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Short Communication

New metal complexes of 4-methyl-7-hydroxycoumarin sodium salt and their pharmacological activity

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Abstract

Complexes of copper (II), zinc (II), nickel (II), cobalt (II) and iron (III) with 4-methyl-7-hydroxycoumarin sodium salt (Mendiaxon, Hymecromone) were synthesized by mixing of equimolar amounts of the respective metal nitrates and 4-methyl-7-hydroxycoumarin sodium salt in water. The complexes were characterized and identified by elemental analysis, conductivities, IR, ^{1}H NMR spectroscopy and mass spectral data. DTA and TGA have been applied to study the compositions of the compounds. Thermal analysis of the complexes indicate the formation of compounds which correspond to the compositions $Met(HL)_2 \cdot nH_2O$, where Met = Cu, Zn, Ni, Co; $n = 2$, 3 or 4 and $Fe(HL)_3 \cdot 5H_2O$. The newly synthesized compounds were assayed for acute intraperitoneal and per oral toxicity, influence on blood clotting time and the most active complex was investigated for spasmolytic activity.

Keywords: Metal complexes of mendiaxon; Anticoagulants; Spasmolytic activity

1. Introduction

Coumarin derivatives are of interest because of their physiological, photodynamic, anticoagulant, spasmolytic and bacteriostatic activity. They are also extensively used as analytical reagents. 7-Hydroxycoumarin is known for its antibiotic and antifungal activities [1]. 8-Substituted-4-methyl-7-hydroxycoumarin [2–4] and 6-substituted-4-methyl-7-hydroxycoumarin [5], have been investigated for complexing ability. These derivatives of coumarin have been found to exhibit anticoagulant and plant growth regulating properties [6]. Racemic sodium Warfarin, is widely used in the prevention of thromboembolic disease [7]. Kerr et al. characterized three novel classes of Warfarin analogs and compared them with the Warfarin enantiomers. All three classes of compounds inhibit vitamin K epoxide reductase, the enzyme inhibited by racemic Warfarin [8]. Ammar et al. studied the interaction of oral anticoagulants Warfarin and Dicumarol with methylxanthines [9]. A series of 7-amino-4-chloro-3-(3-isothioureidopropoxy)isocoumarin derivatives with various substituents at the 7- and 3-positions have been synthesized as inhibitors of several blood coagulation enzymes by Kam et al. [10]. Smirnova et al. reported about new coumarin derivatives used as injectable anticoagulants [11]. The anticoagulant activity of coumarin derivatives was also studied by Wallin [12] and Hart et al. [13].

Yamada et al. evaluated the spasmolytic activity of several coumarin compounds, analogous to aurapten, against Ba (II), acetylcholine and histamine and investigated their structure–activity relationship [14]. The spasmolytic activity of geranyloxycoumarin-related compounds has been described [15]. Chen et al. studied total coumarins in the fruit of *Cnidium monnieri*. In guinea pigs, oral administration of the total coumarins had a protective effect against bronchospasms induced

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by histamine [16]. Aminov et al. investigated some coumarins isolated from the plant Haplophyllum and it was shown that all compounds exhibited spasmolytic and hypotensive activities [17].

Coumarin derivatives are known to have good complexing ability [18]. Although solution chemistry of transition metal complexes of 7-hydroxy-4-methylcoumarin, substituted at position 3, 6 or 8 is reported, no work has been carried out on the synthesis and structural aspects of solid complexes of 4-methyl-7-hydroxycoumarin sodium salt. These data served as a basis of the present study of the possibility of synthesis, isolation and identification of 4-methyl-7-hydroxycoumarin sodium salt complexes with several metals in view of the application of these substances as anticoagulants and spasmolytical agents.

Table 1
Results of the elemental analyses

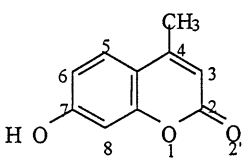
Substance (color)	M.p. (°C)	λ (μS/cm)	Elemental analysis, % (calculated/found)			
			C	H	Met	H_2O
$Cu(HL)_2{\cdot}4H_2O$			49.48	4.53	12.99	14.84
(Green)	163	2.12	50.10	4.07	14.61	15.25
$Zn(HL)_2{\cdot}2H_2O$			53.21	3.99	14.41	7.98
(White)	285	2.33	52.61	3.80	15.84	7.50
$Ni(HL)_2{\cdot}3H_2O$			51.95	4.33	12.70	11.69
(Green)	250	2.14	52.16	3.93	12.30	11.30
$Co(HL)_2{\cdot}4H_2O$			49.89	4.57	12.26	14.97
(Dark grey)	169	2.70	49.75	4.62	12.63	15.00
$Fe(HL)_3{\cdot}5H_2O$			53.65	4.62	8.34	13.41
(Brown)	155	1.98	53.64	4.69	7.15	13.00

$HL = C_{10}H_7O_3$; m.p. (L) > 300°C; λ(L) = 39.6 μS/cm.

Table 2
Results of the IR spectra

Subst.	ν_{OH}	$\nu_{C=O}$	$\nu_{C=C}$				
$C_{10}H_7O_3Na$	3655	1726	1576	1070–976	831	613	484
$Cu(HL)_2{\cdot}4H_2O$	3493	1668	1607	1020–989	841	586	478
$Zn(HL)_2{\cdot}2H_2O$	3493	1671	1607	1022–990	841	586	478
$Ni(HL)_2{\cdot}3H_2O$	3493	1671	1607	1020–989	842	584	478
$Co(HL)_2{\cdot}4H_2O$	3489	1671	1607	1020–989	842	584	478
$Fe(HL)_3{\cdot}5H_2O$	3495	1671	1607	1020–989	841	586	478

Table 3
^1H NMR (100 Mhz, DMSO-d_6)



C_n–H	Multiplicity of the signal	δ (ppm) of the complexes					
		Lig	Cu	Zn	Ni	Co	Fe
C_8–H	s, 1H	5.9	6.8	6.7	6.7	6.7	6.8
C_6–H	D, 1H, $J = 7$ Hz	6.2	6.9	6.8	6.8	6.8	6.9
C_5–H	D, 1H, $J = 7$ Hz	7.2	7.7	7.8	7.6	7.6	7.7
C_3–H	s, 1H	5.5	6.2	6.1	6.0	6.0	6.2
C_4–CH_3	s, 3H	2.1	2.4	2.3	2.4	2.4	2.4

Table 4
Acute i.p. toxicity (LD_{50}) of investigated compounds and hymecromone

Compound	LD_{50} (mg/g)	Range of values (mg/kg)
Hymecromone	189.1	177.8–201.1
$Cu(HL)_2 \cdot 4H_2O$	25.9 *	24.7–27.3
$Zn(HL)_2 \cdot 2H_2O$	392.87 *	234.31–658.74
$Ni(HL)_2 \cdot 3H_2O$	98.8 *	56.7–171.5
$Co(HL)_2 \cdot 4H_2O$	389.2 *	346.4–437.4
$Fe(HL)_3 \cdot 5H_2O$	773 *	696.5–857.9

* $P < 0.05$, statistically significant compared to hymecromone.

Table 5
Acute p.o. toxicity of investigated compounds and hymecromone

Compound	LD_{50} (mg/kg)	Range of values (mg/kg)
Hymecromone	1908.07	1484.75–2452.08
$Cu(HL)_2 \cdot 4H_2O$	> 3000 *	
$Zn(HL)_2 \cdot 2H_2O$	2531.8	2275.6–2816.9
$Ni(HL)_2 \cdot 3H_2O$	> 3000 *	
$Co(HL)_2 \cdot 4H_2O$	2463.99	2216.98–2738.5
$Fe(HL)_3 \cdot 5H_2O$	> 3000 *	

* $P < 0.05$, statistically significant compared to hymecromone.

Table 6
The index of absorption of investigated compounds (calculated by dividing data from i.p. LD_{50} to the data from p.o. LD_{50} and expessed as percentage

Compound	Index of absorption (%)
Hymecromone	9
$Cu(HL)_2 \cdot 4H_2O$	0.8
$Zn(HL)_2 \cdot 2H_2O$	15
$Ni(HL)_2 \cdot 3H_2O$	3
$Co(HL)_2 \cdot 4H_2O$	15
$Fe(HL)_3 \cdot 5H_2O$	26

2. Chemistry

The compounds used for preparing the solutions were Merck products, p.a. grade: $Cu(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$ and $Fe(NO_3)_3 \cdot 9H_2O$. 4-Methyl-7-hydroxy-2H-1-benzopyran-2-one sodium salt was used for the preparation of metal complexes as ligand. 4-Methyl-7-hydroxycoumarin sodium salt was prepared by following procedure: 1.76 g (10 mmol) 4-methyl-7-hydroxycoumarin was added to 0.038 g (9.5 mmol) sodium hydroxide in 30 ml water. The mixture was stirred vigorously at room temperature for an hour until it gets clear. The solution was filtered and the filtrate was evaporated to dryness. The viscous residue was recrystallized from ethylacetate. TLC (hexane–acetone; 2:1). Yield 1.7 g (86%), m.p. above 300°C.

The complexes were synthesized by mixing water solutions of copper (II), zinc (II), nickel (II), cobalt (II) and iron (III) salts and the ligand in amounts equal to metal–ligand molar ratio of 1:2. The reaction mixture was stirred with an electromagnetic stirrer at 25°C for 1 h. The moment the solutions were mixed colored precipitates were obtained. The precipitates were filtered, washed three times with water and dried in a desiccator to constant weight.

The complexes were insoluble in water, methanol and ethanol and well soluble in DMSO. Their physicochemical characteristics such as melting points and molar conductances are presented in Table 1.

3. Experimental

3.1. Chemistry

The carbon and hydrogen content of the compounds were determined by elemental analysis.

The water content was determined by Metrohn Herizall E55 Karl Fisher Titrator and thermogravimetrically.

The experiments of DTA and TGA were carried out using a derivatograph produced by the firm MOM (Budapest). Samples with particle size below 0.25 mm were placed in platinum crucibles. The heating rate was 10°C/min until 900°C. The inert substance was Al_2O_3.

Melting points were determined by using a Boetius melting point apparatus and are uncorrected.

Conductometric measurements were carried out at 25°C on 10^{-3} M solutions in DMSO by using a Metrohn 660 AG-9101 Herisau conductometer with platinum electrode and a cell having a cell constant of 0.79 cm^{-1}.

IR spectra (Nujol) were recorded on a IR-spectrometer FTIR-8101M Shimadzu.

^1H NMR spectra were recorded at room temperature on a Brucker WP 100 (100 MHz) spectrometer in DMSO-d_6. Chemical shifts are given in ppm.

Mass spectra were recorded on a Jeol JMS D 300 double focusing mass spectrometer coupled to a JMA 2000 data system. The compounds were introduced by direct inlet probe, heated from 50 to 400°C at a rate of 100°C/min. The ionization current was 300 mA, the accelerating voltage 3 kV and the chamber temperature 150°C.

General method of synthesis: the complexes were synthesized by mixing water solutions of copper (II), zinc (II), cobalt (II), nickel (II) and iron (III) salts and the ligand in amounts equal to metal–ligand molar ratio of 1:2. The reaction mixture was stirred with an electromagnetic stirrer at 25°C for 1 h. At the moment of mixing of the solutions colored precipitates were obtained. The precipitates were filtered, washed three times with water and dried in a desiccator to constant weight.

3.2. Biological evaluation

Experiments on i.p. and p.o. toxicity and anticoagulation activity were conducted on 84 white male mice, weighted 20 ± 2 g, while those concerning spasmolytic activity were performed on 20 guinea pigs weighted 500–600 g.

4. Pharmacology

Acute intraperitoneal toxicity (i.p. LD_{50}) and oral toxicity (p.o. LD_{50}) of the studied compounds were assessed by dissolving in saline (0.9% NaCl) with one drop of Tween 80, administered to mice i.p. or p.o. route.

The LD_{50} and its confidence interval were determined by using six animals per level of dose, with four or more doses being tested per compound, and with the logarithms of successive dose level differing by a constant amount, and calculated by the method of Litchfield and Wilcoxon [19]. The percentage of mortality was determined at 24 h.

The index of absorption indicates the absorbed ratio from the gastro-intestinal tract and was calculated by dividing data from i.p. to the data from p.o. toxicity and expressed as a percentage.

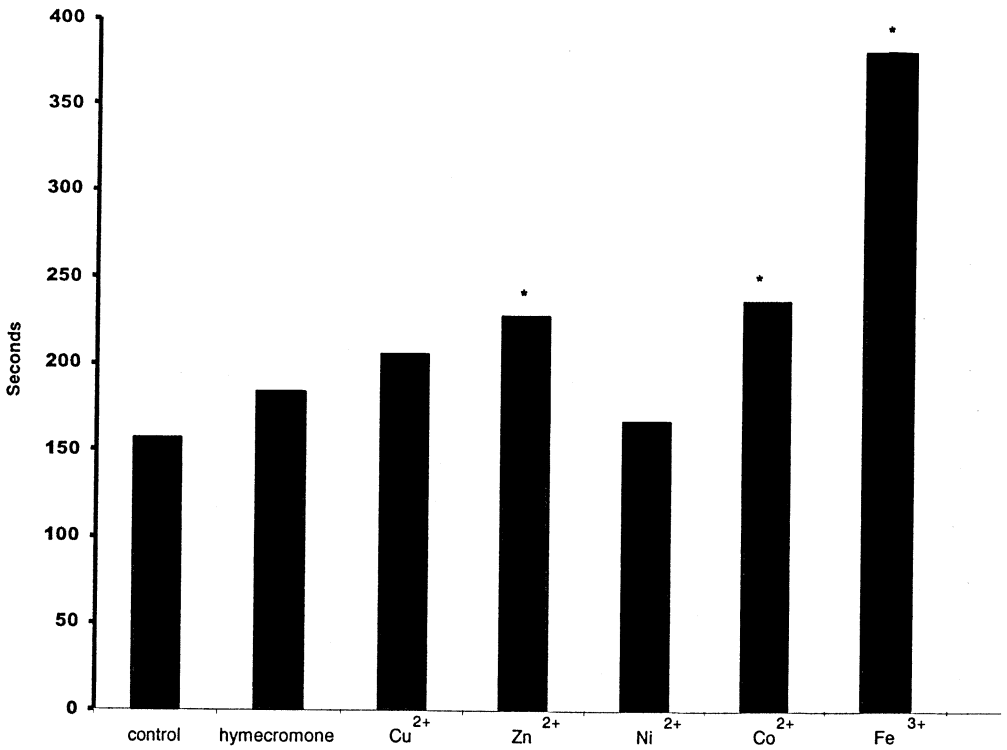


Fig. 1. The influence of investigated compounds on clotting time $*P < 0.05$, statistically significant compared to control.

Table 7
Influence of investigated compounds on acetylcholine-induced contraction

Compound	Concentration (mmol/l)	Inhibition of the contraction (%)	IC_{50}
Hymecromone	1×10^{-6}	0	[a]
	1×10^{-5}	13.4 ± 5	
	3×10^{-5}	22.7 ± 3.6	
	1×10^{-4}	28.9 ± 9.8	
$Fe(HL)_3 \cdot 5H_2O$	1×10^{-6}	17.6 ± 8	1.44×10^{-5}
	1×10^{-5}	38 ± 7.1	
	1×10^{-4}	56.5 ± 3.3	
	3×10^{-4}	100	

Data represented as mean $\pm$ SD; $n = 5$–6.
[a] IC_{50} value could not be calculated, as inhibition of the contraction did not reach 50%.

Table 8
Influence of investigated compounds on serotonine-induced contraction

Compound	Concentration (mmol/l)	Inhibition of the contraction (%)
Hymecromone	1×10^{-6}	0
	1×10^{-5}	16.5 ± 3.8
	1×10^{-4}	19.9 ± 3.6
	3×10^{-4}	35.5 ± 5.1
$Fe(HL)_3\cdot5H_2O$	1×10^{-6}	0
	1×10^{-5}	21.1 ± 4.4
	3×10^{-5}	32.8 ± 4.2
	1×10^{-4}	48.7 ± 3

Data represented as mean $\pm$ SD; $n = 5$–6.
IC_{50} could not be calculated as data did not reach 50% inhibition of the contraction.

The influence of investigated compounds on blood clotting time was determined by the method of Moravitz [20]. The investigation was performed on white male mice, line H, weighting 20 ± 2 g. The compounds were administered per orally in a volume of 0.1 ml/10 g b.w. for three consecutive days in doses of 1/10 of p.o. LD_{50}. On the fourth day (24 h after the last administration) the clotting time was assessed after making a small incision of the sublingual vein and measuring the clotting time of the second drop of blood on clean glass. The results were compared to the control group treated with vehicle only (0.9% NaCl) in the same way as treated group. All treated mice were allowed free access to food and water during the experiment.

The spasmolytic effect of the most active complex (Fe^{3+}) was evaluated on isolated guinea pig ileum. Adult male albino Hartley strain guinea pigs weighing 500–600 g were sacrificed by blow in the head. The animals were not allowed to have food but had water ad libidum 24 h prior the experiment. Isolated ileum was immersed in aerated (95% O_2 and 5% CO_2) Tyrode solution in organ bath of 30 ml volume and kept at 37°C. The strips were suspended between two stainless hooks with one end connected to the tissue holder and the other to a force–displacement transducer. The strips were allowed to equilibrate for 60 min at a resting tension of 1 g and then contracted to reach a plateau with acetylcholine 1×10^{-7} M or serotonin 1×10^{-6} M. After the washing procedure the strips were allowed to rest for 30 min. After the resting time tested compounds were added 5 min prior to the contractile agent (acetylcholine 1×10^{-7} M or serotonin 1×10^{-6} M). Mean inhibitory concentrations IC_{50} (concentration that inhibit the induced contraction in 50%) were calculated using the regression analysis method.

The statistical significance was determined by the Student's t test and was considered significant when $P < 0.05$.

5. Results and discussion

The complexes were characterized by elemental analysis. The metal ions were determined after mineralization and thermogravimetry. The presence of sodium ions was checked by means of flame photometry. The water content in the complexes was determined by Karl Fisher analysis and thermogravimetry. The formation of the complexes was confirmed by IR, ^1H NMR spectroscopy and mass spectrometry. The compounds were investigated by thin layer chromatography and the results showed that the complexes were pure.

Table 1 shows the data of the elemental analysis of the compound obtained serving as a basis for the determination of their empirical formulas and the results of the Karl Fisher analysis. Besides analytical data, in Table 1 the molar conductivities and melting points of the compounds have also been reported . The molar conductance values of all complexes were in the range 1.5–3.0 μS/cm indicating that the complexes are non-electrolytes.

The compositions of the complexes were confirmed by DTA and TGA. At the beginning of the DTA-curves of the complexes there is a clearly manifested endothermic effect ($\sim$100°C), which is due to the hygroscopic moisture released. A steady weight loss is recorded on heating up to $\sim$250°C corresponding to the elimination, respectively, of 4, 2, 3, 4, 5 molecules of water per molecule of copper, zinc, nickel, cobalt, and iron complexes. The amount of this weight loss, determined also by Karl Fisher analysis, is correlated with the intensity of these endothermic effects and with the respective decreases in the mass. On heating the complexes the decomposition step in all the cases corresponds to the loss of molecules of the ligands, which is in agreement with the compositions in Table 1. The exothermal effect (400–500°C) dominates in the thermograms of all the complexes, resulting from the decomposition of the organic matter. A further weight loss recorded up to 850–900°C indicates the formation of thermally stable oxide.

The mode of bonding of the ligand to Cu(II), Zn(II), Ni(II), Co(II) and Fe(III) ions was elucidated by recording the IR spectra of the complexes as compared with those of the free ligand.

IR-spectra of the compounds were recorded in solid state in Nujol in the range from 3600–400 cm^{-1}.

The bands appear in the IR spectrum of the ligand at 3655, 1726, 1603, 1576, 1290, 1140, 1070–976, 831, 613 and 484 cm^{-1}. A band at 1726 cm^{-1} can be attributed to the stretching vibrations of the carbonyl group; two bands at 1603 and 1576 cm^{-1} can be related to the stretching vibrations of the conjugated olefinic system. In all the complexes the $\nu_{(C=C)}$ band at 1607 cm^{-1} remains.

A broad band, characteristic of ν_{OH} of coordinated water was observed in the range of 3500–3400 cm^{-1} in the spectra of all the complexes. The weak band observed at 3655 cm^{-1} in the spectra of the free ligand shifted to lower wavenumbers (3490 cm^{-1}) in the complexes. This assignment is corroborated by the occurrence of the corresponding rocking mode in the range 840–830 cm^{-1}.

The band at 1159 cm^{-1} assigned as ν_{C-OH} is observed at more or less the same position in the complexes, but in the ligand this band is at 1140 cm^{-1}.

The most notable change observed upon complex formation is a shift of the C=O stretch to lower frequency. The $\nu_{C=O}$ band at 1726 cm^{-1} exhibits a shift of 40–50 cm^{-1} to lower wavenumber values on complexation which may be taken as evidence for the participation of the C=O group in coordination.

The C–C and C–O stretch and the C–O–C band are all shifted to higher frequency (1277, 1159 and 1076 cm^{-1}) in the complexes and similar frequency shifts are observed for the other complexes and are attributed to complexation of the positive ion with the carbonyl oxygen [21].

The data of the IR analysis are presented in Table 2 and they are in agreement with the compositions in Table 1.

Metal ion coordination with the ligand and the oxygen atom of the C=O group was shown owing to the data of ^1H NMR spectra. The proton spectra of the compounds recorded at 100 MHz in DMSO-d_6, confirmed the formation of the complexes.

The typical chemical shifts of the ^1H NMR spectra in DMSO-d_6 are presented in Table 3.

The mass-spectral analysis confirmed the metal–ligand ratio in the complexes that have been investigated. For example, Cu-complex MS: m/z (% of intensity): 352 (8); 176 (100); 154 (28); 136 (28); 106 (12) and Zn-complex MS: 352 (7); 176 (100); 154 (31); 136 (32); 106 (16).

The analysis of the data obtained on acute intraperitoneal toxicity (LD_{50}) showed that the complexes of copper and nickel are more toxic compared to hymecromone. The other compounds are statistically significantly less toxic than hymecromone (Table 4).

Analysis of the data obtained on acute per oral toxicity (LD_{50}) showed that all the complexes except the complexes of zinc and cobalt had an acute p.o. toxicity statistically significantly lower than standard substance hymecromone (Table 5).

The index of absorption is shown in Table 6. The least absorption index was for the complex of copper.

The analysis of the data obtained from blood clotting time shows that the complex of iron has the greatest effect on clotting time. The effects of iron, followed by cobalt and zinc complexes are statistically significant, compared to the control (Fig. 1).

In vitro experiments on isolated guinea pig ileum showed that the spasmolytic effect on acetylcholine-induced contraction was more pronounced in complex of iron ($IC_{50} = 1.44 \times 10^{-5}$ M), compared to hymecromone (IC_{50} value could not be obtained, because the higher concentration used 1×10^{-4} M showed only 28.9% relaxation) (Table 7). In experiments with serotonine there were no significant differences between the compound investigated and referent one hymecromone (Table 8).

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