Positive Inotropic Activity (Cardiac Glycosides)

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Contents

General Considerations

Biological standardization of cardiac glycosides was necessary as long as the drugs used in therapy were plant extracts or mixtures of various glycosides. They were standardized in units of an international standard. Some of the pharmacological methods used for these purposes and adopted by many pharmacopoeias have nowadays *historical interest* only. This holds true for the frog method and the pigeon method (Burn et al. 1950).

Particularly, the **frog method** was used for standardization. The method adopted by the US Pharmacopoeia X was the 1 h test. Healthy frogs (*Rana pipiens*) weighing 20–30 g were selected from the cold storage room. One hour before assay, their weight was recorded and they were placed in wire cages with a water depth of 1 cm. The doses of digitalis were calculated so that they approximated 0.015 ml/g body weight. Injections were made into the ventral lymph sac. One hour later, the animals were pithed and the heart removed and examined. Systolic arrest of the ventricle and widely dilated atrium indicated the typical result. Calculations of activity in terms of international units were made from the percentage of dead animals in the test group versus those in the group receiving the international standard.

The **pigeon method** introduced by Hanzlik (1929) and adopted by USP XVII depends on the observation that intravenously injected cardiac glycosides have an emetic action in pigeons. In the original test, adult pigeons weighing

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300–400 g are injected with a solution of the cardiac glycoside into a suitable wing vein in the axillary region. Vomiting occurring within 15 min is regarded as positive result. Two doses of test solution and standard are injected and percentage of vomiting pigeons registered. This four-point assay allows calculation of ED_{50} values and of the potency ratio compared with the standard.

Modifications of other methods, such as the **cat method** introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngaarden (1926), the **guinea pig method** described by Knaffl-Lenz (1926), and the **isolated cat papillary muscle** method introduced by Catell and Gold (1938) still being used for evaluation of synthetic cardiac glycosides and other positive inotropic compounds, are referenced in detail below.

Surveys on the evaluation of cardiac glycosides have been given by Bahrmann and Greef (1981), for the use of the isolated papillary muscle by Reiter (1981) and for other isolated heart preparations by Greef and Hafner (1981). Moreover, the influence on Na^+/K^+-ATPase, an in vitro model specific for cardiac glycosides (Gundert-Remy and Weber 1981), is described.

The mechanisms of action have been reviewed by Scholz (1984) and Grupp (1987).

Analogous to antiarrhythmic agents, Feldmann (1993) proposed a classification system that categorizes inotropic agents according to their supposed mode of action:

Class I: Inotropic agents that increase intracellular cyloAMP, including β-adrenergic agonists and phosphodiesterase inhibitors

Class II: Inotropic agents affecting sarcolemmal ion pumps and channels, in particular cardiac glycosides inhibiting Na^+/K^+-ATPase

Class III: Agents that modulate intracellular calcium mechanisms (no therapeutic inotropic agents in this kind yet available)

Class IV: Inotropic agents having multiple mechanisms of action

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In Vitro Tests

Ouabain Binding

Purpose and Rationale

Cardiac glycosides can be characterized by their binding kinetics (association process, equilibrium binding, and dissociation process) on the ouabain receptor.

Procedure

Heart sarcolemma preparations are obtained from rat or dog heart. From a canine heart or from rat hearts submitted to coronary perfusion, myocytes are isolated by collagenase digestion. The isolated membrane fractions consist mainly of myocyte sarcolemma. [^{3}H] ouabain with a specific radioactivity of about 20 Ci/mmol is incubated with ligands to be tested in 10 ml of binding medium consisting of 1 mM $MgCl_2$, 1 mM inorganic phosphate, and 50 mM Tris–HCl, pH 7.4 at 37 °C for 10 min.

Association process: After temperature equilibration in the presence of either 10 or 100 nM [^{3}H] ouabain, 200 μg of membrane preparation are added to initiate the reaction. At various times, 4.5 ml are removed and rapidly filtered.

Equilibrium binding: At the end of the temperature equilibration carried out in the presence of increasing concentrations of [^{3}H]ouabain ranging from 10 nM to 3 μ M, 40 μg of membranes is added. After 30 min, duplicate aliquots of 4.5 ml are removed and filtered.

Dissociation process: Once equilibrium has been achieved under the experimental conditions used to study association, 10 ml of prewarmed Mg^{2+} plus P_i Tris–HCl solution supplemented with 0.2 mM unlabeled ouabain is added to initiate dissociation of [^{3}H]ouabain. At various times, aliquots of 0.9 ml are removed and rapidly filtered.

All aliquots are filtered under vacuum on HAWP Millipore filters (0.45 μm) and rinsed three times with 4 ml of ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements are determined.

Evaluation

Kinetic parameters for the association and the dissociation process are calculated. The results of equilibrium binding are analyzed by Scatchard plots.

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Influence on Na^+/K^+-ATPase

Purpose and Rationale

The enzyme Na^+/K^+-ATPase is the transport system for Na^+ and K^+ in the cell membranes. The membrane-bound enzyme couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane through a series of conformational transitions between the E_1 and E_2 states of the enzyme. The enzyme is a heterodimer consisting of a catalytic subunit (110 kDa) associated with a glycosylated β subunit (55 kDa). Three alpha (α_1, α_2, α_3) subunits have been identified by cDNA cloning. In the heart, enzyme Na^+/K^+-ATPase is the target of the positive inotropic glycosides. Therefore, it is of interest for the characterization of positive inotropic compounds. The test is based on the determination of phosphate generated from ATP under special conditions. Inhibition of bovine cerebral Na^+/K^+-ATPase prepared according to Schoner et al. (1967) is measured after addition of various concentrations of the test compound compared with those of the standard (Erdmann et al. 1980).

Procedure

Solutions

1.00 ml 133 mM imidazole pH 7.3
0.04 ml 160 mM $MgCl_2$
0.02 ml DPNH (10 mg/ml)
0.04 ml 310 mM NH_4Cl
0.04 ml 100 mM ATP
0.02 ml 40 mM phosphoenol-pyruvate
0.05 ml pyruvate-kinase (1 mg/ml = 150 U/ml)
0.04 ml lactate-dehydrogenase
(0.5 mg/ml = 180 U/ml)
0.20 ml 1 M NaCl
0.01–0.02 ml bovine cerebral ATPase (depending on activity of the enzyme) up to 2.0 ml distilled water

Test

The enzyme activity is started by addition of the ATP solution at 37 °C. After 4 min the inhibitor (various concentrations of the cardiac glycoside) is added. Na^+/K^+-ATPase activity is measured by a coupled optical assay. The reaction is continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of Na^+/K^+-ATPase with 10^{-3} M ouabain.

Evaluation

Inhibition of ATPase is measured after addition of various concentrations of the test compound. Dose-response curves are established and compared with the standard (k-strophanthin). Potency ratios can be calculated.

Modifications of the Method

Brooker and Jelliffe (1972) and Marcus et al. (1975) described an in vitro assay based on displacement of radiolabeled ouabain bound to ATPase by various glycosides. Another method is based on the inhibition of rubidium uptake into erythrocytes (Lowenstein 1965; Belz 1981).

Erdmann et al. (1980) prepared Na^+/K^+-ATPase-containing cardiac cell membranes from rat hearts.

Maixent et al. (1987) described two Na^+/K^+-ATPase isoenzymes in canine cardiac myocytes as the molecular basis of inotropic and toxic effects of digitalis.

The effect of ouabain on Na^+/K^+-ATPase activity in cells of the human rhabdomyosarcoma cell line TE671 was studied by Miller et al. (1993) with a special equipment, the microphysiometer (McConnell et al. 1992).

Critical Assessment of the Method

The in vitro methods being used for determinations of plasma levels of glycosides (Maixent et al. 1995) have been largely substituted by radioimmunoassays specific for individual glycosides.

Nevertheless, the inhibition of Na^+/K^+-ATPase can be used as an indicator of activity of new semisynthetic cardiac glycosides.

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Tests in Isolated Tissues and Organs

Isolated Cat Papillary Muscle

Purpose and Rationale

Isolated cardiac tissue has been chosen to study the decrease of performance after prolonged electrical stimulation and during restoration of force under the influence of cardiac glycosides. Cattell and Gold (1938) described a method using cat papillary muscle.

Procedure

Cats of either sex weighing 2.5–3 kg are used. The animal is anesthetized with ether and the thorax is opened rapidly. The heart is removed, and a papillary muscle from the right ventricle is isolated and fixed in an organ bath containing oxygenated Ringer's solution at 36 °C. One end of the muscle is tied to a tissue holder and the other one to a strain gauge. The muscle is stimulated electrically with 4–6 V, 2 ms duration, and a rate of 30/min. The contractions are recorded on a polygraph. After 1 h, the muscle begins to fail and the force of contraction diminishes to a fraction of control. At this point, the cardiac glycoside is added to the bath, restoring the contractile force to levels approaching control. The standard dose is 300 ng/ml ouabain. The potency of natural and semisynthetic glycosides can be determined with this method. Catecholamines, like adrenaline (10 ng/ml) or isoprenaline (10 ng/ml), are active as well.

Evaluation

The increase of contractile force is calculated as percentage of the predose level. Dose-response curves can be established using various doses.

Critical Assessment of the Method

The use of isolated papillary muscle strips can be recommended for evaluation of inotropic compounds of various chemical classes.

Modifications of the Method

Instead of cat papillary muscle, the isolated left atrium of guinea pigs can be used (see chapter "▶ Studies in Isolated Organs", section "B1-Sympatholytic Activity in Isolated Guinea Pig"). For testing cardiac glycosides, the calcium content in the Ringer's solution is reduced to 50 %.

Andersom (1983) compared responses of guinea pig paced left atria to various positive inotropic agents at two different calcium concentrations (1.25 and 2.50 mM). Consistently good results were obtained at the lower calcium concentration with isoproterenol, ouabain, amrinone, and 3-isobutyl-1-methylxanthine.

Böhm et al. (1989) studied positive inotropic substances like isoprenaline and milrinone in isolated cardiac preparations from different sources. They used isolated papillary muscles from Wistar–Kyoto rats and from spontaneously hypertensive rats and also human papillary muscle strips from patients with moderate heart failure (NYHA II–III) and compared the effects with

papillary muscle strips from patients with severe heart failure (NYHA IV). They recommended that new positive inotropic agents should be screened in human myocardial tissue from patients with heart failure.

Labow et al. (1991) recommended a human atrial trabecular preparation for evaluation of inotropic substances.

Böhm et al. (1989) tested positive inotropic agents in isolated cardiac preparations from different sources, e.g., human papillary muscle strips from patients with severe heart failure (NYHA IV), human papillary muscle strips from patients with moderate heart failure (NYHA II–III), human atrial trabeculae, isolated papillary muscles from Wistar–Kyoto rats, and isolated papillary muscles from spontaneous hypertensive rats. They suggested that positive inotropic effects should be screened in isolated myocardium from patients with heart failure.

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Isolated Hamster Cardiomyopathic Heart

Purpose and Rationale

Special strains of Syrian hamsters develop cardiomyopathy. These animals can be used for evaluation of cardiotonic drugs

Procedure

Hamsters with cardiomyopathy (Bio 14/6) at the age of 50 weeks are used. Controls are normal Syrian hamsters (FIB hybrids) at the same age. The animals are pretreated with heparin (5 mg/kg i.p.), and 20 min later the heart is prepared according to the method of Langendorff and perfused with heart Ringer's solution under 75 mm H_2O hydrostatic pressure. The preparation is allowed to equilibrate in the isolated state for 60 min at 32 °C with a diastolic preload of 1.5 g. The force of contractions is recorded isometrically by a strain gauge

transducer on a polygraph, e.g., Heliscriptor He 19 recorder (Hellige GmbH, Freiburg, Germany). From these signals, the heart rate is measured by a chronometer. The coronary flow is measured by an electromagnetic flowmeter. Compounds are injected via the aortic cannula into the inflowing heart Ringer's solution.

Evaluation

The contractile force and the coronary flow in hearts from diseased and normal animals are registered before and after application of the test drugs. Mean values and standard deviation are calculated before and after drug application and statistically compared using Student's *t*-test.

Modification of the Method

Jasmin et al. (1979) showed after prolonged in vivo administration beneficial effects of a variety of cardiovascular drugs, including verapamil, prenylamine, dibenamine, and propranolol.

After chronic administration (4 or 12 weeks subcutaneously), Weishaar et al. (1987) found beneficial effects of the calcium channel blocker diltiazem but not by the administration of digitalis.

In contrast, in the experiments of Ottenweller et al. (1987), hamsters treated orally with digoxin survived and showed significant amelioration of the pathological syndrome of heart failure.

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Potassium Loss from the Isolated Guinea Pig Heart

Purpose and Rationale

Cardiac glycosides induce a net loss of potassium from cardiac tissue due to their inhibition of the Na^+/K^+-ATPase. Therefore, potassium is increased in the effluent of the isolated guinea pig heart. This phenomenon can be used as parameter for the activity of digitalis-like compounds (Lindner and Hajdu 1968).

Procedure

The isolated heart of guinea pigs according to Langendorff is prepared as described in chapter "▶ Coronary Drugs", section "Heart-Lung Preparation". The coronary outflow is measured by counting the drops of the effluent by a photocell. The effluent is collected in a funnel with a thin upward-shaped outlet allowing to withdraw small fluid samples for analysis by a flame photometer. A pump attached to a four-way valve changes the samples to the flame photometer every 15 s in the following sequence: effluent Tyrode solution from the heart, distilled water, Tyrode solution used for perfusion, and distilled water. The potassium content of affluent and effluent Tyrode solution is compared and registered on a Varian recorder. The difference is attributed to the potassium outflow from the heart. The dose-response curve is flat in the therapeutic range, much steeper in the toxic range.

Evaluation

The following parameters are recorded and calculated:

- Coronary flow [ml/min]
- Contractile force
- Potassium loss [mVal/min]

Critical Assessment of the Method

A good correlation was found between the measured potassium loss and the positive inotropic effect of cardiac glycosides. The method is suitable for the quick determination of efficacy of digitalis-like substances and facilitates the discrimination from other positive inotropic compounds like adrenaline.

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In Vivo Tests

Cardiac Toxicity in Cats (Hatcher's Method)

Purpose and Rationale

The purpose of the method, originally introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngaarden (1926), was to establish "cat units" for cardiac glycoside preparations. Hatcher and Brody defined "the cat unit as the amount of crystalline ouabain which is fatal within about 90 min to a kilogram of a cat when the drug in injected slowly and almost continuously into the femoral vein." Time to cardiac arrest after intravenous infusion of a solution with defined concentration of the standard was used as reference and the unknown solution of the test preparation compared with the standard. The method can be used for testing natural and semisynthetic glycosides.

Procedure

Cats of either sex weighing 2–3.5 kg are temporarily anesthetized with ether. Anesthesia is maintained with 70 mg/kg chloralose given intravenously. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula is inserted. ECG is recorded from lead II. Then, intravenous infusion of the test solution is started. The end point is cardiac arrest which should be reached within 30–60 min by proper adjustment of the concentration of the infused solution.

Modifications of the Method

Hatcher's original method has been modified by many authors. The method using **guinea pigs**, introduced by Knaffl-Lenz (1926), is in its essentials similar to the cat method.

Guinea pigs weighing 400–600 g are anesthetized with urethane (1.75 g/kg i.m.) The animal is secured on an operating table and the trachea is cannulated. The jugular vein is cannulated for infusion of the test preparation. Cardiac arrest is recorded from ECG lead II.

Dogs and guinea pigs were used by Dörner (1955).

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Decay Rate and Enteral Absorption Rate of Cardiac Glycosides

Purpose and Rationale

The basic principle of Hatcher's or Knaffl-Lenz's method is suitable to determine decay rates of cardiac glycosides. The decay of efficacy can be due to excretion or metabolic degradation of the glycoside.

Procedure

Beagle dogs of either sex weighing 820 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula inserted. The vena femoralis is cannulated for continuous infusion of a defined concentration (μg/kg/min) of the test compound. ECG is recorded from lead II. The signs of first toxic effects, e.g., extra systoles, AV-block, are recorded. At this time, the infusion is terminated and the total dose/kg of the applied glycoside registered. After 4, 8, 12, or 24 h the infusion procedure is repeated. Within this period of time the glycoside administered with the first dose is only partially metabolized or excreted. Therefore, the dose needed for observation of ECG changes during the second infusion will be lower than in the first experiment.

Evaluation

The dose required in the second experiment for induction of ECG changes is equal to the amount of metabolized or excreted glycoside. This value is expressed as percentage of the amount required in the first experiment and indicates the decay rate of the glycoside. Testing after various time intervals, the decay rate can be visualized graphically and half-life times be calculated.

Modifications of the Method

Rhesus monkeys have been used since their response to cardiac glycosides is more similar to that of man than that of dogs (Lindner et al. 1979).

The basic principle of Hatcher's or Knaffl-Lenz's method is also suitable to determine *enteral absorption of cardiac glycosides*. Again, for this purpose dogs are preferred instead of cats or guinea pigs. The dose to induce cardiac arrest is determined in 3–6 dogs. To other dogs, the same test compound is given intraduodenally at a dose below the intravenous lethal dose. Ninety or 180 min afterward, the intravenous infusion with the same infusion speed and the same concentration of the test compound as in the previous experiments is started and time until cardiac arrest determined. The higher the duodenal resorption of the compound, the lower the dose of the intravenous infusion will be. For evaluation, the intravenous dose needed in the second experimental series (with enteral predosing) is subtracted from the dose of the first series (without enteral predosing) and indicates the amount of absorbed compound. This value is expressed as percentage of the value of the first series and indicates the absorption rate.

The efficacy and safety of a novel Na^+/K^+-ATPase inhibitor has been tested in dogs with propranolol-induced heart failure by Maixent et al. (1992).

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