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Different methods for measuring endogenous digitalis-like factor(s)[1]

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Abstract

It is well recognized that one of the difficulties in the search for endogenous ligand(s) with digitalis-like properties (endogenous digitalis-like factor(s), EDLF) in mammals has been the lack of a unique, specific method for the accurate measurement of EDLF. Using C_{18} solid-phase extracts of plasma from normal adults and various patient groups, and purified extracts from umbilical cord plasma by affinity resin chromatography and HPLC, different methods to measure EDLF were evaluated. These were: (a) a human placenta radioreceptor assay (RRA) developed on the premise that competition for cardiac glycoside receptors was an absolute requirement for EDLF; (b) the inhibition of ^{86}Rb uptake in human erythrocytes to estimate the potassium transport by the sodium pump; (c) an enzyme immunoassay specific for ouabain recently introduced in the market (DuPont Ouabain EIA Reagent Pack). The human placenta RRA was found to have the same ease of application as immunoassay, but could have major advantages in detecting active molecules, being "biologically more meaningful". Ouabain immunoreactivity correlated with EDLF values obtained by RRA, but in some instances the two assays were completely unrelated. Moreover, the high specificity of the DuPont antibody for ouabain ($< 3\%$ cross reactivity with digoxin) could be disadvantageous to detect EDLF not strictly resembling ouabain. The ^{86}Rb uptake inhibition method correlated with RRA for EDLF purified by HPLC. It tested the complete enzymatic cycle and could therefore better reflect the in-vivo inhibitory activity of EDLF. However, it appeared not suitable for the routine EDLF evaluation in clinical studies since it was susceptible to sample osmolarity and required daily isolation of human erythrocytes possibly from the same donor. Results of the present study demonstrate that every assay has its limitations, and would suggest the use of multiple assays for EDLF detection.

Keywords: Endogenous digitalis-like factor(s); Ouabain; Radioreceptor assay; Enzyme immunoassay; Erythrocyte ^{86}Rb uptake; Human placenta

1. Introduction

Considerable, but still controversial, evidence indicates the presence in mammalian tissues of endogenous counterpart(s) to digitalis glycosides

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(EDLF; endogenous digitalis-like factor(s)) which inhibit cell membrane Na,K–ATPase through binding to the digitalis receptor on this enzyme [1,2]. This factor(s) might be relevant to sodium homeostasis and to the development of hypertensive diseases [2].

It is generally recognized that one of the difficulties in EDLF research has been the lack of a unique, specific screening assay for EDLF [3,4]. On the assumption that EDLF must reproduce the major biological actions of cardiac glycosides, inhibition of Na,K–ATPase activity, inhibition of potassium (or sodium) transport in intact cells, and inhibition of ^{3}H-ouabain binding to receptors have been employed. Radioimmunoassay for digoxin has also been extensively utilized [5–7], but the possible physiological role of digoxin-like immunoreactivity had been highly controversial [8,9]. Recently, Hamlyn's group [10,11], in view of their mass spectrometry data indicating that EDLF in human plasma is identical to ouabain, have proposed an immunoassay specific for ouabain to detect EDLF [11]. An ouabain enzyme immunoassay (EIA) reagent pack using Hamlyn's antibody has now been introduced in the market (NEK-072, DuPont–NEN) [12].

The aim of this study was to evaluate three different methods to measure EDLF in human plasma: (a) a human placenta radioreceptor assay (RRA) developed by the present authors [12] on the premise that competition for cardiac glycoside receptors was an absolute requirement for EDLF; (b) the inhibition of ^{86}Rb uptake in human erythrocytes [13] as a method to test the complete enzymatic cycle; (c) the DuPont Ouabain EIA Reagent Pack. For comparison, C_{18} solid-phase extracts of plasma from normal adults, newborn infants (umbilical cord) and various patient groups, and pools of umbilical cord plasma extracted and purified by affinity resin chromatography and HPLC were employed.

2. Materials and methods

2.1. Chemicals

[^{125}I]-digoxin (sp. act. 2200 Ci mmol^{-1}) was purchased from DuPont–New England Nuclear, Boston, MA. ^{86}RbCl (sp. act. 1 Ci g^{-1}) was purchased from Amersham International, Aylesbury, UK. Antidigoxin Fab fragments (Digibind) were obtained from Wellcome. C_{18} SepPak cartridges and C_{18} μBondapak column were purchased from Waters Associates, Milford, MA. The Ouabain EIA Reagent Pack was purchased from DuPont–New England Nuclear. Tween-20 and tetramethylbenzidine were from Sigma Chemical Co., St. Louis, MO. Sepharose CL-6B was from Pharmacia, Uppsala, Sweden. All other chemicals were of HPLC grade and were purchased from Merck.

2.2. Plasma samples

Peripheral blood samples were drawn with consent from healthy volunteers and patients with different diseases into heparinized tubes. Cord blood was collected from the placental vein immediately after normal delivery from women with uncomplicated pregnancies. None had ever taken digitalis drugs. Plasma was stored at −20°C until extracted.

2.3. Extraction procedures of plasma samples

2.3.1. C_{18} solid-phase extraction

Plasma (2 ml) was passed through disposable C_{18} SepPak cartridges preconditioned with 4 ml of acetonitrile followed by 20 ml of 0.1% trifluoroacetic acid (TFA), washed with 10 ml of 0.1% TFA, and eluted with 3 ml of 25% acetonitrile. Recovered eluate was evaporated to dryness under vacuum, and stored at −20°C until assayed.

2.3.2. Extraction and purification by HPLC of umbilical cord plasma

Pooled heparinized umbilical cord plasma was treated according to the following procedure [15]. Lyophilization, extraction in 0.6 volumes of methanol, extraction through SepPack C_{18} cartridges with methanol (10 ml original plasma per cartridge), immunoaffine chromatrography (in batch for 30 min at 4°C, onto 5 × 40 mm column until no absorbance at 210 nm could be detected in the washes; elution with methanol) on antidigoxin Fab fragments bound to activated Sep-

harose CL-6B (10 mg Fab fragments for about 8 ml of packed Sepharose activated with 0.6 g BrCN, overnight at 4°C), extraction on SepPack C_{18} cartridges with methanol, and final purification with HPLC (300 × 7.8 mm C_{18} μBondapak column, mobile phase acetonitrile–methanol–water (14:14:72, v/v/v; flow 1.5 ml min^{-1}). 15 HPLC fractions (2 ml) were collected, dried under vacuum, and stored at −20°C until assayed. Before reinjecting the next sample, the column was flushed with methanol for 1 h and re-equilibrated with mobile phase for 30 min.

2.4. Human placenta RRA

The assay was performed as previously described [13,16]. Briefly, human placenta membranes (300 μg protein), obtained by differential centrifugation from term placentas, and ^{125}I-digoxin (250 pM) were incubated with or without ouabain standards (10–200 nM) or sample extracts in total volume of 500 μl of 5 mM Na_2HPO_4, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.4 at 37°C, BSA 0.1%. Bound and free radioactivity were separated by rapid filtration through glass fiber filters, and retained radioactivity was counted in γ-spectrometer. Nonspecific binding was assessed with 10 μM ouabain and was subtracted from total binding to calculate specific binding. Protein content was assayed by the Pierce BCA Protein Assay Reagent.

2.5. ^{86}Rubidium uptake inhibition assay.

Washed erythrocytes from healthy donors were incubated for 3 h at 37°C in Ringer's solution with or without sample extracts or 0.1–100 nM ouabain. After a further incubation for 1 h with 1 μCi per tube of ^{86}RbCl, the cells were washed with ice-cold isotonic saline, and radioactivity taken up by the cells was determined by γ-counter. ^{86}Rb uptake inhibition was calculated as a percentatge of pump activity on Ringer's solution.

2.6. DuPont Ouabain EIA Reagent Pack

The DuPont Ouabin EIA was used according to the manufacturers' instructions [12], employing 50 mM phosphate buffered saline containing 2% BSA and 0.05% TweeN-20 as assay buffer, a ready-to-use solution of Tetramethylbenzidine for color development, and 0.5 M sulphuric acid as stop solution. The ouabain standard curve in assay buffer (0.01–5 nM) was freshly prepared from a 10 mM stock solution.

3. Results and discussion

There is increasing recognition that endogenous digitalis-like factor(s) do exist in mammals [1,2]. Two substances have been recently isolated from human materials and identified as ouabain [17] and digoxin [18], but some criticism has been raised against these findings [19]. As yet, a specific method for the accurate measurement of EDLF is not available.

Using crude plasma extracts and highly purified extracts from umbilical cord plasma, two biological methods (a radioreceptor assay and an assay measuring sodium-pump-mediated potassium transport), and immunoassay specific for ouabain were analyzed.

Since pure EDLF is not available to be used as a standard, and to better compare the results obtained by the biological methods with those obtained by the ouabain immunoassay, ouabain was employed to construct the dose-response curves. EDLF data, therefore, were expressed as ouabain equivalents (o.e.).

Fig. 1 shows typical ouabain standard curves and corresponding imprecision profiles for the human placenta RRA, the DuPont Ouabain EIA Reagent Pack, and the ^{86}Rb uptake inhibition method in human erythrocytes. The analytical performance of the assays is reported in Table 1.

As expected, the highest sensitivity was found in the immunoenzymatic method, and the lowest in the ^{86}Rb uptake inhibition assay. Assay sensitivity of the DuPont Ouabain EIA was higher than that reported for other ouabain immunoassays [19–21], where values from 10 fmol per tube [20] to 160 fmol per tube [19] have been found. The sensitivity of the DuPont Ouabain EIA, however, decreased with time, probably reflecting the instability of protein-ouabain immobilized to the microplates.

The reproducibility of this immunoassay, by contrast, appeared to be lower than that reported for other immunological methods [21,22], even if a standardized evaluation of the analytical performance has not been generally presented. Interestingly, although the reproducibility of RRA is generally slightly worse than that of immunoassay, reflecting the greater complexity of the biological system, intra- and inter-assay RSDs of the DuPont Ouabain EIA were similar to those found by RRA (see Table 1).

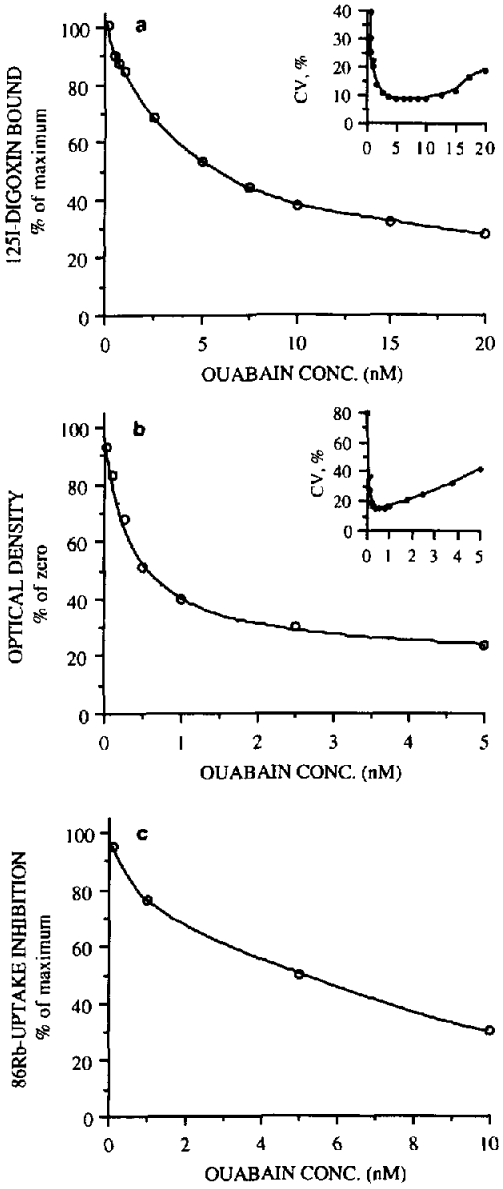

Fig. 1. Typical ouabain standard curves and imprecision profiles (insets) of human placenta RRA (a), DuPont Ouabain EIA Reagents Pack (b), and ^{86}Rb uptake inhibition in human erythrocytes (c).

In eight normotensive volunteers not taking digoxin or other drugs, EDLF concentration by RRA was 175 ± 66 pM o.e. (mean $\pm$ SEM), similar to that found in a larger number of normal adults ($n = 21$, 204 ± 34 pM o.e.). Immunoreactive ouabain was detectable in only four of the eight subjects. In these four subjects, the level of ouabain was 90 ± 26 pM.

Compared with healthy adult subjects, cord blood plasma contained significantly higher concentrations of EDLF, independent of the method utilized to detect EDLF. In two pools from 4–10 subjects, EDLF values by RRA were 420 ± 183 pM o.e. and ouabain immunoreactivity was 214 ± 63 pM; EDLF concentration by ^{86}Rb uptake inhibition assay ranged from 100 to 300 pM o.e. Considering all data as a whole, a weak but significant correlation was found between the values obtained by the DuPont Ouabain EIA (x) and those obtained by RRA (y) ($y = 1.25x + 100.6$; $r = 0.47$, $p = 0.0001$, $n = 71$). The intercept was significantly different from zero. It is worth noting that six plasma samples from two of the normal adults had bioactivity in the RRA and read zero in the Ouabain EIA, suggesting that EDLF in these two subjects was not ouabain. Other specimens (9/71) from different patients had no activity by RRA reading up to 80 pM ouabain immunoactivity.

A significant correlation was also found between the percentage of ^{86}Rb uptake inhibition (y) and RRA (x) for an EDLF purified from pooled umbilical cord plasma by immunoaffinity chromatography and HPLC ($y = 0.20x + 4.73$; $r = 0.86$, $p < 0.001$, $n = 14$). Interestingly, it had previously been found [23] that the HPLC fractions assayed by the human placenta RRA revealed a major peak coincident with the one observed by ^{86}Rb uptake inhibition measurements.

When the influx of $^{86}RbCl$ in human erythrocytes was tried to be employed with crude plasma extracts such as C_{18} solid-phase extracts, however,

Table 1
Analytical performance of human placenta RRA, DuPont Ouabain EIA Reagent Pack, and ^{86}Rb uptake inhibition in human erythrocytes

Method	Assay sensitivity (nM)	IC_{50} (nM)	%RSD	
			Intra-assay	Inter-assay
Human placenta RRA	0.32	5.9	4.5% (1–6 pmol per tube); $n = 10$	25% (0.7–2.5 pmol per tube); $n = 13$
DuPont Ouabain EIA	0.03	0.15	18% (12 fmol per well); $n = 5$	34% (57 fmol per well); $n = 3$
^{86}Rb uptake inhibition	1	3	11.9% (0.8 pmol per tube); $n = 4$	–

haemolysis of cells was sometimes observed. The ^{86}Rb uptake inhibition method, in fact, showed susceptibility to variations in environmental osmolarity. Moreover, it had previously been found that this method was not able to distinguish between ouabain and digoxin, despite the difference in their potency as cardioactive compounds. Ouabain and digoxin standard curves, in fact, were almost perfectly superimposable [24]. In the human placenta RRA, in contrast, ouabain was more effective than digoxin in inhibiting binding with IC_{50} values of 5.9 ± 0.3 nM and 8.4 ± 0.2 nM respectively.

In conclusion, the human placenta RRA was found to have the same ease of application as immunoassay, but could have major advantages in detecting active molecules, being "biologically more meaningful".

Ouabain immunoreactivity as detected by the DuPont Ouabain EIA Reagent Pack correlated with EDLF values obtained by RRA, but in some instances the two assays were completely unrelated. Moreover, the high specificity of the DuPont antibody for ouabain (< 3% cross reactivity with digoxin) could be disadvantageous to detect EDLF not strictly resembling ouabain.

The ^{86}Rb uptake inhibition method correlated with the RRA for EDLF purified by HPLC. It tested the complete enzymatic cycle and could therefore better reflect the in-vivo inhibitory activity of EDLF. However, it was susceptible to environmental osmolarity, and required daily (or every few days) isolation of human erythrocytes possibly from the same donor to avoid inter-individual variations. Thus, it appeared not suitable for routine EDLF evaluation in clinical trials.

Taken together, these results demonstrate that every assay has its limitations, and would suggest the use of multiple assays for EDLF detection.

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