Biochemical Pharmacology, Vol. 31, No. 2, pp. 267-271, 1982.
Printed in Great Britain.

0006-2952/82/020267-05$03.00/0

WATER VERSUS ACETONE-HCl EXTRACTION OF DIGITALIS-LIKE FACTOR FROM GUINEA-PIG HEART

Alain De Pover, Gilberto Castañeda-Hernández and Théophile Godfraind

Université Catholique de Louvain-Faculté de Médecine
Laboratoire de Pharmacodynamie Générale et de Pharmacologie
Avenue E. Mounier, 73 - U.C.L. 7350 - B-1200 Bruxelles (Belgium)

(Received 4 September 1981)

Factors exhibiting some digitalis-like properties have been identified in several mammalian tissues including plasma of volume expanded dog (1-5). By definition, a true digitalis-like factor should present all the main characteristics of cardiac glycosides :

1) it should interact with Na,K-ATPase showing
   a) reversible inhibition
   b) competition with KCl and dependence of this effect on Na/K ratio
   c) competition with [^{3}H]ouabain binding
   d) species sensitivity (namely a weak activity in rat heart as compared to guinea-pig).
2) it should inhibit the Na-K pump and evoke a positive inotropic effect in isolated heart preparations.

In addition, it could interact with some of the specific cardiac glycosides antibodies, competing with the radioligand in a way similar to the glycoside itself.

In most of the extraction procedures previously reported, the tissues were homogenized in acetone-HCl. As shown below, acetone-HCl extracts contain non-specific irreversible Na,K-ATPase inhibitors. Therefore, we have designed two extraction procedures excluding acetone-HCl. In procedure I, the tissue was homogenized in water and digitalis-like factors were extracted from the post-microsomal supernate. In procedure II, the tissue was homogenized in 0.3 M sucrose and the factor was recovered from the post-nuclear particulate material after exposure to hypotonic shock. The two water extracts interacted with antidigoxin antibodies and inhibited Na,K-ATPase in a way similar to digoxin.

METHODS

Flow diagrams of the procedures for isolating extracts from guinea-pig heart are illustrated in Fig. 1.

Procedure I: 30 g of tissue was homogenized in 300 ml of deionized water. The homogenate was frozen overnight at -30° C, thawed and centrifuged successively at 1000 g for 10 min and 100000 g for 1 h.

- The 100000 g x 1 h supernate was lyophilized and the dry matter suspended in 50 ml of methanol. The mixture was stored for 1 h at -30° C and centrifuged at 1000 g for 10 min. After removal of the pellet, methanol was evaporated under low pressure. The dry residue was taken up in 5 ml of water and treated with 50 ml of petroleum benzine. The organic phase was removed and the aqueous phase applied to a 400-ml column of Amberlite^{R} MB3 (Fluka, Switzerland) equilibrated with 4 M pyridine-acetate (pH 7.0). The eluate was evaporated under low pressure and the dry matter suspended in 1 ml of 0.1 M acetic acid. The mixture was applied to a 25 x 300 mm column of Sephadex ^{R} G-25 (Pharmacia, Sweden) equilibrated with 0.1 M acetic acid (20 ml/h). 2-ml fractions were collected and assayed for immunoreactivity with antidigoxin antibodies. Peak fractions were pooled and lyophilized. The final extract was taken up in 0.5 ml of deionized water. Its ionic content was measured by atomic absorption spectroscopy.

- The 100000 g x 1 h pellet was treated with acetone-1N HCl according to Carraway and Leeman (6).

Procedure II: 120 g of tissue was homogenized in 500 ml of 0.3 M sucrose. After washing with 500 ml of sucrose, the 100000 g x 1 h pellet was suspended in 1200 ml of water, frozen overnight at -30° C, thawed and centrifuged at high speed. The supernate was lyophilized and treated as in procedure I, except that before ion exchange chromatography, the residue was taken up in 50 ml of diethylether or acetone in order to remove sucrose.

Radioimmunoassay: 50 µl of extract (10-1000 fold diluted) was incubated at 20° C for 30 min with ^{125}I-labelled digoxin analogue and rabbit specific antidigoxin antibodies (Diagnostic Product Corp., California). The antibodies were precipitated by addition of anti γ-globulin antibodies and polyethyleneglycol. After centrifugation and removal of the supernate, radioactivity of the pellet was counted in a γ-counter.

Enzyme and [^{3}H]ouabain binding assays: preparation of guinea-pig heart Na,K-ATPase and of microsomal fractions containing Ca,Mg-ATPase, and assay of specific [^{3}H]ouabain binding to Na,K-ATPase were performed as described previously (7, 8). The ATPase activities were assayed according to Bais (9).

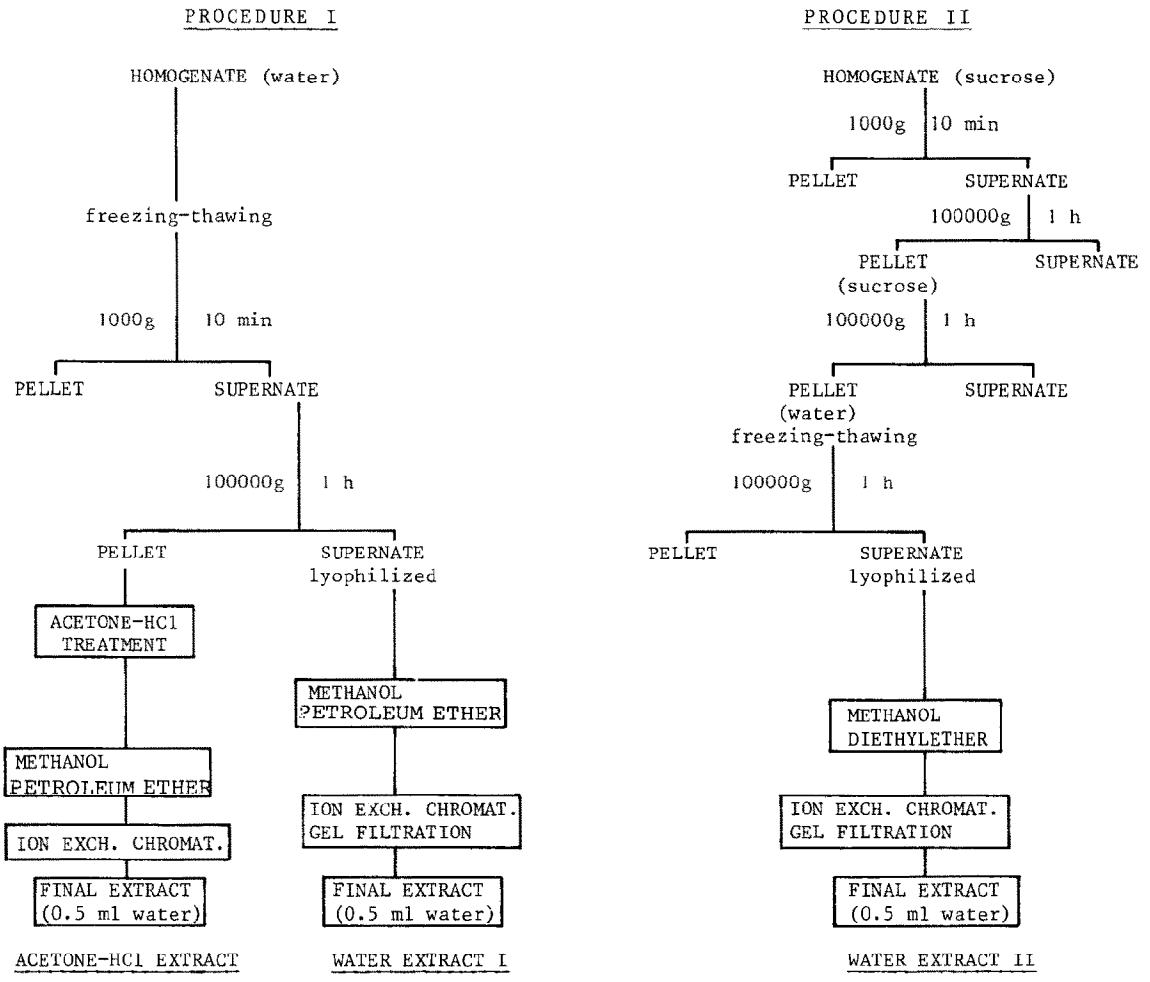

Fig. 1: Flow diagrams of the procedures for isolating guinea-pig heart extracts.

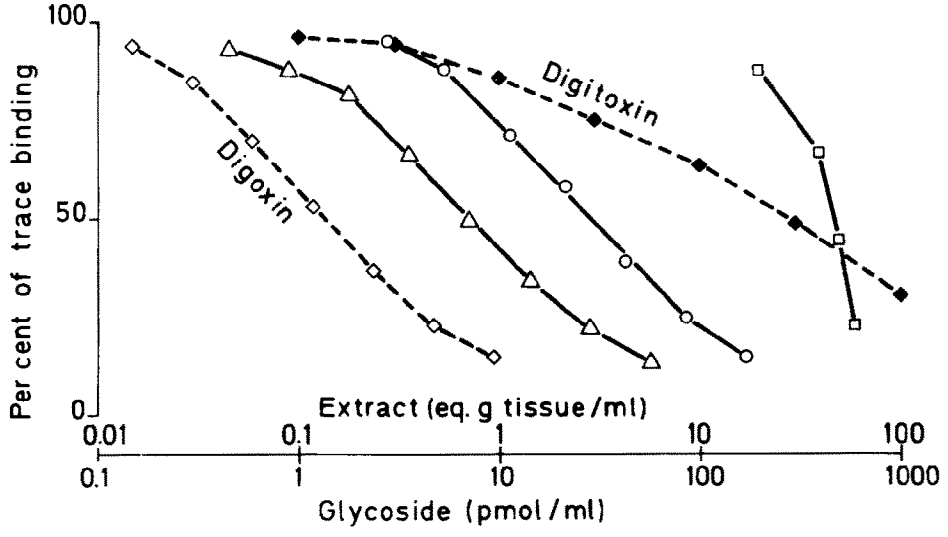

Fig. 2: Radioimmunoassay : effect of water extract I (△), water extract II (○), acetone HCl extract (□), digoxin and digitoxin on [^{125}I]antigen binding to antidigoxin specific antibodies. 3 extract preparations were tested (S.E.M. within the symbols).

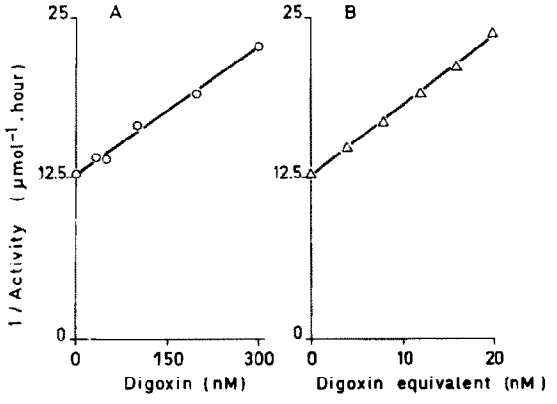

Fig. 3. : Effect of digoxin (A) and of water extract II (B) on guinea-pig heart Na,K-ATPase (Dixon plot). The enzyme (about 25 µg/ml) was incubated for 30 min at 37° C in 0.1 ml medium containing 3 mM KCl, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM [γ^{32}P]ATP (Amersham, England), 1 mM EGTA, 20 mM Tris-maleate (pH 7.4) and various concentrations of inhibitor. The amount of water extract II is expressed as digoxin equivalent, which was estimated by radioimmunoassay (Fig.2). Data are means from triplicate determinations in a typical experiment.

RESULTS AND DISCUSSION

The acetone-HCl extracts from particulate fractions of guinea-pig heart were yellowish suspensions in contrast to the crystal-clear colourless water extracts. The concentration of material reacting with antidigoxin antibodies was much higher in water extracts than in acetone-HCl extracts (Fig. 2). Sigmoidal displacement curves, parallel to the digoxin curve, were obtained only with the water extracts. Extracts I and II contained, respectively, 2 and 0.4 pmol of digoxin equivalent per g of wet tissue. The activities of the extracts were destroyed after mineralization.

The acetone-HCl extracts inhibited Na, K-ATPase, Mg-ATPase and Ca, Mg-ATPase. The inhibition of Na, K-ATPase was maximal when these extracts were preincubated with the enzyme at 0°C in the absence of ATP. 50-fold dilution of the preincubation mixture did not reduce the inhibitory effect. The specific binding of [^{3}H]ouabain was not inhibited by the extracts. These results suggest the presence of non-specific, irreversible inhibitors in acetone-HCl extracts.

Although the yield of digoxin-like factor was higher in water extracts I than in extracts II, we investigated in detail the inhibition of Na, K-ATPase by extracts II. Procedure II allowed removal of most of the cytosolic constituants (i.e. nucleotides) which can interfere in enzyme assay. The ionic content of extracts I was about 0.2, 0.3, 3 and 10 mM respectively for Mg, Ca, K and Na; but extracts II were cation-free.

The features of Na, K-ATPase inhibition by water extracts II were similar to those previously reported for ouabain (8, 10) :

(1) Dose-dependence of Na, K-ATPase inhibition by extracts II and by digoxin is illustrated in Fig. 3. Dixon plots are straight lines suggesting that inhibition obeys to mass-action law. Assuming a mole to mole competition ratio in radioimmunoassay, it can be calculated that the endogenous factor is 10-fold more potent than digoxin.

(2) The inhibitory effect was obtained within 15 min incubation and was antagonized by KCl. The effect of 12 nM digoxin equivalent decreased from 47 % to 28 % and 22 % respectively in the presence of 0.5, 3 and 5 ml KCl.

(3) The specific binding of 20 nM [^{3}H]ouabain decreased from 6.9 to 4.9, 3.2 and 2.2 pmol per mg protein respectively in the absence or the presence of 4, 12 and 16 nM digoxin equivalent. The digoxin concentration which inhibits 50 % of specific binding was 150 nM. This is consistent with the above observation that the endogenous factor is 10-fold more potent than digoxin.

(4) Rat heart Na, K-ATPase was insensitive at doses which inhibit 50 % of guinea-pig heart Na, K-ATPase.

(5) Mg-ATPase and Ca, Mg-ATPase were not inhibited.

These results suggest the presence in water extracts II of a true digitalis-like factor. Its molecular weight was estimated to about 600 daltons from gel filtration. Further experiments are still required to conclude that the heart contains a physiologically active endogenous digoxin since all the criteria defined above are not yet fulfilled.

ACKNOWLEDGEMENTS

G. CASTANEDA-HERNANDEZ is fellow of the A.R.C.I. We thank A. SEGHERS for skilful technical assistance, P. SIMON for the drawings and T. PAELINCK-HEULENS for typing the paper.

This work was supported by the F.R.S.M. (grant n° 3.9001.79) and by the F.D.S. of U.C.L.

REFERENCES

1. S.D. Hillyard, E. Lu and C. Gonick, Circulation Res., 38, 250 (1976)
2. M.C. Fishman, Proc. Natl. Acad. Sci. USA, 76, 4661 (1979)
3. G.T. Haupert, Jr, and J.M. Sancho, Proc. Natl. Acad. Sci. USA, 76, 4658 (1979)
4. K.A. Gruber, J.M. Whitaker and V.M. Buckalew, Jr, Nature, 287, 743 (1980)
5. T. Godfraind and G. Castañeda-Hernández, Arch. int. Pharmacodyn. Ther., 250, 316 (1981)
6. R. Carraway and S.E. Leeman, J. Biol. Chem., 248, 6854 (1973)
7. T. Godfraind, A. De Pover and N. Verbeke, Biochim. Biophys. Acta, 481, 202 (1977)
8. A. De Pover and T. Godfraind, Biochem. Pharmacol., 28, 3051 (1979)
9. R. Bais, Anal. Biochem., 63, 271 (1975)
10. A. Schwartz, G.E. Lindenmayer and J.C. Allen, Pharmacol. Rev., 27, 1 (1975)