplatin. By the AAS method the advanced conditions for functionalization of ceramic substitute biomaterials with cisplatin were found. Prolonged release of the drug from the functionalized material will provide a local concentration of the drug sufficient to suppress tumor growth in the problem area.

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