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Short communication

Atomic absorption spectrometric determination of the iridium content in tumor cells exposed to an iridium metallodrug

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ABSTRACT

An electrothermal atomic absorption spectrometric method to quantify the iridium content of HT-29 colon carcinoma cells exposed to iridium metallodrugs was developed. Optimisation of the procedure involved the evaluation of pyrolysis and atomisation conditions (optimal values were 1400 °C for pyrolysis and 2400 °C for atomisation) and the addition of appropriate additives. The presence of cellular components as well as the addition of nitric acid and hydrochloric acid led to enhanced absorption signals and suggested the use of matrix matched calibration. The described method allows the measurement of iridium in cell suspensions in the low μg/L range (linear dynamic range: 10–450 μg/L) with a detection limit of 11.2 μg/L. The applicability of the method was tested by means of a novel iridium metallodrug. First results on the complex [$IrCl_3$(DMSO)(phen)] indicated a low cellular uptake (21.2 μM at incubation with 100 μM) of this iridium species in HT-29 cells compared to other metal containing antitumor drugs.

1. Introduction

Based on certain therapeutic disadvantages (such as nephrotoxic side effects and resistance development) of established platinum anticancer drugs (e.g. cisplatin) there have been increasing efforts in the development of novel non platinum metallodrugs. Very promising new representatives, which are currently undergoing clinical trials, are complexes containing ruthenium or gallium central atoms. [1,2] Among the – concerning drug development – not widely used central atoms iridium has also attracted attention as its complexes show generally high stability and are, in general, kinetically inert [3], properties which are desirable in modern drug development. Together with the possibility that the iridium center might enable specific interactions with biological targets, which are not possible with "normal" organic drugs, these complexes fulfill the basic requirements to generate new innovative drugs. However, the inertness of iridium complexes may also be the reason for only a very limited number of available reports on the biological activity of iridium species [4–6].

Recently, we have reported about in-vitro cytotoxic iridium polypyridyl complexes exhibiting IC_{50} values (meaning the concentration that causes a 50% reduction of tumor cell growth) in the low micromolar to submicromolar level [7]. The activity of these agents depended significantly on the nature of the polypyridyl ligand and indicated a significant influence of parameters such as cellular drug uptake and biodistribution. Consequently, analytical methodologies with low detection limits are required for further development of iridium species concerning their biodistribution and target identification. However, reports on the quantification of iridium in biological samples are extremely rare [8,9].

Due to its low detection limits, electrothermal atomic absorption spectrometry (ET-AAS) offers a valuable tool for the quantification of metal concentrations in biological tissues, which has been successfully used in the bioanalytics of various metallodrugs (for some recent representative examples see the reference section [7,10–14]).

In order to establish a methodology enabling intensive bioanalytical studies on iridium complexes we developed an ET-AAS based procedure for the quantification of iridium in cellular tissues and demonstrated its applicability for the quantification of the cellular drug uptake in HT-29 colon carcinoma cells on the example of a novel iridium metallodrug ([$IrCl_3$(DMSO)(phen)]).

2. Experimental

2.1. Reagents

Chemicals and reagents were purchased from Sigma, Aldrich and Fluka. Phosphate buffered saline (PBS) pH 7.4; cell culture medium: minimum essential medium eagle was supplemented

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with 2.2 g $NaHCO_3$, 110 mg/L sodium pyruvate and 50 mg/L gentamicin sulfate and adjusted to pH 7.4. Prior to use 10% (v/v) fetal calf serum were added. Iridium stock solution: 1 mg/mL iridium in 5% (m/v) HCl (Acros); Triton X-100 solution: Triton X-100 (1%, m/v); additive solutions: hydrochloric acid (18%, m/v), nitric acid (13%, m/v), ascorbic acid (10 g/L), palladium standard solution 3 mg/L as obtained by dilution from 1 g/L palladium in 10% (m/v) HCl (Acros), lanthanum nitrate (4 mg/L); The purity of the iridium metallodrug [$IrCl_3$(DMSO)(phen)] was investigated by elemental analysis (Vario EL, Elementar Analysensysteme GmbH). Measured values (C, H, N) did not differ more than 0.12% from the calculated ones. Results on the synthesis and structural characterisation of [$IrCl_3$(DMSO)(phen)] will be reported elsewhere (M. Scharwitz, I. Ott, R. Gust, A. Kromm, W.S. Sheldrick, unpublished results).

2.2. Protein quantification

Protein quantification was performed using the Bradford method as described in more detail recently [13].

2.3. Apparatus

A Vario 6 graphite furnace atomic absorption spectrometer (AnalytikJena AG) was used for the iridium measurements. Iridium was measured at a wavelength of 208.9 nm with a bandpass of 0.5 nm. A deuterium lamp was used for background correction. Samples were injected at a volume of 25 μL into standard graphite tubes ("AAS IC-Standardrohr", AnalytikJena AG). The temperature program in the graphite furnace is given in Table 1. During the temperature program the graphite tube was purged with a constant argon gas flow, which was only halted during the zeroing and atomisation steps. The mean integrated absorbances (integr. abs.) of duplicate injections were used throughout the study.

2.4. Cell culture and preparation of cell pellets

HT-29 human colon carcinoma cells were maintained in cell culture medium (see above) at 37 °C/5% CO_2 and passaged twice a week according to standard procedures. Cell pellets were prepared and isolated from approximately 70% confluent 175 cm^2 cell culture flasks as described in more detail recently [13].

2.5. Samples and standards for method optimisation

Cellular lysates were prepared by resuspending an isolated cell pellet in 1–5 mL twice distilled water followed by treatment with a sonotrode (Bandelin Sonopuls GM70, 60 W, 20 kHz, operated at 70% of maximum for 6–8 cycles). Aliquots for the protein measurements were removed and stored at −20 °C until further use. Matrix containing iridium standard solutions in the 10–500 μg/L concentration range were prepared by appropriate dilutions of the 1 mg/mL stock solution (see general) using blank cellular lysate. Aqueous standards were prepared analogously using twice distilled water. Each 20 μL Triton-X 100 solution (for more effective solubilisation of the membrane components of the used cell suspensions) and additive solution (see general) were added to each 200 μL of the samples. All solutions were prepared freshly prior to use.

Table 1
Graphite furnace program

Step	T (°C)	Rate (°C/s)	Hold (s)
Drying	90	10	40
Drying	105	7	30
Drying	120	15	20
Drying and initial pyrolysis	500	50	30
Main pyrolysis	Various (optimum 1400)	200	20
AZ (zeroing)	= Pyrolysis	0	3
Atomisation	Various (optimum 2400)	Maximum	6
Tube cleaning	2600	1000	5

2.6. Analytical performance

The characteristic mass was determined as that mass of iridium causing 1% integrated absorbance. The limit of detection (LOD) was calculated as that concentration corresponding to the mean absorbance of separate blank samples augmented by the three-fold value of the respective standard error. The background equivalent concentration (BEC) was calculated as the concentration intercept of the calibration curve. Values for characteristic mass, LOD and BEC were obtained from each three separate samples with different matrix protein contents (between 1.11 and 1.67 g/L); The dynamic linear range was determined using standard concentrations ranging from 0 to 500 μg/L (0, 10, 20, 40, 60, 100, 150, 200, 300, 350, 400, 450 and 500 μg/L) with a matrix protein concentration of 1.07 g/L. Linearity of the calibration curve was assumed if the correlation coefficient (r^2) between integrated absorbance values and concentrations of the standards was above 0.995. The precision was determined as the error in signal intensity of 12 subsequent injections (matrix protein concentration: 1.54 g/L.)

2.7. Sample preparation for cellular uptake studies

For cellular uptake studies cells were grown until at least 70% confluency in 175 cm^2 cell culture flasks. Stock solutions of [$IrCl_3$(DMSO)(phen)] in dimethylsulfoxide (DMSO) were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMSO concentration: 0.1% (v/v), final [$IrCl_3$(DMSO)(phen)] concentration: 100 μM). The cell culture medium of the cell culture flasks was replaced with 10 mL of the cell culture medium solutions containing the drug and the flasks were incubated at 37 °C/5% CO_2 for 6 h. The cell pellets were isolated, resuspended in 1–5 mL twice distilled water, lysed by using a sonotrode (see above) and appropriately diluted using twice distilled water. An aliquot was removed for the purpose of protein quantification. Samples and standards were adjusted to the same protein concentration by dilution with twice distilled water (matrix matched calibration). Prior to ET-AAS analysis to each 200 μL of the cell suspensions each 20 μL Triton X-100 (1%, m/v) and hydrochloric acid (18%, m/v) were added. Cellular uptake was expressed as microgram iridium per milligram cell protein. Data were obtained from three independent experiments. Conversion of the iridium/mg protein value to the micromolar cellular concentration was performed according to a previously described procedure [13].

3. Results

ET-AAS analytical methods have a high impact in metal complex research due to their comparable low detection limits and high throughput capacity. However, proper analysis of complex samples is often hampered by effects of the sample matrix. Besides optimisation of thermal sample pretreatment procedures the choice of appropriate additives plays an important role in ET-AAS method development [15,16].

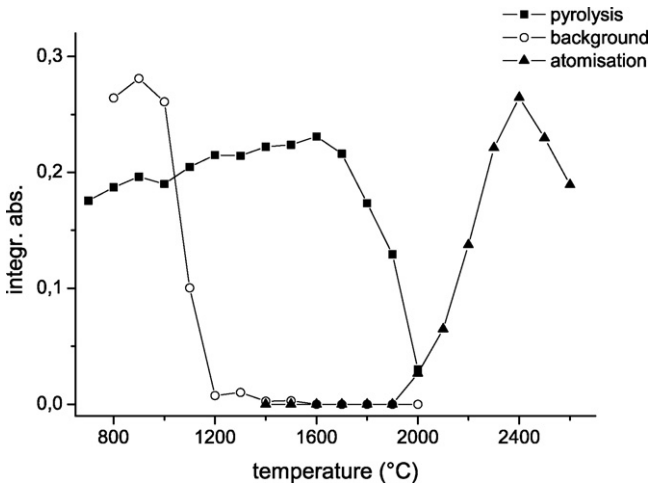

Fig. 1. Optimisation of pyrolysis and atomisation conditions of a 150 μg/L iridium probe (sample matrix protein content: 0.47 g/L).

3.1. Optimisation of pyrolysis and atomisation conditions

Thermal sample preconditioning and pyrolysis are critical steps in the development of appropriate temperature programs in the graphite furnace prior to atomising and measuring the vaporised metal atoms. The furnace parameters for iridium samples were adjusted from previously used ET-AAS methods applied for the analysis of cellular lysates [13]. Temperatures for pyrolysis and atomisation were optimised according to a method suggested by Welz and Sperling [17] and recently used by us for the analysis of gold containing samples [13].

According to this method, the pyrolysis temperatures are raised in a stepwise manner (keeping the atomisation temperature unchanged) until signal loss occurs. In a second step the atomisation temperatures are increased keeping the (optimised) pyrolysis temperature unchanged.

Evaluation of the pyrolysis conditions (see Fig. 1) showed a slight increase of signal intensity from 700 to 1600 °C followed by drastically decreased absorbance values at temperatures above 1700 °C, which can be attributed to early atomisation of iridium during the pyrolysis step and subsequent removal of the atomic species by the argon flow passing through the graphite tube at this stage of the procedure. Pyrolysis below 1200 °C was accompanied by significant background absorbances (as measured by means of a deuterium lamp), which may have led to suboptimal corrections of the data measured in the corresponding temperature range below 1200 °C. Accordingly, for the following experiments the pyrolysis temperature was kept at 1400 °C.

Optimisation of the atomisation temperature revealed a steep enhancement of signal intensity at temperatures above 2000 °C with a maximum at 2400 °C.

3.2. Influence of modifiers, digesting agents and cell components/analytical performance

In order to maximise the absorbances the influence of the addition of various modifiers and digesting agents (hydrochloric acid, nitric acid, ascorbic acid, palladium chloride and lanthanum nitrate) was evaluated in aqueous samples in comparison to samples containing different concentrations of cellular matrix. For this purpose the protein concentration of the used cell suspensions served as a measure of the matrix content. All samples were prepared freshly directly before use. Triton X-100 was added to avoid sedimentation of cell components (e.g. membranes).

In the aqueous samples the digestive agents hydrochloric acid and nitric acid led to an approximately 50% enhancement in absorption signal intensity in comparison to the samples without additives (see Fig. 2). In contrast addition of ascorbic acid, lanthanum nitrate and palladium chloride did not cause any significant changes. The same trend was observed in the matrix containing samples with a slight preference for hydrochloric acid over nitric acid.

Interestingly, components of the cellular matrix led to a strong signal induction as all matrix containing samples afforded higher absorbance values than their aqueous (matrix free) counterparts. In order to further evaluate this signal enhancing matrix effect the absorbance values of a series of aqueous and corresponding cell suspension containing iridium standards were determined. Both standard series afforded linear calibration ranges ($r^2 > 0.998$ in both experiments) up to absorbance values of approx. 0.7. In good agreement with the absorbance

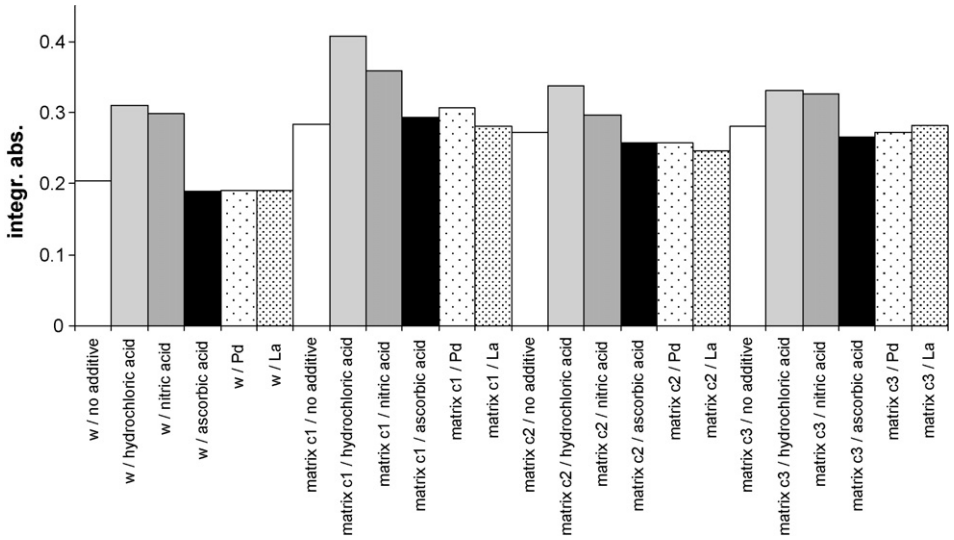

Fig. 2. Influence of various additives on iridium (150 μg/L) absorbance signals in aqueous (w) and matrix containing media (c1, 1.61 g/L; c2, 0.58 g/L; c3, 0.33 g/L); no additive: double distilled water was added instead of additive solution; hydrochloric acid: 1.5% (m/v), nitric acid: 1.1% (m/v), ascorbic acid: 0.83 g/L, Pd: palladium chloride: 0.25 g/L, La: lanthanum nitrate: 0.33 g/L.

Table 2
Analytical performance parameters

Parameter	Value
Characteristic mass (pg)	0.152 ± 0.016
Limit of detection (LOD, μg/L)	11.2 ± 4.4
Background equivalent concentration (BEC, μg/L)	12.2 ± 1.9
Linear dynamic range (μg/L)	10–450
Precision (50 μg/L, %)	1.0
Precision (200 μg/L, %)	1.6
Precision (400 μg/L, %)	3.0

increase noted above, the slope of the matrix containing standards was 1.6-fold higher than that of the aqueous standards (2.3×10^{-3} integr. abs./(μg/L) for the matrix containing samples and 1.4×10^{-3} integr. abs./(μg/L) for the aqueous samples).

Based on the above mentioned results concerning the use of additives hydrochloric acid was added to all the samples used in the following experiments. Table 2 summarises the analytical performance of the method.

3.3. Analysis of samples spiked with an iridium metallodrug

Next, the applicability of the above described sample pretreatment and apparative conditions on the analysis of samples spiked with an iridium metallodrug was evaluated. [$IrCl_3$(DMSO)(phen)] was chosen as an example for a series of novel iridium complexes, for which we have determined interesting antiproliferative properties in cell culture experiments. The complex exhibited an IC_{50} value of 4.6 ± 0.2 μM on incubation with HT-29 colon carcinoma cells (M. Scharwitz, I. Ott, R. Gust, A. Kromm, W.S. Sheldrick, unpublished results).

Initially, pyrolysis and atomisation conditions were investigated as described above. Similar to the experiments using iridium standard solutions pyrolysis optimisation showed a decrease of absorbance values above 1800 °C and minimal background at temperatures above 1200 °C. Variation of the atomisation temperatures afforded a maximum at between 2300 and 2400 °C (data not shown). This proved that the metallodrug could be analysed appropriately using the same furnace parameters as the iridium standards (pyrolysis, 1400 °C; atomisation, 2400 °C).

Recovery rates were determined for samples spiked with the metallodrug corresponding to iridium concentrations from 50 to 400 μg/L. Analysis of the samples using iridium standards containing the same matrix concentration (1.99 g/L) showed that the iridium content of the samples could be completely recovered (recovery rates: 108% for 50 μg/L, 101% for 100 μg/L, 103% for 200 μg/L and 94% for 400 μg/L.) Therefore, the iridium concentration of cells exposed to iridium metallodrugs can be quantified by matrix matched calibration.

3.4. Quantification of the cellular uptake of an iridium metallodrug

The iridium uptake of HT-29 colon carcinoma cells exposed for 6 h to 100 μM of the iridium metallodrug [$IrCl_3$(DMSO)(phen)] was determined according to the above described results. Thus, after isolation and lysis of the cells the matrix content of all samples and standards was set to the same level by dilution with double distilled water (final matrix protein concentrations: 1.46 and 1.72 mg/mL) and hydrochloric acid was added as a digesting agent. Three independently performed experiments afforded cellular iridium levels of 21.2 ± 5.2 ng/mg protein. Based on the knowledge of the mean cellular diameter and mean cellular protein content the iridium per milligram protein value can be transformed to the molar cellular concentration (1.0 ng iridium/mg cell protein correspond to 1.0 μM) [13]. Accordingly, the molar concentration of the metallodrug in the cells was 21.2 μM.

4. Discussion

An ET-AAS based method to quantify iridium in cell suspensions was developed. Components of the cellular matrix and the acids HCl and HNO_3 led to an enhancement of absorption signal intensity compared to matrix and/or additive free aqueous samples. The effect of the inorganic acids can be related to a more effective sample digestion. However, the same cell matrix caused the adverse effect – a decrease of absorbance intensities – when gold containing samples were analysed. For the gold analysis ascorbic acid was found to be a suitable modifier as it allowed pyrolysis at higher temperatures [13]. In the case of the presented iridium measurements the pyrolysis temperature of modifier free samples could be set up to 1600 °C without significant loss of analyte (see Fig. 1), which may explain why the ascorbic acid modifier showed no beneficial effect for the iridium determinations. The effective background reduction by pyrolysis at 1400 °C expectedly also limited the influence of other modifiers such as palladium chloride and lanthanum nitrate, which act mainly by catalytic modes such as reduction of analyte oxides or interaction with the graphite surface [15,16].

Recovery experiments showed that the complex [$IrCl_3$(DMSO)(phen)] could be appropriately analysed using the described conditions. However, this is most probably not a general fact and for structurally different iridium metallodrugs the performance of initial recovery experiments is recommended.

The described method allows quantification of the iridium content in cell suspensions in very low concentrations (low μg/L range) that are suitable for regular bioanalysis of iridium metallodrugs. Lower detection limits for iridium in biological samples could only be reached by inductively coupled plasma mass spectroscopy (ICP-MS). Thus, Begerow et al. [8] reported the measurement of iridium in the low ng/L range in blood samples by using double focusing magnetic sector field inductively coupled plasma mass spectrometry.

The cellular uptake was quantified exemplarily for 100 μM of the metallodrug [$IrCl_3$(DMSO)(phen)] and afforded a cellular iridium concentration of 21.2 μM. Obviously, the intracellular concentration of this compound is significantly below its extracellular concentration. Consequently, inefficient cellular drug uptake has to be considered as a possible reason together with kinetical inertness for low biological activity of many chloridoiridium species [3–6].

For anticancer drugs containing other transition metals very differing uptake efficacies have been described. On the one hand, especially high uptake rates have been reported for certain ruthenium or hexacarbonyldicobalt species or titanocene dichloride. For example, in experiments with hexacarbonyldicobalt complexes the intracellular concentrations exceeded the extracellular ones up to 150-fold [7,18,19]. On the other hand for established platinum anticancer drugs (e.g. cisplatin) only up to six-fold accumulation inside the cells has been reported. [20,21]

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