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IN-VITRO EFFECTS OF CADMIUM, CHROMIUM, MANGANESE AND ZINC ON THE ANTIMICROBIAL ACTIVITY OF CHLORAMPHENICOL

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ABSTRACT: Heavy metals have been shown to interact with various antibiotics resulting in differing spectrum of activity than that of the parent drug. In the present study the nature of antimicrobial activity exhibited by chloramphenicol in the presence of Cd, Cr, Mn and Zn at 37°C against *S. typhii, S. aureus, E. coli, P. vulgaris and K. pneumoniae* were evaluated. Broth dilution method of antimicrobial susceptibility testing was used to determine the minimum inhibitory concentration (MIC) of chloramphenicol against the organisms, while the cup-plate agar diffusion technique was used to quantify antimicrobial activity of the free drug and drug-metal mixtures. Results obtained for the interactions showed both decrease and increase in chloramphenicol activity depending on the type and concentration of the metal involved, and also on the organism. The resultant change in spectrum and profile of activity can result in unpredictable clinical efficacy of this drug and they should be avoided where possible.

KEYWORDS: chloramphenicol, heavy metals, complexation, interaction, zone of inhibition

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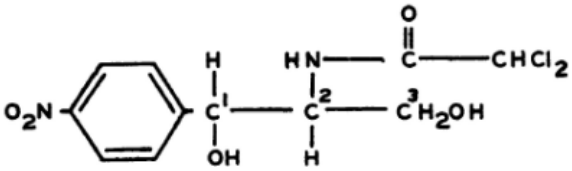
INTRODUCTION

Chloramphenicol is a broad spectrum antibiotic which was first isolated from cultures of *Streptomyces venezuelae* but is now produced synthetically. The preferred chemical name is D-threo-2,2-dichloro-N-[3-hydroxy-a-(hydroxymethyl)-p-nitrophenethyl]-acetamide (Figure 1). It is a derivative of dichoroacetic acid with a nitrobenzene moiety attached and it acts by interfering with bacterial protein synthesis. It is mainly bacteriostatic with a broad spectrum of action against both Gram positive and Gram negative bacteria, as well as some other organisms including rickettsiae and chlamydias [1]. The risk of life threatening adverse effects (particularly bone marrow aplasia) and resistance, has severely limited the clinical usefulness of chloramphenicol [1]. It is however experiencing resurgence in use in some countries due to resistance to other safer antibiotics and its superiority in fighting certain anaerobic infections and infections of the central nervous system [2].

Figure 1: Chloramphenicol



Metal complexes with active pharmaceuticals in which the drug molecules play the role of a ligand have been reported [3,4,5]. The nitro group, alkyl hydroxyl groups and the amide nitrogen in chloramphenicol also act as suitable ligand and metal binding sites for formation of dative covalent bonds with heavy metals [6,7,8,9]. As a result of such interactions, metal ions have been reported to significantly alter the activity of different drugs especially antibiotics [10,11,12].

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Heavy metals such as Co, Cu, Fe, Ni, Mn and Zn exist in trace amounts as essential elements in biological systems and play important roles in biochemical reactions of living systems [13]. Such essential trace metals as well as others with no essential biological function (e.g. Al, Cd, Cs, Hg, Pb and Sn) are all increasingly being found at very high concentrations in biological systems of humans, animals and even microbes due to increased industrial use accompanied by improper disposal [14].

Although there have been reports on the activity of drug-metal complexes, most researchers used pre-synthesized complexes requiring special conditions (e.g high temperatures and refluxing for long periods) for their studies [11,15,13]. Such requirements may however not be met in living systems such as the human body. Also, little or no work has been done on the specific effects of Cd, Cr, Mn, or Zn on the activity of chloramphenicol in solution on the growth of the microorganisms used in this study.

The aim of the present study is to evaluate the changes in antimicrobial activity of chloramphenicol after interaction with Cd, Cr, Mn and Zn at simulated body temperature (37°C) against *Escherichia coli, Staphylococcus aureus, Salmonella typhii, Proteus vulgaris, Klebsiella pneumoniae.*

MATERIALS AND METHODS

Materials

Chloramphenicol reference standard was obtained as a gift donation from Doyin Pharmaceuticals, Ltd., Nigeria. Chloride salts of Cd, Cr, Mn and Zn were obtained from the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The organisms used in this study include *Escherichia coli* ATCC 11775, *Salmonella typhii, Klebsiella pneumoniae, Staphylococcus aureus* ATCC 021001 *and Proteus vulgaris* obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The culture media used were Nutrient Agar (Antec Diagnostic Products, United Kingdom), Nutrient Broth No. 1 (Fluka Chemical Company, Spain), and Mueller Hinton Agar (Oxoid, Bassingtoke, United Kingdom).

Preparation of Solutions

A quantity (0.100g) of each of the metal salts were weighed out separately and carefully dissolved in small beakers with minimal amount of distilled deionized water. The solutions were then transferred to a 100ml volumetric flask, made up to mark and shaken vigorously. These solutions (stock solutions) were used in preparing the required concentration for the interaction studies. A stock solution of chloramphenicol reference standard was prepared in a similar way and used for the antibiotic susceptibility determination and interaction studies.

Cultures and Media

All organisms used were purified by sequential streaking and single colony isolation on nutrient agar and then placed on agar slants for subsequent use [16]. All the media were autoclaved at 15 psi pressure for 15 minutes at 121°C. Sterile nutrient broth was used in the determination of the MIC of the drug on all organisms. The Mueller Hinton solution was dispensed in 200ml aliquots for use in the bio-assay trays.

Standardization of Cultures

The density of viable cells in the inoculums is the most important variable that influences the results of susceptibility tests [12]. The organisms used were standardized by streaking pure samples of the organisms on nutrient agar plates and incubating overnight at 37°C in a bacteria incubator, after which two or three colonies of the organism were emulsified in sterile, deionized distilled water. The bacterial suspension was diluted and visually matched with McFarland 0.5 turbidity standard before each use [17].

Antimicrobial Susceptibility

The MIC of chloramphenicol against each of the organisms was determined by the broth dilution technique [18]. Serially diluted logarithmic concentrations of the drug ranging from 128 µg/ml to 0.0625 µg/ml were inoculated with standardized overnight cultures of the organisms and incubated at 37°C for 18 – 24 hours. The lowest concentration of the drug that was able to inhibit growth of the organism was taken as the MIC [16,19].

Sterilization and use of Bioassay plates

Bioassay plates were sterilized at 160°C for 1 hour. Prior to use, Mueller Hinton Agar was aseptically prepared and seeded with 2ml of standardized overnight culture of organism. A sterile cork borer was then used to create 36 evenly distributed wells on the plate [20].

Table 1: Antimicrobial Susceptibility of the Organisms to Chloramphenicol

Organisms	Concentration of Chloramphenicol used (µg/ml)											
	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
E. coli	S	S	S	S	R	R	R	R	R	R	R	R
S. aureus	S	S	S	S	S	S	R	R	R	R	R	R
S. typhii	S	S	S	S	S	S	S	S	R	R	R	R
P. vulgaris	S	S	S	R	R	R	R	R	R	R	R	R
K. pneumoniae	S	S	R	R	R	R	R	R	R	R	R	R

Key: R – Resistant; S – Susceptible.

Interaction of Antimicrobial agent with Metal salts

Varying concentrations of the metal salts were prepared (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/ml.) from the stock solutions and varying concentrations of the antibiotic were also prepared (1, 4, 16, 32, 64 µg/ml) according to the MIC obtained for each organism. 5ml of each of the metal solutions and the antibiotic solution were then interacted in a 1:1 ratio for 30 minutes at 37°C on a water bath after which they were then immediately applied into the wells on the bioassay plates before incubation.

Application of Samples

0.1ml of sample (consisting of the pure antibiotic and the interaction mixture in different instances) was then applied in each well and the plates were allowed to pre-diffuse for about 30 minutes before incubating at 37°C for 18 hours in a bacterial incubator. After incubation, the diameter of zones of inhibition around each well was measured using a vernier caliper with white light against a dark non-reflective background.

RESULTS AND DISCUSSION

Results obtained for the antimicrobial susceptibility test of chloramphenicol on the organism showed MICs of 1, 4, 16, 32 and 64µg/ml against *Salmonella typhii, Staphylococcus aureus, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumonia* respectively (Table 1). The average zones of inhibition of chloramphenicol against all the organisms observed at the respective MICs are shown in Table 2. The antimicrobial actions of chloramphenicol when interacted (at the observed MICs against the various organisms) with different concentrations of the metals are shown in Tables 3–7. Synergistic changes in the zones of inhibition were observed in a few cases as a result of such interactions. In majority of the cases however, chloramphenicol activity was either antagonized or completely abolished.

Table 2: Zones of Inhibition at MICs of Chloramphenicol against Various Organisms

Organism	Concentration of Chloramphenicol (µg/ml)	Average zone of inhibition (mm ± SEM) (n = 4)
S. typhii	1	14.25 ± 0.14
S. aureus	4	17.25 ± 0.43
E. coli	16	24.00 ± 0.58
P. vulgaris	32	15.50 ± 0.29
K. pneumonia	64	14.25 ± 0.20

Complexation of a drug by a metal may have any number of effects on the activity of a drug, including retention of activity, decreased activity [11] or even an increase in activity [21]. The effect on activity would in any of such cases depend on the site of

Table 3: Zones of Inhibition of Solutions of Metals Interacted with Chloramphenicol at the MIC (4 μg/ml) against *Staphylococcus aureus*

Metal Concentration (μg/ml)	Zones of Inhibition (mm ± SEM) (n = 4)			
	Cadmium	Chromium	Manganese	Zinc
2	No Zone	15.5 ± 0.29*	No Zone	17.0 ± 0.08
4	No Zone	15.0 ± 0.58*	14.0 ± 0.23*	No Zone
8	13.0 ± 0.29*	19.0 ± 0.41	18.0 ± 0.34	No Zone
16	12.0 ± 0.41*	18.0 ± 0.08	No Zone	13.5 ± 0.29*
32	No Zone	No Zone	No Zone	No Zone

* = Significant decrease in activity (P<0.05)

Table 4: Zones of Inhibition of Solutions of Metals Interacted with Chloramphenicol at the MIC (1 μg/ml) against *Salmonella typhii*

Metal Concentration (μg/ml)	Zones of Inhibition (mm ± SEM) (n = 4)			
	Cadmium	Chromium	Manganese	Zinc
0.5	16.0 ± 0.13**	No Zone	No Zone	No Zone
1.0	No Zone	No zone	No Zone	No Zone
2.0	No Zone	19.0 ± 0.29**	16.5 ± 0.29**	No Zone
4.0	16.0 ± 0.08**	No Zone	No Zone	No Zone
8.0	No Zone	No Zone	No Zone	No Zone

** = Significant increase in activity (P<0.05)

attachment of the metal and its consequent impact on the activity of the antimicrobial agent.

Chloramphenicol undergoes a curled configuration in solution with hydrogen bonding between C_1 and C_3 forming a 6-membered ring including C_2 (fig. 1). Such a configuration is suitable for interaction with polar groups of a protein chain since the two OH groups and nitrogen of the peptide link would all be pointing outward [22]. The propanediol side chain including the hydrogen atoms on C_2 and C_3 and the amide nitrogen are proposed to be points of attachment with an enzyme [23] while the para-nitrophenyl group is not essential for activity but may be important in modifying the propanediol portion in such a way as to elicit pharmacological effect without in itself being involved in attachment to the enzyme [24]. Consequently, any drug-metal interaction which involves the propanediol side chain and its amide nitrogen will result in a change in activity while drug-metal interactions involving only

Table 5: Zones of Inhibition of Solutions of Metals Interacted with Chloramphenicol at the MIC (16 μg/ml) against *Escherichia coli*

Metal Concentration (μg/ml)	Zones of Inhibition (mm ± SEM) (n = 4)			
	Cadmium	Chromium	Manganese	Zinc
8.0	No Zone	21.0 ± 0.71*	No Zone	No Zone
16.0	21.0 ± 0.40*	14.5 ± 0.20*	18.0 ± 0.09*	No Zone
32.0	14.5 ± 0.29*	No Zone	No Zone	No Zone
64.0	No Zone	No Zone	16.0 ± 0.04*	16.5 ± 0.2*
128.0	No Zone	No Zone	18.5 ± 0.29*	No Zone

* = Significant decrease in activity (P<0.05)

Table 6: Zones of Inhibition of Solutions of Metals Interacted with Chloramphenicol at the MIC (64 μg/ml) against *Klebsiella pneumoniae*

Metal Concentration (μg/ml)	Zones of Inhibition (mm ± SEM) (n = 4)			
	Cadmium	Chromium	Manganese	Zinc
32.0	16.5 ± 0.29**	15.5 ± 0.65	19.0 ± 0.20**	16.5 ± 0.29**
64.0	28.5 ± 0.65**	23.0 ± 1.29**	14.5 ± 0.29	15.0 ± 0.15**
128.0	18.0 ± 0.20**	14.0 ± 0.15	13.5 ± 0.20	14.0 ± 0.04
256.0	18.5 ± 0.20**	15.0 ± 0.41	15.5 ± 0.09**	15.5 ± 0.20**
512.0	No Zone	15.5 ± 0.20**	15.0 ± 0.09**	15.5 ± 0.09**

** = Significant increase in activity (P<0.05)

the para-nitrophenyl moiety may not lead to a loss of activity but may even lead to increased activity.

Heavy metals in themselves also have their specific effects on microbes and some microorganisms have even developed ways of handling high concentrations of heavy metals in their environment by means such as efflux, accumulation in special organelles, complexation of such metal to other microbial organic metabolic waste products, and reduction of such metals to less toxic states [14].

The susceptibility of the organisms to the chloramphenicol-metal mixtures varied depending on the metal involved and the organism in question. The chloramphenicol-Cd mixture when tested against *S. aureus* showed a general significant decrease in activity ($P < 0.05$) leading eventually to loss of activity. Chloramphenicol-Cr mixture showed an initial significant decrease in activity ($P < 0.05$) on *S. aureus* which increased (probably due to complexation at the para-nitrophenyl group) and then a complete

Table 7: Zones of Inhibition of Solutions of Metals Interacted with Chloramphenicol at the MIC (32 μg/ml) against *Proteus vulgaris*

Metal Concentration (μg/ml)	Zones of Inhibition (mm ± SEM) (n = 4)			
	Cadmium	Chromium	Manganese	Zinc
16.0	No Zone	15.0 ± 0.08	13.5 ± 0.29*	No Zone
32.0	No Zone	No Zone	No Zone	14.0 ± 0.08*
64.0	16.0 ± 0.08	14.0 ± 0.08*	14.0 ± 0.09*	14.5 ± 0.29
128.0	16.5 ± 0.29	14.0 ± 0.12*	15.0 ± 0.08	No Zone
256.0	16.5 ± 0.12**	No Zone	15.0 ± 0.09	16.5 ± 0.29

* = Significant decrease in activity (P<0.05)
** = Significant increase in activity (P<0.05)

loss of activity at the highest metal concentration (probably due to complexation with metal ions at the active site of the drug). All concentrations of Mn interacted with chloramphenicol resulted in its loss of activity except at Mn concentrations of 4 and 8 μg/ml which showed significant decrease and an insignificant increase (P< 0.05) in activity respectively. Chloramphenicol-Zn mixture showed a general decrease in activity at all concentrations with complete loss of activity at 4, 8 and 32 μg/ml (Table 3).

A complete loss of chloramphenicol activity against *S. typhii* was observed when the drug was interacted (at the MIC) with all the metals at various concentrations (Table 4). The only exceptions were observed at metal concentrations of 0.5 and 4 μg/ml for Cd, and at 2 μg/ml for both Cr and Mn where activity was significantly increased (P< 0.05) as indicated by increases in the zones of inhibition.

A general decrease in chloramphenicol activity against *E. coli* leading to complete loss of activity at most of the metal concentrations interacted with the drug was observed (Table 5). Even at concentrations where some activity was seen, the zones of inhibition were significantly (P< 0.05) smaller than that observed at the MIC (24.00 μg/ml) of chloramphenicol against *E. coli*.

Interacting chloramphenicol with all the metals appears to have increased the susceptibility of *K. pneumonia* to the drug (Table 6). This is reflected in the significant increase (P< 0.05) in zones of inhibition observed in 60% of the interaction mixtures tested against *K. pneumonia* compared with that observed at the MIC (14.25 μg/ml) of the drug alone.

The activity of the chloramphenicol-metal complexes against *P. vulgaris* was irregular and depended on the interacting metal and its concentration (Table 7). The chloramphenicol-Cd mixture showed an initial loss followed by increase in activity with increasing concentration. The chloramphenicol-Cr mixture showed a general decrease in activity with increasing concentration. Chloramphenicol interaction mixtures with both Mn and Zn exhibited inconsistent activity with increasing concentrations.

These apparently erratic results exhibited by the various chloramphenicol-metal interaction mixtures against all the organisms can be explained by considering the structure activity relationship of chloramphenicol. Since the para-nitrophenyl group is highly electronegative and not sterically hindered, at low concentrations, the heavy metals will more readily bind at this site and since this site does not significantly affect activity, the mixture will retain activity and show zones of inhibition. At higher concentrations of metal however, the metals will saturate the para-nitrophenyl groups available and start interacting with the propane side chain which then results in a decrease in activity of the mixture. At even higher

concentrations, the excess metal ions left with no interaction sites will exhibit their own antimicrobial action leading to an apparent increase in zones of inhibition, since the free metals in themselves have their specific effects on microbes [14].

Apart from the nature of the drug-metal complexes formed as a result of such interactions, differing results for the metal-antibiotic mixture against different organisms are not uncommon [11,12] and are as a result of the biochemical differences between organisms. For instance, Gram positive organisms such as *S. aureus* are known to have thick walled peptidoglycan layers which in addition to physically excluding materials from the cell may also be involved in chemical interactions with any charged particles hence limiting the metal ions to the outer cell wall [25].

The foregoing shows that the presence of heavy metals such as Cd, Cr, Mn and Zn can significantly affect the activity of antibiotics such as chloramphenicol. Interaction of chloramphenicol with heavy metals may lead to either a decrease or an increase in activity depending on the type and concentration of the metal involved in the interaction, and also as a result of the biochemical differences between organisms. The resultant change in spectrum and profile of activity can result in unpredictable clinical efficacy of this drug. Thus the co-administration of chloramphenicol with preparations of these metals should be avoided where possible.

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