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Copper Complex of Diphenylamine Mercapto Carboxylic Acid And Its Anti-Inflammatory Activity

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ABSTRACT

Diphenyl amine-2-mercapto-2'-carboxylic acid and its Cu (II), Ni(II), Co(II) and Zn(II) complexes have been synthesized and characterized by their elemental analyses, molecular weight determination, molar conductance, infrared and electronic spectra and magnetic measurements. In acute anti-inflammatory test Cu (II) complex was found to be more potent than Ibuprofen in normal and adrenalectomized rats but less effective in sub acute and chronic anti-inflammatory tests. It inhibited PGE_2 like substances (25%) and Castor oil induced diarrhea (30%), but did not protect the mice against arachidonic acid induced mortality. However, it had very low degree of gastric irritation and without analgesic effect. Metal complexes had very low degree of gastric irritation so it can be safely used for the treatment of inflammation.

KEY WORDS: Metal complex, NSAID'S, Prostaglandin, Anti-inflammatory agent

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INTRODUCTION

Inflammation is a multi mediated response of tissue and cells to an injury or injurious agents involving physiological, morphological and biochemical changes. As inflammation, especially chronic inflammatory diseases, affect the quality of patient's life, extensive search for a new drug effective to treat inflammation and other orthopathies has got added impetus. Unfortunately, most of the anti-inflammatory (AI) drugs, available today, reveal a high incidence of gastric irritation, apart from the effects on kidney, liver, bone marrow and skin. Therefore, there is a need for a non-steroidal anti-inflammatory drug (NSAID) effective in rheumatoid arthritis, osteoarthritis, gout and related diseases with reduced side effects as compared to the existing clinically effective drugs. Newer COX-2 inhibitors like Rofecoxib and Valdecoxib, have also been reported to have side effects specially on kidney and on vascular integrity [1]. The newer drugs for early aggressive treatment of rheumatoid arthritis like Leflunomide, Infliximab and Etanercept also are not well tolerated [2,3]. The cyclic acetal like derivatives of salicylic acid and salicylamide has been reported to be anti-inflammatory [4].

Classical pharmacological studies have measured the ability of a chemical compound to reduce the symptoms of inflammation. It will be beneficial to eliminate the symptoms either by suppressing normal or correcting an impaired inflammatory response. However the suppression may lead to more serious consequences e.g. by corticoids and therefore, an agent which corrects the impaired response without serious toxicity, should have potential for therapeutic usefulness. The metal complexes meet these later criteria and have been shown to be effective in treatment of connective tissue diseases. It has been suggested that in human, the rheumatoid arthritis may be the result of a deficiency or lack of superoxide dismutase enzyme [5] which mostly contain Copper, required for dismutase activity [6].

Inspired by the clinical success of Gold salts, Copper and Zinc chelates of known AI compounds have been extensively investigated for use as potential AI agents. It has been established that Copper chelates of known AI drugs show more AI activity than the parent compound[7,8] and exhibit intrinsically low ulcerogenicity[9] whereas Zinc has been used in rheumatoid arthritis and is beneficial in ulcer healing[10,11].Nickel protects aspirin induced mucosal damage[12] and Cobalt complexes have been reported to exhibit anti-inflammatory activity from this laboratory [13,15].

Keeping above facts in view and in search for an ideal AI agent devoid of common side effects, many compounds and its complexes involving Copper, Nickel, Cobalt and Zinc as metal ions have been synthesized and evaluated for its anti-inflammatory activity. The compound and chelates were subjected to primary screening against carrageenan induced rat paw edema test. On primary screening, it has been found that Zinc complex of Diphenylamine-2, 2'-dicarboxilic acid is active and has been reported earlier [16].

Another Copper chelate of the Diphenyl amine-2-mercapto-2'-carboxylic acid (DPMC) is also active, therefore, it has been further investigated and the results are reported here.

MATERIALS AND METHODS

All the chemicals used were of AR grade either, BDH or E. Merck.

SYNTHESIS OF DIPHENYL AMINE-2-MERCAPTO-2'-CARBOXYLIC ACID

Diphenyl amine-2-mercapto-2'-carboxylic acid (DPMC) was synthesized by condensing an equimolar amount of O-chlorobenzoic acid and Thiophenol in the presence of Copper oxide, in slightly alkaline media. The compound was decolorized with activated charcoal on boiling. It was dried under vacuum.

SYNTHESIS OF METAL COMPLEXES:

The solid metal complexes of Cu(II), Ni(II), Co(II) and Zn(II) were prepared by refluxing the DPMC with respective metal acetate in 1:1 ratio for three hours. On concentrating and cooling the reaction mixture, a colored precipitate was obtained. The precipitate was filtered under suction, washed first with water, then alcohol and finally with ether and dried under vacuum.

PHYSICAL MEASUREMENTS:

The synthesized ligand and its metal complexes were analyzed for Carbon, Hydrogen, Nitrogen and Sulfur by micro analytical techniques and metal contents in the complexes were estimated by standard methods [17].

Molecular weight of metal complexes was determined by cryoscopic method in Dimethyl sulfoxide (DMSO). The molar conductance of metal complexes was measured in DMSO on a Toshniwal conductivity bridge. Infrared spectra were recorded on Perkin-Elmer spectrophotometer model-521. The electronic spectra of ligand and metal complexes were recorded on cary-14 spectrophotometer using pure

DMSO as reference. Magnetic measurements were carried out at room temperature by Gouy's method using $CuSO_4.5H_2O$ as a calibrant. The values were corrected for diamagnetism by Pascal constants.

PHARMACOLOGICAL ACTIVITY:

The standard drugs and Copper complex were administered subcutaneously as suspension in saline containing 1.4% Poly vinyl alcohol.

CARRAGEENAN INDUCED RAT PAW EDEMA TEST:

The anti-inflammatory action was assessed according to the method of Winter et.al[18]. The standard drug was Ibuprofen (Boots, India). Overnight fasted rats (Wistar) of either sex weighing 140-160 gm were arranged in group of six each. Edema was induced by injecting 0.1 ml or 1% carrageenan (Marine colloids Inc., USA) suspension in normal saline into the plantar aponeurosis of right paw. The paw volume was measured immediately and 4 hours after the injection of carrageenan by a volume differential meter (M 7101, Ugo Basile,). The percentage inhibition was calculated.

CARRAGEENAN INDUCED RAT PAW EDEMA IN ADRENALECTOMIZED RATS

Male Wistar rats (140-160 gm) were bilaterally adrenalectomized under light ether anesthesia by the method reported by Gaunt [19]. Water was replaced with normal saline for drinking. Two days after surgery the rats were divided into groups of 6 each. Edema was induced by carrageenan and measured as in normal rats. The percentage inhibition was calculated.

COTTON PELLET GRANULOMA TEST

Inhibition of granuloma tissue formation was assessed by the method reported earlier[20]. Sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously on either side of the midline dorsally under light ether anesthesia in male Wistar rats. The Copper complex, Naproxen & Ibuprofen were administered each day for six days. On the 7th day the rats were sacrificed and the pellets were dissected out and dried to a constant weight at 80^{o}C. The mean weight of granulation tissue formed around each pellet of the group was calculated.

ADJUVANT ARTHRITIS (ESTABLISHED)

Male Wistar rats (160±20 gm) were injected with 0.1 ml of a fine suspension of Freunds adjuvant complete (Difco) into the plantar aponeurosis of right paw and the paw was left untreated for 14 days[21]. On day 14th, the rats which showed 45 to 55% edema of the injected paw, were grouped into four groups of eight each. The Naproxen and Copper complex were administered daily from 14th day to 28th day. The paw volume of both, injected and un-injected paw was measured every alternate day using water Plethysmometer (M 7150 Ugo Basile). The percentage inhibition was calculated and data were assessed statistically using student 't' test. The secondary lesions were assessed in the ear, forelimbs, hind limbs and tail.

CASTOR OIL INDUCED DIARRHEA

Effect of Copper complex on Castor oil (Amrutanjan Ltd., Hyderabad) induced fluid loss was assessed by the method of Awouters et.al [22]. Overnight fasted male (Charles Foster Strain) rats,

weighing 180 ± 20 gm were used. Vehicle/drug was administered subcutaneously, 1/2 hour prior to 1 ml castor oil orally. The treated rats were kept in groups of two in metabolic cages (Techniplast, Gazeda, Italy) for collection of Castor oil induced gastro-intestinal evacuation.

Paper sheets of uniform weight were kept beneath each metabolic cage for fecal collection and were assessed at the end of 6 hours. The percentage inhibition was calculated. The rats were again used after fifteen days in the cross over test.

PROSTAGLANDIN'S (PG'S) ESTIMATIONS

Prostaglandin's were extracted in the inflammatory exudates by the method of Higgs et al[23]. The exudates were transferred to a graduated tube and treated with 5.0 ml of absolute acetone. It was stirred and centrifuged at 0^{o}C. The supernatant liquid after addition of 2 volumes of n-hexane was stirred and centrifuged. The lower aqueous layer was acidified to pH 3.5 with citric acid. The PG's were extracted into ethyl acetate. The ethyl acetate layer was evaporated to dryness under reduced pressure and reconstituted in Kreb's solution for the bioassay. PG's were bio assayed on rat fundus strip[24]. PGE_2 (Sigma) was used as standard and the contents were estimated by matching assay.

ANALGESIC ACTIVITY

Analgesic activity was assessed in prescreened mice using Acetic acid, BDH[25] or Phenylquinone, (Sigma)[26]. The prescreened Swiss albino mice were divided into three groups of six each. The mice were fasted for sixteen hours before the experiment. The mice were given Ibuprofen, or Copper complex, 1/2 hour before the injection of Acetic acid (50 mg/kg) or Phenylquinone (2 mg/kg) intraperitoneally. The data were reported as all or none i.e. number of writhing per minute for each mice treated with vehicle or respective treatment groups. The number of writhing movements shown by each mouse was counted for 20 minutes using manually operated digital counter. The percentage inhibition was calculated.

ARACHIDONIC ACID INDUCED MORTALITY IN MICE

The test was conducted as per method of Kohler et.al[27]. Arachidonic acid (Sigma), 50 mg/kg was administered into the tail vein in a volume of 10 ml/kg in Swiss albino mice. For determining the inhibitory activity of Copper complex or Naproxen or vehicle, these were administered *s.c.* to groups of 5 mice, one hour before arachidonic acid challenge. The percentage mortality and percentage protection in each group was noted 24 hours after arachidonic acid challenge.

ULCEROGENIC TEST

Experiments were carried out in 24 hours fasted male/s female (non-pregnant) rats (Charles Foster Strain) weighing between 140-175 gm. Phenylbutazone was used for comparison. Water was allowed *ad libitum* before and during the experiment. Copper complex or Phenylbutazone was given orally as a suspension in saline containing 1.4% poly vinyl alcohol and sacrificed 6 hours after the treatment. After opening the abdomen, the stomach was removed, cut open along the greater curvature, washed with saline and examined under stereoscopic binocular microscope (Meopta) for scoring the lesions under blind conditions[28]. The lesions were scored as

0 - No lesion; 1 - Hemorrhagic; 2 - Mucosal Ulceration; 3 - Deep ulceration 4 - Perforated ulcer.

The ulcerogenic index (UI) was calculated as follows :

$$\textbf{Ulcerogenic index (UI)} = \frac{\text{ADU X \%RU}}{100}$$

RESULT AND DISCUSSIONS

ELEMENTAL ANALYSES, MOLECULAR WEIGHT DATA AND MOLAR CONDUCTANCE MEASUREMENTS

All the metal complexes were found thermally stable and insoluble in water. They varied in their solubility in various organic solvents. The low molar conductance value (0.97-2.02 $ohm^{-1}cm^2mol^{-1}$) indicated their non-electrolytic nature due to charge neutralization of the metal ion (M) with ligand (L). The 1:1 ML stoichiometry was concluded from their elemental analysis and molecular weight measurement data, which are well in agreement with the theoretical values. Thermal dehydration and infrared spectra confirmed the presence of water molecules.

MAGNETIC PROPERTIES

The magnetic moments of Cu (II), Ni (II) and Co (II) complexes, calculated from the corrected magnetic susceptibilities have been studied. The observed magnetic moment for Copper complex was found 1.8 B.M., which is well in agreement with the spin only value[29]. The observed magnetic moment value, 3.09 B.M. for Nickel complex is within range, expected for octahedral complex. The magnetic moment value for Cobalt complex is found 4.88 B.M. reported for high spin octahedral complexes.

ELECTRONIC SPECTRA

The copper complex shows a single broad band around 13833 cm^{-1} due to the distorted octahedral environment around the metal ion [30]. The three bands observed at 9980, 17185 and 15850 cm^{-1} for

nickel complex may be due to $^3A_{2g} \rightarrow {}^3T_{2g}$ (F), $^3A_{2g} \rightarrow {}^3T_{1g}$ (F) and $^3A_{2g} \rightarrow {}^3T_{1g}$ (P) transitions respectively in an octahedral geometry [31]. The electronic spectra of Cobalt complex shows three absorption band at 8050, 17675 and 20085 cm^{-1} respectively which may be assigned to $^4T_{1g} \rightarrow {}^4T_{2g}$ (F), $^4T_{1g} \rightarrow {}^4A_{2g}$ (F) and $^4T_{1g} \rightarrow {}^4T_{1g}$ (P) transitions showing distorted octahedral geometry around metal ion. On the basis of elemental analyses, infrared spectra, molar conductance and molecular weight determination data, the Zinc complex was proposed to have an octahedral geometry.

INFRARED SPECTRA:

For the sake of brevity, only shifted or altogether new peaks appearing in the spectra of metal complexes have been discussed. The Diphenyl amine-2-mercapto-2'-carboxylic acid shows a band around 3200 cm^{-1} which is shifted to the lower frequency region in the case of its complexes, suggesting the coordination through N of -NH group. The infrared spectra showing band at 1675 cm^{-1}, which is shifted to the lower frequency region in case of metal complexes, confirm the coordination of the ligand to the metal ion through carboxylic group. The infrared band appearing at 2535 cm^{-1} in the case of ligand is due to the presence of -SH group, which was found absent in the case of metal complexes, indicates the deprotonation and coordination through S of -SH group. Stretching modes occurring at 3500-3600 cm^{-1} and bending modes H-OH appearing around 1580 cm^{-1} reveals the presence of water molecules in the complexes. It has been confirmed by the thermal gravimetric analysis. The appearance of band at 460-470 cm^{-1} (M-N), 400-420 cm^{-1} (M-O) and 360-380 cm^{-1} (M-S) support the coordination through N, O and S donor sites of the ligand[32].

PHARMACOLOGICAL ACTIVITY

Dr. F. Dudley Hart [33] once stated that " A perfect NSAID should be as potent as large doses of corticosteroids but without their endocrine action. It should be as nontoxic, as placebo, leaving the GIT unaffected at both ends with no toxic effect on blood, eye, liver and kidney. He further stated that it is doubtful, if it has ever been or ever will be produced, even in heaven". This quote prompted us to develop an ideal NSAID and therefore we have synthesized about 200 compounds and their metal complexes that were primarily screened for their AI activity against carrageenan induced edema. The Copper complex inhibited 58.4% of carrageenan induced edema and hence it was subjected for further AI tests. As the ligand and its metal complex were insoluble in water, they were suspended in normal saline using 1% Poly vinyl alcohol as a nonionic suspending agent. In our experience, it has been observed that more consistent results were obtained by giving the complex by subcutaneous route. For this very cause, this route was preferred over oral or intraperitoneal route.

Copper complex was found to be more potent than Ibuprofen in normal and adrenalectomized rats in carrageenan induced rat paw test (Table 1) but was less effective than Ibuprofen and Naproxen (33%) in cotton pellet granuloma test and 50%

as effective as Naproxen in adjuvant arthritis (Established) test in rats at 25 mg/kg dose (Table 2 & 3). It has been suggested that metal complexes specially Copper and Zinc may cause a decrease in synthesis of pro inflammatory prostaglandin's viz PGE_2 with concomitant increase in anti-inflammatory prostaglandin (PGF_2)[34] and therefore, can inhibit,Castor oil induced diarrhea. Copper complex inhibited PGE_2 like substances (25%), though this inhibition was less than Naproxen and also inhibited 30 % of Castor oil induced diarrhea (Table 4 & 5). However Copper complex did not have any analgesic effect in primary screening against either Acetic acid or Phenylquinone induced writhings in mice (Table 6 & 7). There are reports that administration of arachidonic acid, triggers formation of either prostaglandin or thromboxane, which induces platelet thrombi and death due to constriction of pulmonary vessels[35]. Copper complex surprisingly did not protect the mice against arachidonic acid induced mortality (Table 8). Copper and Zinc ions may be beneficial in treatment of inflammatory conditions and low doses of these metals can be administered safely to protect the stomach against the ulcerogenic actions of NSAID'S [36]. As such, gastric irritation is now considered, at the preliminary stages of development of an NSAID as one of the major cause to reject the compound, if it shows high degree of gastric irritation. Copper complex exhibited very low incidence of gastric irritation (Table 9) and hence can be designated as a safe NSAID in relation to gastric irritation.

Table 1 : Effect of Copper complex in normal and adrenalectomized rats against Carrageenan induced rat paw oedema.

S. No	Pretreatment (Dose mg/kg s.c)	Normal rats		Adrenalectomized rats	
		Paw volume in in(ml) mean ± SE	% Inhibition	Paw volume in(ml) mean ± SE	% Inhibition
1	Vehicle	1.30±0.04	-	1.02±0.20	-
2	Copper complex (50)	0.54±0.05	58.46	0.55±0.30	46.0
3	Ibuprofen (50)	0.62±0.04	52.30	0.58±0.40	43.20

n = 6 in each group

Table 2 : Effect of Copper complex on Cotton pellet granuloma in rats.

S. No	Pretreatment (Dose mg/kg s.c)	Weight of dry Granuloma mg± SE	% Inhibition
1	Control	250.4±5.6	-
2	Copper complex (50)	220.0±7.8	12.00
3	Naproxen (25)	205±4.0	18.00
4	Ibuprofen (50)	203±5.3	18.61

n= 10 in control experiments and 8 in treated groups

Table 3 : Effect of Copper complex on adjuvant arthritis (Established) test in rats.

S. No	Drug dose mg/kg s.c	Days : Oedema developed in (ml) mean ± SE							
		0 14th	2 16th	4 18th	6 20th	8 22nd	10 24th	12 26th	14 28th
1	Control	1.41 ±0.12	1.89 ±0.15	1.85 ±0.12	1.82 ±0.09	1.84 ±0.12	1.87 ±0.12	1.82 ±0.17	1.70 ±0.6
2	Copper complex (25)	1.35 ±0.15*	1.57 ±0.13*	1.51 ±0.13**	1.41 ±0.15*	1.30 ±0.16*	1.50 ±0.13**	1.31 ±0.16**	1.25 ±0.16*
3	Naproxen (04)	1.39 ±0.12**	1.32 ±0.12***	1.07 ±0.09***	0.98 ±0.07***	0.95 ±0.08**	0.77 ±0.06***	0.86 ±0.08***	0.81 ±0.07***
4	Naproxen (08)	1.32 ±0.12**	1.20 ±0.14***	1.03 ±0.08***	0.95 ±0.06***	0.85 ±0.08***	0.70 ±0.08***	0.73 ±0.70***	0.73 ±0.10***

n=8 in each group- * p<0.05, ** p<0.01 , *** p<0.001

Table 4: effect of copper complex on castor induced diarrhea

S. No	Pretreatment (Dose mg/kg s.c.)	Mean evacuation in gm ± SE in 6 hours	% Inhibition
1	Control	6.00±0.16	-
2	Copper complex	4.15±0.38	30

n= 14 in both groups.

Table 5 : Effect of Copper complex on PGE_2 like substance in the inflammatory exudate.

S. No	Pretreatment (Dose mg/kg s.c X 3 days	PGE_2 like substance mg/ml± SE	%Inhibition
1	Control	40.8± 0.35	-
2	Copper complex (50)	30.6± 0.46	25.0
3	Naproxen (25)	15.5± 0.28	62.0

N= 6 in each group

Table 6 : Effect of Copper complex on acetic acid inducted writhing in mice.

S .No	Pretreatment(Dose mg/kg s.c.)	Writhing mean±SE per minute	% protection
1	Control	11.00±1.6	-
2	Ibuprofen (50)	8.20±1.5	25
3	Copper complex (50)	12.16±2.02	nil

Table 7: Effect of Copper complex on 0.2% Phenylquinone induced writhing test in mice.

S. No	Pretreatment(Dose mg/kg s.c.)	Writhing mean±SE per minute	% protection
1	Control	14.0±1.2	-
2	Ibuprofen (50)	1.6±0.2	80
3	Copper complex (50)	23.2±5.2	Nil

N=5 in each group

Table 8: Effect of Copper complex on Arachidonic acid induced mortality in mice.

S.No	Pretreatment (Dose mg/kg s.c)	No of mice dead/ total	% Moratlity	% Protection
1	Control	5/5	100.0	-
2	Copper complex (50)	5/5	100.0	0.0
3	Naproxen (25)	0/5	0.0	100.0

Table 9 : Effect of Copper complex on Ulcerogenic potential.

S. No	Pretreatment (Dose mg/kg PO)	Average degree of Ulceration (ADU	Percentage of rats with ulcer (%RU)	Ulcerogenic index(UI)
1	Vehicle	-	-	-
2	Copper complex(50)	1.4	50	0.7
3	DPMC(50)	2.0	90	1.8
4	Phenylbutazone(50)	2.0	100	2.0

N= 10 in each group

The mechanism of action of these coordination compounds as AI and antiulcer agent is yet not well understood. However, it is well known that repair at sites of inflammation including ulcer, requires the extra cellular maturation or cross linking of the extra cellular components, collagen and elastin. Since the enzyme, Lysyl oxidase responsible for this is a Copper dependent enzyme, this aspect of wound or tissue repair assumes central significance with regard to the plausible role of Copper coordination compounds. There are many reports, which adopt the structures of existing NSAID's to increase the stability constant towards metal ions and thus favor the increased passage of metal complex through the gastric mucosa [36]. However it is true that metal complexes certainly deserves more extensive attention and bio-investigation to develop an ideal NSAID devoid of common side effect

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