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B-Agonist Intoxication from Meat Ingestion

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Abstract: β-agonist drugs are illegally used for auxinic purposes in very high doses. Their accumulation in significant amount in the liver or meat of the treated farm animals is evident. Acute intoxication has also been diagnosed in several countries including Italy. Pressure of the official controls pulsed to synthesise as new compounds to escape analytical screening procedures. Here we have reported two new compounds named "A" and "G4", found in animal feeding. Chemical structure was studied through nuclear magnetic resonance imaging (NMR) and infra-red (IR) spectroscopy. The pharmacodynamic of β-1 and β-2 adrenergic agonist activity was evaluated on isolated guinea pig atrium and trachea.

Key words: Clenbuterol, Contamination, Food

Introduction

β_2-agonist adrenergic drug as CLENBUTEROL is used in human therapy chiefly for bronchodilatation to improve lung functions (1-6). It is however, illegally used at higher dosages as growth promoting agent (1-6). It has lipolytic and protein synthesis stimulating side effects (1-6). Officers appointed by the Court of Justice of Milan (Italy) found in some animal farms unlabeled bottles and syringes (1-6). They forwarded all those materials to our Institute, with the request to characterise the chemicals and their pharmacological properties, in order to improve control strategy and to assess the possible risk for farm workers and for the consumers (1-6).

Materials and Methods

The synthetic approach to the final compounds, marked G4 and Letter A, was accomplished through the preliminary preparation of N-[2,6-dichloro-4-(2-oxiacetyl)-phenylformamide] (1).

Substances: Carbachol chloride, clenbuterol , propranolol, ICI-118,551, isoprenaline hydrochlorides, and ascorbic acid were purchased from Sigma Chem. Co (St Louis, MO, USA). Concentration of substances is expressed as free base. Compounds G4 and A were synthesised as pure compounds. All substances were dissolved in ethyl alcohol 50%-95% v/v, and stored at 4°C. For biological tests the alcoholic solution was diluted in Krebs solution. The solvent at the maximum concentration used (1% v/v) in preliminary test did not show any interference to the biological response.

Animals: Male guinea pigs weighing 350-400 g purchased from the breeding farm Rodentia (Brescia, Italy) were used. All experiments were carried out according to the guidelines of the European Community Council on animal care, and were approved by the Bioethical Committee of the National Health Institute of Italy.

Guinea Pig Atrium: Following sacrifice through cervical dislocation, the heart of the animal was quickly dissected and the right atrium was isolated and mounted in an organ bath containing Krebs solution maintained at 30°C. Ascorbic acid was added at a concentration of 200 μg/ml. Atrial beats were recorded with an isometric transducer (Ugo Basile – Varese, Italy) coupled to a recorder (Ugo Basile – Varese, Italy). Rate of atrial contraction was determined by counting beats per minute (bpm). The mean of three consecutive counts was considered. Before beginning each experiment, the preparation was stimulated with isoprenaline (0.9×10^{-7} M), and the atrial bpm count was recorded. The effects induced by test substances on the heart rate were evaluated by adding them in increasing doses, cumulatively to the preparation. The response was expressed as a percentage of increase with respect to the basal frequency. In the experiments carried out in the presence of the non selective β-blocking agent, propranolol (1.15×10^{-6} M). This latter compound was added to the bath 5 min before the addition of the test

substance.

Guinea-Pig Trachea: Following animal sacrifice through cervical dislocation the trachea was removed, cleared of the adhering tissues, cut into two zigzag strips, suspended in an organ bath containing Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2 , and attached to an isotonic force displacement transducer (Ugo Basile – Varese, Italy) under 1 g of resting tension. Movements were recorded with a microdynamometer (Ugo Basile). The preparation was allowed to gain the spontaneous tone until a steady-state level was reached (30-60 min). At this point the preparation was incubated with carbachol ($3.4x10^{-7}$ M) until the contraction reached the steady state. Then the complete relaxation of the isolated preparation was obtained by adding isoprenaline ($2.4x10^{-7}$ M). This procedure was repeated until the response to carbachol was constant. The relaxant effect of test substances was evaluated by adding increasing doses of test substances cumulatively to the contracted preparation. In the experiments performed with the selective β_2-blocking agent ICI-118,551 ($3.6x10^{-7}$ M) the compound was added in the bath 10 min before the addition of test substance. Responses were expressed as a percentage of the relaxation.

Statistical Analysis: The potency of clenbuterol, compound A and compound G4 was expressed as EC_{50} ± SEM, which were computed with the method of Wizard regression (Sigma Plot 7.0).

Results

Biological Activity

Guinea-Pig Atrium: Clenbuterol increased the heart rate frequency in isolated guinea-pig atrium starting from the concentration of $3.6x10^{-10}$M, the maximal increase was about 30% of the basal rate. Compounds A and G4 appeared similar to clenbuterol in potency and efficacy, being their EC_{50} values of the same order of magnitude, between $1,96x10^{-9}$ M and $2,59x10^{-9}$ M. Propranolol blocked completely the response to all the substances tested.

Guinea-Pig Trachea: The reference substance clenbuterol induced relaxation of the tracheal preparation in a concentration-dependent manner.
Compounds A and G4 showed also a concentration-dependent myorelaxant effect, but they were about 100 times less potent than clenbuterol. The relaxing effect of these three compounds was antagonised by the selective β-2 antagonist, ICI-118,551, which induced a shift to the right of the concentration-response curves.

Table 1: Myorelaxant effect (expressed as EC50 ± SE) of Clenbuterol, Compound A and Compound G4 on isolated guinea-pig trachea contracted with carbachol ($3.4x10^{-7}$). n = 5 (**P < 0.01 vs basal value)

Substances	EC_{50} ± SE M
Clenbuterol	$4.38 \pm 0.72 \times 10^{-8}$**
Compound A	$6.21 \pm 0.81 \times 10^{-8}$**
Compound G4	$3.70 \pm 0.65 \times 10^{-8}$**

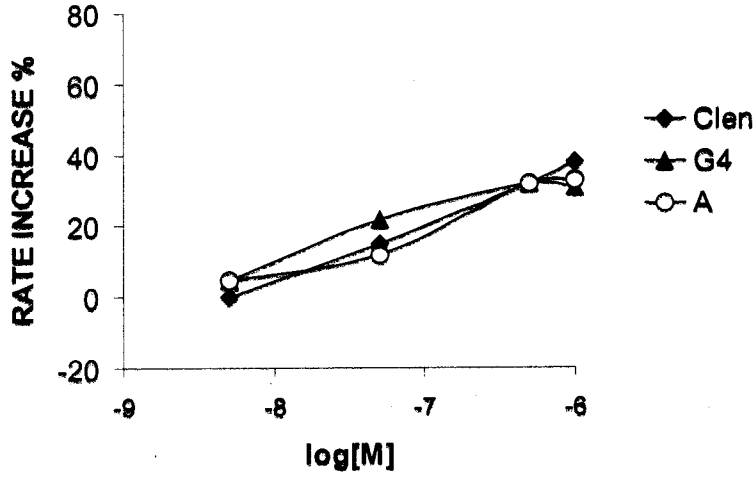

Fig. 1: Effects of Clenbuterol, Compound A and compound G4 on heart rate of the isolated guinea-pig atrium. n = 7

Conclusion

The test compounds are characterised by a more pronounced lipophylicity. This is attained through substitution on secondary amino group of the alkylic chain for compound A and substitution of the ter-butylic group with a benzene ring for compound G4, with respect to clenbuterol structure. This determines a more prolonged persistent effect on cell membrane, thus enabling the drug to elicit an enduring stimulation of the adrenergic receptor. The molecular features thus determine the modified pharmacological activity, with a more pronounced activity on β_1 *versus* β_2 receptors, as shown by present data on isolated atrium and trachea. An increased risk, both for the people handling such drugs, and/or the consumers consuming compounds A and G4 in edible tissues, is therefore expected, if we consider that the lipolytic effects of clenbuterol could be addressed to a closer drug crossreactivity between β_2 and β_3 receptors, than that between β_1 and β_3. Therefore, the compounds need to be administered in doses much higher than clenbuterol, to induce the same β_2 (i.e., "metabolic") effects. The pronounced β_1 agonist potency which is recorded in the isolated atrium, the same order of magnitude as clenbuterol can provoke more severe cardiovascular clinical symptoms than those recorded for clenbuterol intoxication.

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References

Boatto, G., C. Testa, G. Brambilla, B. Neri, M. Fiori, D. Cuccaro, G. Manca, 2000. Synthesis and characterisation of new beta agonists of probable illicit use in animal productions. In: Residues of Veterinary drugs in food. L.A. van Ginkel, A. Ruiter (Eds), Veldhoven, NL, 8-10 pp.237-41

Brambilla, G., T. Cenci, F. Franconi, R. Galarini, A. Macri, F. Rondoni, M. Strozzi, A. Loizzo, 2000. Clinical and pharmacological profile in a clenbuterol epidemic poisoning of contaminated beef meat in Italy. Toxicology Letters ;114:47-53

Brambilla, G, A. Loizzo, L. Fontana, *et al.*, 1997. Food poisoning following consumption of clenbuterol-treated veal in Italy. J. Am. Med Ass ; 278: 635

Italian Ministery of Health, 1997. Results of the National Residue Plan 1996. Protocol N. 600.7/24490/AG13,

Kuiper, H. A., M. Y. Noordam, M. M. H. van Dooren-Flips, R. Schilt, H. Roos, 1998. Illegal use of ß-adrenergic agonist: European Community. Journal of Animal Science ;76:195-207.

Martinez-Navarro J. F., 1990. Food poisoning related to consumption of illicit beta-agonist in liver. The Lancet ; 336: 1311.