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Research Paper :

Complexes of diphenyl amine mercapto carboxylic acid : A new anti-inflammatory agent

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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 subacute and chronic anti-inflammatory tests. It inhibited PGE_2 like substance (25%) and Castor oil induced diarrhoea (30%) but did not protect the mice against arachidonic acid induced mortality. However, it had very low degree of gastric irritation but without analgesic effect.

Key words : Metal complex, NSAID'S, Prostaglandins, Anti inflammatory agent

Inflammation is a multi mediated response of tissue and cells to an injury or injurious agents involving physiological, morphological and biochemical changes. As inflammation, specially chronic inflammatory diseases, affect the quality of patient's life. Extensive search for a new drug effective to treat inflammation and other orthopathies have got added impetus. Unfortunately, most of the anti-inflammatory (A1) 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 steroid 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.

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[1], which mostly contain Copper, required for dismutase activity[2].

Inspired by the clinical success of gold salts, copper and zinc chelates of known AI compounds, they 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[3] and exhibit intrinsically low ulcerogenicity[4], whereas, zinc has been used in rheumatoid arthritis[5] and is beneficial in ulcer healing[6]. Nickel protects aspirin induced mucosal damage[7] and cobalt complexes have been reported to exhibit AI activity from this laboratory[8-10].

Keeping above facts in view and in search for an ideal AI agent devoid of common side effects, the Diphenyl amine-2-mercapto-2'-carboxylic acid (DPMC) 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 oedema test. It has been found that copper chelate of DPMC is most active among all the compounds. 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 (Fig. 1) was synthesized by condensing a equimolar amount of O-chlorobenzoic acid and Thiophenol in the presence of

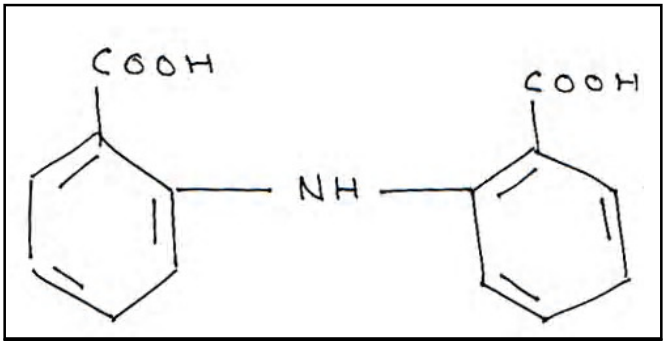

Fig. 1 : Diphenyl amine-2,2'-dicarboxylic acid

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 Diphenyl amine-2-mercapto-2'-carboxylic acid (DPMC) with respective metal acetate (Fig.2) 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, than alcohol and finally with ether and dried under vacuum.

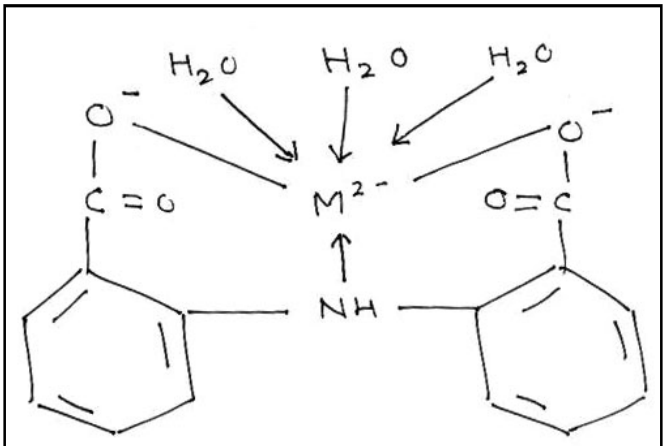

Fig. 2 : Structure of metal complex

Physical measurement :

The synthesized ligand and its metal complexes were analysed for carbon, hydrogen, nitrogen and sulphur by micro analytical techniques and metal contents in the complexes were estimated by standard methods[11].

Molecular weight of metal complexes were determined by cryoscopic method in Dimethyl sulfoxide (DMSO). The molar conductance of metal complexes was measured in DMSO using Toshniwal conductivity meter. 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 calibrate. The values were corrected for diamagnetism by Pascal constants.

Pharmacology :

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

Carrageenan induced rat paw oedema test :

The anti-inflammatory action was assessed according to the method of Winter *et al.*[12] . The standard drugs were Naproxen (Searl, India) and Ibuprofen (Boots, India).

Overnight fasted rats (Wistar) of either sex weighing 140-160 g were arranged in group of six each. Oedema 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 were measured immediately and 4 hours after the injection of carrageenan by a volume differential meter (M 7101, Ugo Basile, Milan, Italy). The percentage inhibition was calculated.

Carrageenan induced rat paw oedema in adrenalectomized rats :

Male Wistar rats (140-160 g) were bilaterally adrenalectomized under light ether anesthesia by the method of Schultzer[13]. Water was replaced with normal saline for drinking. Two days after surgery the rats were divided into groups of 6 each. Oedema was induced by carrageenan and measured as in normal rats. The percentage inhibition was calculated.

Cotton pellet granuloma test (CP) :

Inhibition of granuloma tissue formation was assessed by the method of Winter and Porter[14]. 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 and 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°C. The mean weight of granulation tissue formed around each pellet of the group was calculated.

Adjuvant arthritis (Established) :

Male Wistar rats (160-20 g) 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. On day 14th, the rats which showed 45 to 55% oedema of the injected paw were grouped into four of eight each. The Naproxen and Copper complex were administered daily from 14th day to 28th day. The paw volume of both, injected and uninjected paw was measured every alternate day using a water Plethsmometer (M 7150 Ugo Basile, Milan, Italy).

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 Coppper complex on Castor oil (Amrutanjan Ltd., Hyderabad) induced fluid loss was assessed by the method of Awouters *et al.*[16]. Overnight fasted male (Charles Foster Strain) rats were used weighing 180 ± 20 g. Vehicle/drug was administered SC 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 faecal collection and was 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.

Prostagladins (PG's) estimations :

Prostagladins were Extracted in the inflammatory exudate by the method of Higgs and Salman[17]. The exudate was transferred to a graduated tube and treated with 5.0 ml of absolute acetone. It was stirred and centrifuged at 0^0C. 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 bio assay.

PG's were bio assayed on rat fundus strip[18]. 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)[19] or Phenylquinone (Sigma)[20]. The prescreened Swiss albino mice were divided into three groups. The mice were fasted for sixteen hours before the experiment. The mice were given Ibuprofen, Naproxen or Copper complex 1/2 hour before the injection of Acetic acid (50 mg/kg) or Phenylquinone (2 mg/kg) intraperitoneally. The data was 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.*[21]. 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 and female (non-pregnant) rats (Charles Foster Strain) weighing between 140-175 g. 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 and examined under stereoscopic binocular microscope (Meopta) for scoring the lesions under blind conditions[22]. The lesions were scored as follows :

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

The ulcerogenic index (UI) was calculated as follows:

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

RESULTS AND DISCUSSION

The results obtained from the present investigation are below :

Elemental analyses, molecular weight data and molar conductance measurement :

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 analyses and molecular weight measurement data, which are well in agreement with the theoretical values. The presence of water molecules were confirmed by thermal dehydration and infrared spectra.

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[23]. 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[24].

Electronic spectra :

The copper complex shows a single broad band around 13833 cm^{-1} due to the distorted octahedral environment around the metal ion[25]. 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[25]. 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[26]. 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 indicating the deprotonation and coordination through S of -SH group[27]. The presence of water molecules in the complexes is revealed by stretching modes occurring at 3500-3600 cm^{-1} and bending modes H-OH appearing around 1580 cm^{-1}.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[28].

Pharmacology :

Dr. F.Dudley Hart[29] 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, lever 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 many compounds and their metal complexes which were primarily screened for their AI activity against carragennan induced oedema. The Copper complex inhibited 58.4% of carrageenan induced oedema 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 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 vary cause this route was preferred over oral or intraperitonial route.

Copper complex was found more potent than Ibuprofen in normal and adrenalectomized rats in carrageenan induced rat paw test (Table 1) but was less

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

Pretreatment (Dose mg/kg SC)	Normal rats		Adrenalectomized rats	
	Paw volume in mean ml ± SE	% inhibition	Paw volume in mean ml ± SE	% inhibition
Vehicle	1.30 ± 0.04	-	1.02 ± 0.20	-
Copper complex (50)	0.54 ± 0.05	58.46	0.55 ± 0.30	46.0
Ibuprofen (50)	0.62 ± 0.04	52.30	0.58 ± 0.40	43.20

n = 6 in each group

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 and 3). It has been suggested that metal complexes specially copper and zinc may cause a decrease in synthesis of pro inflammatory prostaglandins

Table 2 : Effect of copper complex on cotton pellet granuloma in rats

Pretreatment (Dose mg/kg SC)	Weight of dry Granuloma mg ± SB	% Inhibition
Control	250.4 ± 5.6	-
Copper complex(50)	220.0 ± 7.8	12.00
Naproxen(25)	205.0 ± 4.0	18.00
Ibuprofen(50)	203.8 ± 5.3	18.61

n = 10 in control experiments and 8 in treated groups

inflammatory conditions and low doses of these metals can be administered safely to protect the stomach against the ulcerogenic actions of NSAID'S[32]. 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.

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

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

Drug dose (mg/kg SC)	Days : Oedema developed in mean ml ± SE							
	0 14th	2 16th	4 18th	6 20th	8 22th	10 24th	12 26th	14 28th
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±0.16
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*
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***
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±0.07***	0.73±0.10***

*, ** and *** indicate significance of values at P=0.05, 0.01, 0.001, respectively n=8 in each group

viz., PGE_2 with concomitant increase in anti-inflammatory prostaglandin (PGF_2)[30] and therefore can inhibit Castor oil induced diarrhoea. Copper complex inhibited PGE_2 like substances (25%) though this inhibition was less than Naproxen and also inhibited 30 % of Castor oil induced diarrhoea (Table 4 and 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 and 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[31]. Copper complex surprisingly did not protect the mice against arachidonic acid induced morality (Table 8). Copper and zinc ions may be beneficial in treatment of

Table 4 : Effect of copper complex on castor induced diarrhoea

Pretreatment (Dose mg/kg SC)	Mean evacuation in g ± SE in 6 hours	% Inhibition
Control	6.00 ± 0.16	-
Copper complex (50)	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

Pretreatment (Dose mg/kg SC x 3 days)	PGE_2 like substance mg/ml ± SE	% Inhibition
Control	40.8 ± 0.35	-
Copper complex(50)	30.6 ± 0.46	25.0
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

Pretreatment (Dose mg/kg SC)	Writhing Mean ± SE	% Protection
Control	11.00 ± 1.6	-
Ibuprofen (50)	8.20 ± 1.5	25
Copper complex (50)	12.16 ± 2.02	nil

n = 6 in each group

enzyme[33], 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

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

Pretreatment (Dose mg/kg SC)	Writhing Mean ± SE	% Protection
Control	14.0 ± 1.2	-
Ibuprofen(50)	1.6 ± 0.2	80
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

Pretreatment (Dose mg/kg SC)	No. of mice dead/total	% mortality	% Protection
Control	5/5	100.0	-
Copper complex (50)	5/5	100.0	0.0
Naproxen (25)	0/5	0.0	100.00

Table 9 : Effect of copper complex on Ulcerogenic potential

Pretreatment (Dose mg/kg SC)	Average degree of Ulceration (ADU)	Percentage of rats with ulcer(%RU)	Ulcerogenic index(UI)
Vehicle	-	-	-
Copper complex (50)	1.4	50	0.7
DPMC(50)	2.0	90	1.8
Phenylbutazone(50)	2.0	100	2.0

n = 10 in each group

increase the stability constant towards metal ions and thus favour the increased passage of metal complex through the gastric mucosa[32]. However it is true that metal complexes certainly deserves more extensive attention and bio investigation to develop an ideal NSAID devoid of common side effects.

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