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# Investigations into the interaction between tumor-inhibiting ruthenium(III) complexes and nucleotides by capillary electrophoresis

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Dedicated to Prof. Ernst-G. Jäger on the occasion of his 65th birthday

**Abstract**

Ruthenium(III) complexes of the general formula $HL[RuCl_4L_2]$, with two *trans*-standing heterocyclic ligands L bound to ruthenium via nitrogen, show remarkable activity in different tumor models. To obtain a deeper insight into the mode of action of this class of anticancer compounds, we investigated the interaction of HIm *trans*-$[RuCl_4(im)_2]$ (im, imidazole) and HInd *trans*-$[RuCl_4(ind)_2]$ (ind, indazole) with all four nucleoside monophosphates in buffered solution by means of capillary electrophoresis. A preference for GMP- and AMP-coordination was found. A decrease of the pH resulted in a significantly increased amount of bound nucleotide. This feature seems to be interesting with regard to the lower pH values in solid tumors. 

*Keywords:* Cancer; Ruthenium(III); Nucleotides

## 1. Introduction

Today, ruthenium complexes appear to be one of the most promising alternatives to platinum complexes in the research field of metal anticancer compounds. Especially ruthenium(III) complexes of the general formula $HL[RuCl_4L_2]$ with two *trans*-standing heterocyclic ligands L bound to ruthenium via nitrogen, show good antitumor activity in different tumor models [1–5].

In most cases the target molecule for metal-based anticancer agents appears to be DNA. Especially for cisplatin (*cis*-diamminedichloroplatinum(II)) it is widely accepted that its interaction with DNA occurs via bifunctional binding to N7 of adjacent purine bases, preferentially d(GpG) sites [6]. These cross-links lead to inhibition of DNA replication and apoptosis [7–11]. Similar reactions are expected for ruthenium compounds. Mestroni et al. [12] and Cauci et al. [13] suggested that *cis*- and *trans*-$RuCl_2(DMSO)_4$ interact in vivo with DNA. Studies of the interaction with nucleotides and DNA in vitro suggested that binding between ruthenium and DNA mainly occurs at N7 of guanine on the major groove of the double helix [12–16]. In this work we focused on the investigation of the interaction of HIm *trans*-$[RuCl_4(im)_2]$ (im, imidazole) and HInd *trans*-$[RuCl_4(ind)_2]$ (ind, indazole) (see Fig. 1) with nu-

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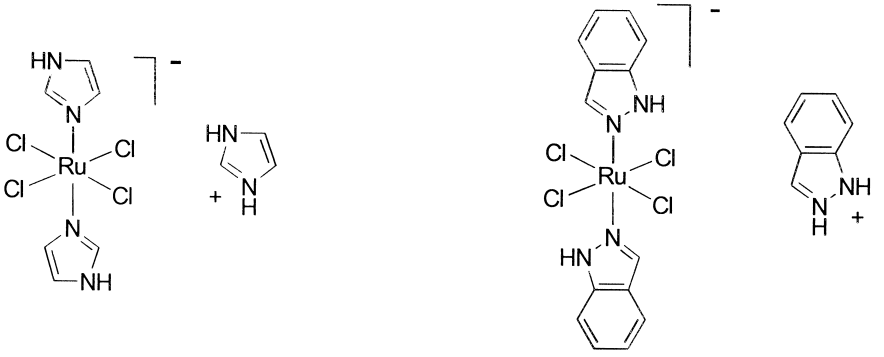


Fig. 1. HIm *trans*-[$RuCl_4(im)_2$] (left) and HInd *trans*-[$RuCl_4(ind)_2$] (right).

cleoside monophosphates applying capillary electrophoresis. CE proves to be an attractive alternative to more common separation techniques like HPLC or spectroscopic methods for these kinds of investigations. Studies using NMR are limited due to the paramagnetism of Ru(III) compounds. Some of the most important characteristics of CE are operation in aqueous media, short analysis times, high efficiency and automated instrumentation. Physiological conditions were simulated by using a phosphate buffer system and keeping the temperature constant at 37°C. Previous studies [17] have shown a strong pH-dependence of the hydrolysis rate of HInd *trans*-[$RuCl_4(ind)_2$]. Therefore we performed measurements also at pH 6.0. This is of interest with regard to the hypoxic milieu and the lower pH value in solid tumors.

## 2. Experimental

### 2.1. Capillary electrophoresis

All analyses were performed on a Hewlett-Packard $^{3D}$Capillary Electrophoresis system equipped with a diode-array detector (DAD). Fused-silica capillaries (50 μm I.D., effective length 41.5 cm) were purchased from Hewlett-Packard (Waldbronn, Germany). Before the first use the capillary was flushed with sodium hydroxide solution (1 *M* and 0.1 *M* HPCE grade; Fluka, Buchs, Switzerland) and bidistilled water (Merck), each for 10 min. Afterwards the capillary was conditioned with separation buffer (sodium phosphate buffer, 50 m*M* pH 6.0 or 7.4) for a minimum of 60 min. Before each analysis the capillary was flushed with separation buffer for 3 min. Temperature of the capillary as well as of the sample tray was kept constant at 37°C. All samples were dissolved in the separation buffer of lower concentration (10 m*M*) to make use of the stacking effect. Injection was performed by pressure ($p=10$ mbar, $t=15$ s). During the separation mode the voltage was kept constant at 30 kV. Alterations of the standard separation mode are described in the corresponding figure legends. Figures show detection at 200 nm. To optimize constant migration times, the separation buffer was replenished for all five runs.

### 2.2. Compounds

HIm *trans*-[$RuCl_4(im)_2$] [18] and HInd *trans*-[$RuCl_4(ind)_2$] [19] were prepared as described elsewhere. The purity was verified by CE, NMR and elemental analysis.

### 2.3. Sample preparation

Solutions of each nucleoside monophosphate (1 or 0.5 m*M* GMP, AMP, CMP and TMP) were prepared in 10 m*M* phosphate buffer of pH 6.0 or 7.4. HIm *trans*-[$RuCl_4(im)_2$] (1 m*M*) and HInd *trans*-[$RuCl_4(ind)_2$] (0.5 m*M*) were dissolved in the prepared solutions containing the nucleotide in an ultrasonic bath to obtain a molar ratio of 1:1. The higher concentration of HIm *trans*-[$RuCl_4(im)_2$] was chosen due to less UV absorption of the complex. Immediately after dissolving, the first aliquot was taken and analyzed by directly injecting into CE and

recording online at 200 nm and the maxima of the major analytes. Samples were incubated at 37°C. Samples of HInd *trans*-[$RuCl_4(ind)_2$] were filtered due to precipitation by a 0.2-μm filter (Millipore, Bedford, MA, USA) prior to each analysis.

To investigate the binding behavior under competitive conditions, all four nucleotides (1 or 0.5 m*M* each) were incubated with HIm *trans*-[$RuCl_4(im)_2$] (1 m*M*) and HInd *trans*-[$RuCl_4(ind)_2$] (0.5 m*M*) in 10 m*M* phosphate buffer (pH 6.0 or 7.4) in a ratio of 1:1. Data were collected at 200 and 254 nm. For external calibration GMP, AMP, CMP and TMP were dissolved in the incubation buffer (0.05 m*M*–2 m*M*) and analyzed under equal conditions (calibration curve: $r^2 = 0.998–0.999$).

### 2.4. Chemicals

Disodium salts of guanosine 5′-monophosphate (GMP), adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP) and thymidine 5′-monophosphate (TMP) as well as sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Fluka (Buchs, Switzerland). All solutions were filtered prior to analysis by a 0.2-μm filter (Millipore) and degassed in an ultrasonic bath.

## 3. Results and discussion

Peak identities of the nucleoside monophosphates and the hydrolysis products were established on the basis of their migration behavior and spectral properties. Binding of platinum compounds to nucleotides results in a significant shift of the absorption maximum of the UV-spectra to lower energy [20,21]. Comparable results for the interaction of ruthenium compounds with nucleotides are still unknown.

At a pH value of 6.0, GMP as well as the other nucleotides are only onefold negatively charged due to a dissociation exponent of the P–OH group of about 6.25 [22]. Therefore, their electrophoretic mobility is lower than the one of the complex anion *trans*-[$RuCl_4(im)_2]^-$. At a pH of 7.4, nucleotides are twofold negatively charged resulting in a slower migration time than the complex anion.

Due to short reaction times and immediate precipitation after dissolving at a pH of 7.4, samples of HInd *trans*-[$RuCl_4(ind)_2$] were analyzed only at pH 6.0, where precipitation occurs first after ~5 h of incubation at 37°C.

The hydrolytic behavior of these ruthenium compounds was investigated earlier [17], therefore it is not discussed here in detail.

### 3.1. Interaction of nucleoside monophosphates with ruthenium compounds

#### 3.1.1. HIm trans-[$RuCl_4(im)_2$]

HIm *trans*-[$RuCl_4(im)_2$] was incubated with each nucleoside monophosphate corresponding to a molar ratio of 1:1. After incubation with GMP at a pH of 7.4 (Fig. 2C), two peaks could be detected (peaks 2 and 4) additionally to the hydrolysis products (indicated with $H_1$ and $H_2$), the imidazolium ion $HIm^+$, the complex anion *trans*-[$RuCl_4(im)_2]^-$ and the $GMP^{2-}$. In Fig. 3A the relative decrease of the peak area of $GMP^{2-}$, $HIm^+$ and *trans*-[$RuCl_4(im)_2]^-$ is shown. The peak area of free GMP decreased to 65% of the initial value during the first 3 h of incubation, accompanied by a rise of peak 4 that reached its maximum after 3 h (Fig. 3B). During further incubation, the value of free GMP remained constant at 65%, whereas peak 4 decreased and a new peak (Fig. 2C, peak 2), migrating faster, appeared. This appearance of an intermediate product probably indicates coordination of a second GMP. Both formed species are negatively charged and migrate faster than $GMP^{2-}$ and *trans*-[$RuCl_4(im)_2]^-$. They possess a similar UV maximum at 252 nm indicating coordination of GMP. In contrast to investigations with cisplatin [23], the typical shift of the UV maximum from 252 to 258 nm could not be observed. The presence of GMP had no influence on the rate constant of decomposition of *trans*-[$RuCl_4(im)_2]^-$ ($k = 12.5 \pm 0.5 \times 10^{-5}$ $s^{-1}$). The decrease of the peak area of $HIm^+$ to 22% of the initial value accompanied by a rise of a positively charged species (Fig. 2, peak $H_1$) indicates ligand exchange reactions of a chloride with an imidazole ion. This corresponds to preliminary investigations of the hydrolysis of HIm *trans*-[$RuCl_4(im)_2$] [17]. It is expected that complexes with more than two coordinated imidazoles are significantly less reactive to GMP. Consequently a

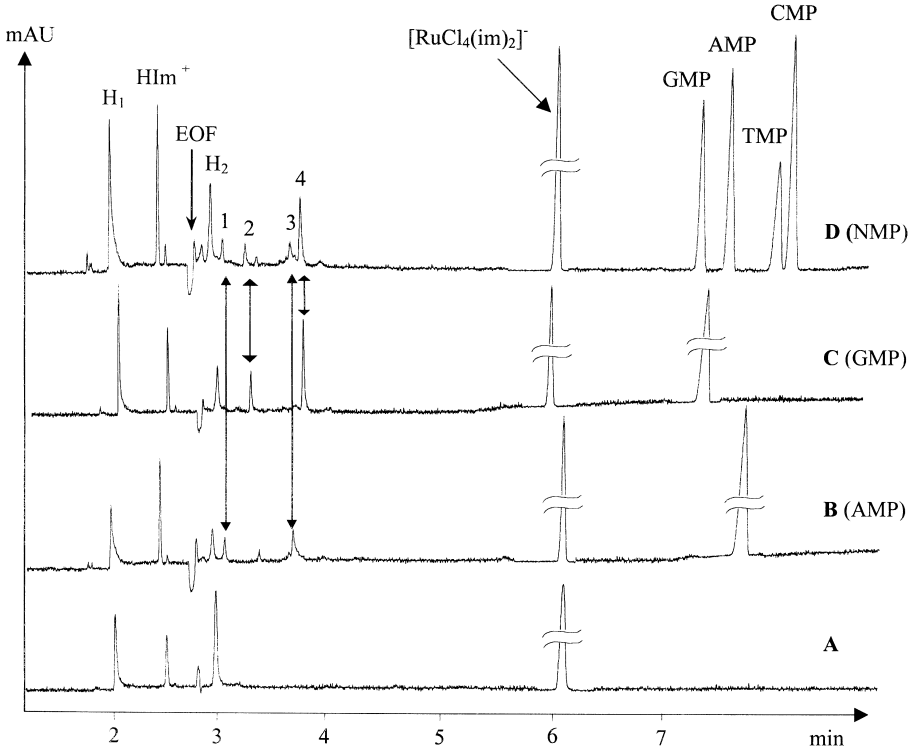


Fig. 2. Electropherograms of HIm *trans*-$[RuCl_4(im)_2]$ incubated for 2.5 h in phosphate buffer at pH 7.4 (A), in the presence of AMP (B), GMP (C) or all four nucleotides (D) at 37°C. Hydrolysis products are indicated with H.

competition between coordination of the imidazole and the nucleotide may occur.

Investigations in phosphate buffer at pH 6.0 led to a different pattern of the electropherogram. Besides the $HIm^+$ and the hydrolysis product, the peak for $GMP^-$ and *trans*-$[RuCl_4(im)_2]^-$, a positively charged complex–GMP-adduct with a $\lambda_{MAX}$ at 252 nm could be detected. The peak reached its maximum after 5 h of incubation. Corresponding to the appearance of the GMP-adduct, the amount of free GMP decreased to 30% of the initial value. At pH 6.0, the presence of GMP enhances the rate constant of the decomposition of *trans*-$[RuCl_4(im)_2]^-$ somewhat from $11.8\pm0.8\times10^{-5}$ to $14.3\pm0.2\times10^{-5}$ $s^{-1}$. The peak area of $HIm^+$ decreased insignificantly to about 90% of the initial value.

Reaction of HIm *trans*-$[RuCl_4(im)_2]$ with AMP at a pH of 7.4 was found to be quite similar as with GMP. Fig. 2B shows an electropherogram recorded after 2.5 h of incubation. Two additional peaks with a $\lambda_{MAX}$ at 266 nm could be observed (peak 1 and peak 3). Pure AMP possesses an absorption maximum at 259 nm, a shift to lower energy occurred corresponding to investigations with cisplatin [24], where coordination of AMP resulted in a $\lambda_{MAX}$-shift from 259 to 264 nm. Peak 3 reached a maximum after 3 h. Subsequently the peak area decreased and peak 1 appeared (Fig. 3D). The amount of free AMP decreased to 75% within 3 h and remained constant at this value (Fig. 3C).

Coordination of AMP at a pH of 6.0 showed a different behavior. Only one, uncharged species (migrating with the EOF) with a $\lambda_{MAX}$ at 266 nm could be detected. The peak reached its maximum after 5 h of incubation accompanied by a decrease of free AMP to about 55% of the initial value.

Investigations with CMP resulted in a small decrease of free CMP to about 80% at pH 6.0 and about 90% at pH 7.4. The value of free nucleotide remained constant after 6 h (pH 6.0) and 3 h (pH

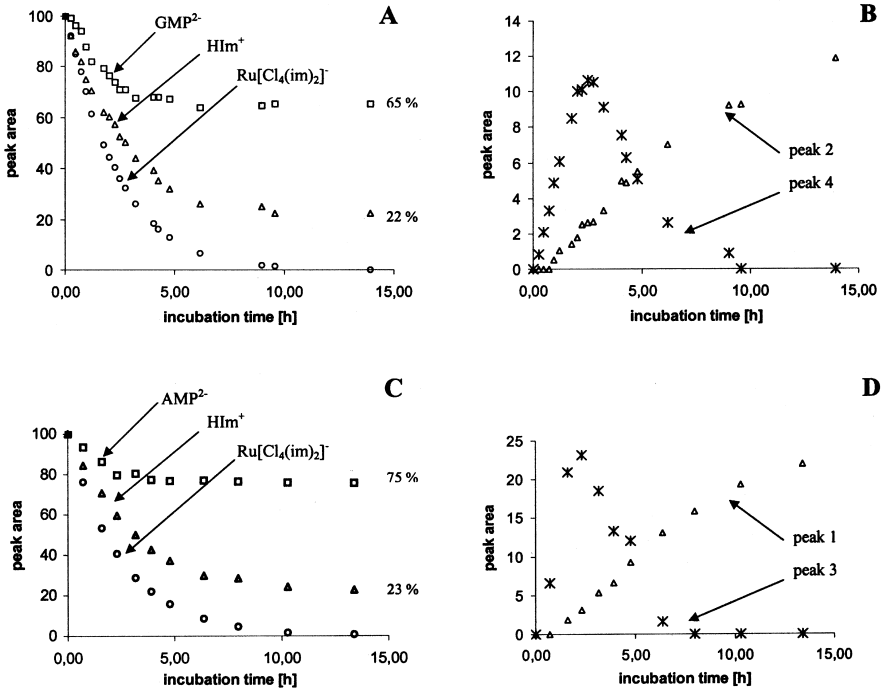


Fig. 3. Relative decrease of the peak area of the nucleotide, $[RuCl_4(im)_2]^-$ and the imidazolium ion during incubation of HIm *trans*-$[RuCl_4(im)_2]$ with GMP (A) and AMP (C) (1:1) in phosphate buffer pH 7.4 at 37°C. Adduct formation of HIm *trans*-$[RuCl_4(im)_2]$ with GMP (B) and AMP (D) in phosphate buffer pH 7.4.

7.4) of incubation. An adduct could not be detected due to small amounts. Analogous investigations [24] with cisplatin showed that bonding to N3 of CMP occurs only if there is no other target or in case of excess of the platinum compound.

The peak areas of TMP remained constant during the whole study. TMP has to be deprotonated at N3 to coordinate metal complexes. This does not occur under physiological conditions.

A comparison of the amount of bound nucleotide after incubation with HIm *trans*-$[RuCl_4(im)_2]$ at different pH values is shown in Fig. 4. The values of free nucleotide remained constant after ~6 h at pH 6.0 and 3 h at pH 7.4.

In all experiments, a decrease of the pH to 6.0 resulted in a significantly increased amount of bound nucleotide. Approximately twice as much was coordinated than at pH 7.4. This feature seems to be interesting with regard to the lower pH in solid tumors. A possible explanation may be the increased formation of trisimidazole species at higher pH

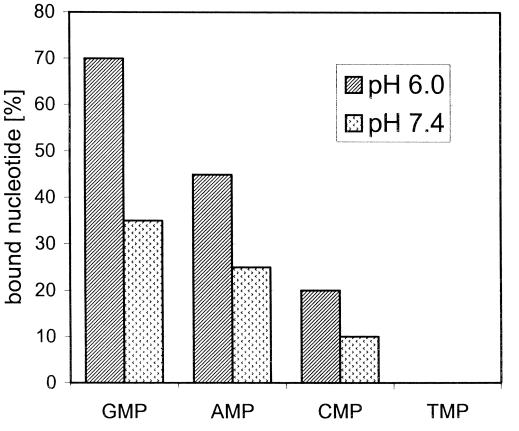


Fig. 4. Comparison of the relative amount of bound nucleotide after 6 h of incubation of HIm *trans*-$[RuCl(im)_2]$ with each mononucleotide (molar ratio 1:1) at pH 6.0 and 7.4.

values as a competitive reaction to coordination of the nucleotides.

### 3.1.2. HInd trans-[$RuCl_4(ind)_2$]

It has to be noticed that only soluble species can be detected by applying this method. In case of the indazole complex precipitation occurred after 5 h of incubation in phosphate buffer pH 6.0 at 37°C. At pH 7.4 precipitation occurred immediately after dissolving. The nature of the precipitate stays unclear. Incubation of HInd *trans*-[$RuCl_4(ind)_2$] with GMP (molar ratio 1:1) in phosphate buffer pH 6.0 resulted in formation of a negatively charged adduct ($\lambda_{MAX}$ = 250–260 nm, exact determination impossible due to small amounts) additionally to the negatively charged hydrolysis product. This species migrated slower than the EOF. In the presence of GMP, the peak of the hydrolysis product was much smaller than in the case of the hydrolysis investigations under comparable conditions, likely indicating its reaction with GMP. The value of free GMP decreased to 60% during 24 h of incubation and remained constant at this value. Investigations with AMP have shown to be quite similar, the value of free AMP also decreased to about 60% during incubation. Incubation with CMP resulted in a decrease of free CMP to about 80% of the initial value. An identification of reaction products was not possible in any case due to small amounts and fast precipitation. Neither GMP nor AMP nor CMP did influence the decomposition rate of *trans*-[$RuCl_4(ind)_2$]$^-$ ($k = 3.4 \pm 0.1 \times 10^{-5}$ $s^{-1}$). As expected, no TMP coordination was found. The peak area remained constant during the whole time of incubation.

### 3.2. Interaction of nucleoside monophosphates with HIm trans-[$RuCl_4(im)_2$] under competitive conditions

The preference of the four nucleotides GMP, AMP, CMP and TMP for the ruthenium complex has been investigated. The nucleotides were used in equimolar amounts to obtain a competitive situation for the complex. The investigations were performed at different ratios of complex:NMP (1:1 and 4:1).

Fig. 5 shows the electropherograms of HIm *trans*-[$RuCl_4(im)_2$] incubated with all four nucleotides

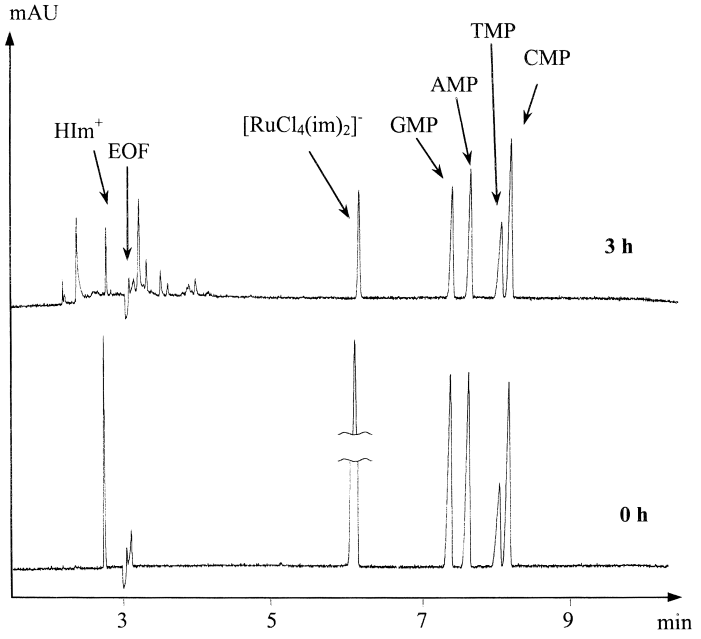


Fig. 5. Electropherogram of a competitive study of nucleoside monophosphates with Him [$RuCl_4(im)_2$] in phosphate buffer at pH 7.4 (molar ratio complex:NMP = 4:1).

corresponding to a molar ratio of 4:1 (complex-:NMP) at the beginning of the study and after 3 h of incubation at 37°C in phosphate buffer of pH 7.4. During 3 h of incubation, the value of free GMP decreased to 50% and the one of AMP to 60% of the initial values. Incubation of HIm *trans*-[$RuCl_4(im)_2$] with NMP in a molar ratio of 1:1 resulted in 75% GMP and 80% AMP remaining free in solution. The peak areas of CMP and TMP remained constant in any case. One can conclude that, analogous to investigations with cisplatin, coordination of CMP occurs only if there is no other target molecule or in case of an excess of the complex.

In Fig. 2 the electropherogram of the competitive study (D) is compared with the one of a sample incubated in buffered solution without nucleotides to identify peaks due to hydrolysis (A). Electropherograms of samples incubated with AMP and GMP alone are shown in Fig. 2B and C. A comparison of the peaks migrating after the EOF enables assignment of the adducts formed by interaction with nucleotides. Peaks 1 and 3 showed similar migration behavior and absorption spectra as the peaks of the sample incubated with AMP alone, whereas peaks 2 and 4 can be assigned to be complex–GMP adducts. Peaks resulting from the hydrolysis of the complex have been marked with H.

In accordance with the investigations above, a decrease of the incubation pH value to 6.0 resulted in an increase of the amount of bound nucleotide. Electropherograms of a competitive study of the imidazole complex, corresponding to a ratio complex:NMP = 4:1, at pH 6.0 are shown in Fig. 6. Compared to pH 7.4, after 6 h of incubation nearly the whole amount of GMP disappeared, resulting in formation of one adduct migrating faster than the EOF indicating a positive net charge of the species. An absorption maximum was found at 252 nm. Besides this complex–GMP adduct, formation of a neutral AMP-adduct with an absorption maximum at 265 nm could be observed. In the case of using an excess of complex, the amount of free AMP decreased to 20% and that of CMP to about 70% of the initial value. No complex–CMP-adduct could be detected and the peak area of TMP remained constant. In the case of incubation of HIm *trans*-[$RuCl_4(im)_2$] with NMP corresponding to a molar ratio of 1:1, the peak area of GMP decreased to 20% and

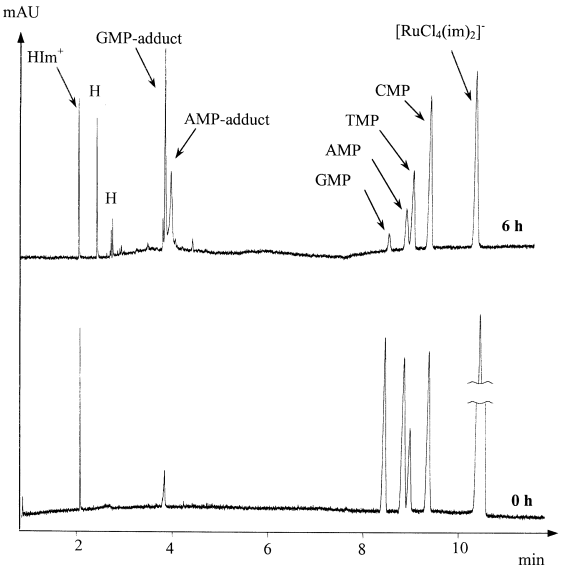


Fig. 6. Electropherograms of a competitive study of nucleoside monophosphates with HIm *trans*-[$RuCl(im)_2$] at pH 6.0 (molar ratio complex:NMP = 4:1). Hydrolysis products are indicated with H.

the one of AMP to 75% of the initial value. The peak areas of CMP and TMP remained constant during the whole study.

## 4. Conclusion

The suitability of capillary electrophoresis for investigations into the interaction of mononucleotides with Ru(III) complexes has been demonstrated. Especially for paramagnetic Ru(III) complexes this technique is an attractive alternative to NMR for this kind of investigation. An advantage of HPCE compared to HPLC is the possibility to use the same aqueous buffer for incubation and separation. Therefore, physiological conditions can be simulated easily. Because the separation principle is based on the electrophoretic mobility of the analytes, HPCE is a suitable tool for analyzing different charged species. In an electropherogram, cations, anions and neutral species can be distinguished easily which is not possible in the case of reversed-phase chromatography.

Independent of the pH value, a preference for GMP and AMP coordination was found. Binding of

CMP occurs only if there is no other target molecule. Under competitive conditions no coordination of CMP was found. TMP has to be deprotonated to coordinate metal complexes. This does not occur under physiological conditions. Consequently the peak area of TMP remained constant during incubation in all cases. The reactions occurred within several hours, much faster compared to cisplatin. The values of free nucleotide remained constant after ~6 h (pH 6.0) and 3 h (pH 7.4) of incubation at 37°C. The kind as well as the rate of adduct formation with NMP depends on the pH value of the buffer system. The amount of bound nucleotide after incubation with HIm *trans*-[$RuCl_4(im)_2$] depends significantly on the pH value (Fig. 4). A decrease of the pH to 6.0 resulted in a significantly increased amount of bound nucleotide. Approximately twice as much was coordinated compared to pH 7.4. This feature seems to be interesting with regard to the lower pH values in solid tumors. The fact that the amount of free imidazole decreases strongly at higher pH values accompanied with a rise of a positively charged species indicates coordination of an imidazole. Consequently a competition between coordination of an imidazole and a nucleotide occurs.

The presented methodology offers several application possibilities. It is known that in the anticancer complex HIm *trans*-[$RuCl_4(im)_2$] aquation occurs stepwise by sequential loss of two chlorides [17,25,26]. Therefore it might be interesting if the presence of chloride ions in the physiological relevant concentrations affect the kind and/or the rate of adduct formation with the NMP. Studies to investigate this are in progress.

Several biologically occurring redox agents such as ascorbic acid or glutathione are expected to be capable of reducing Ru(III) complexes. Ru(III) complexes may serve as prodrugs, which are activated by reduction in vivo to coordinate biomolecules [27]. First investigations concerning the influence of the presence of ascorbic acid onto the binding behavior of HIm *trans*-[$RuCl_4(im)_2$] towards GMP showed a remarkable increase of the rate constant of the complex decomposition from $14.3\pm0.2\times10^{-5}$ $s^{-1}$ to $48.6\pm1.1\times10^{-5}$. The amount of bound GMP did not change significantly but differences in the kind of adduct formation could be observed. This work also showed the limits of detection by UV–DAD. The major disadvantage of this kind of detection is the lack of structural information about the adducts. A solution of this problem might be coupling of CE with mass spectrometry.

## 5. Nomenclature

| | |
|---|---|
| EOF | electroosmotic flow |
| CE | capillary electrophoresis |
| im | imidazole |
| ind | indazole |
| NMP | nucleoside monophosphate |

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