ENZYME MODULATION AS AN APPROACH TO CLINICAL ANALYSIS: INVESTIGATIONS ON THE FEASIBILITY OF THE METHOD AND THE PROBLEMS ENCOUNTERED

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1. Introduction

Enzymic determination of metabolites in biological fluids and tissues is becoming increasingly important because of the greater specificity of such procedures compared to chemical methods [1]. To date such enzymic assays are based on the stoichiometric conversion of the compound to be measured (i.e. the compound is a substrate of the enzyme used). The dependence on a stoichiometric equivalence between the measured response and the substance to be assayed limits the sensitivity of such procedures and may render them impractical. A different enzymic approach, which has received little or no attention, may be based on modulation of enzyme activity by the metabolite in question. This modulatory approach is potentially more sensitive than classical enzymic procedures since it is amplifying rather than stoichiometric. To test the feasibility of such an approach we used bovine liver glutamate dehydrogenase (EC 1.4.1.3) in the estimation of total oestrogens in pregnancy urine. This enzyme is modulated by a number of steroid hormones [2].

Abbreviations:
Oestrone: 3-hydroxy-1,3,5 (10)-oestratrien-17-one;
oestriol: 3,16α, 17β-dihydroxy-1,3,5 (10)-oestratriene;
oestradiol-17β: 3,17β-dihydroxy-1,3,5 (10)-oestratriene;
pregnanediol: 3α, 20α-dihydroxy-5β-pregnane;
progesterone: 4-pregnene-3,20-dione;
GLC: gas–liquid chromatography.

2. Materials and methods

The sources of materials used were as follows: oestrone, oestriol, oestradiol-17β, progesterone, xanthine, hypoxanthine, uric acid, α-ketoglutarate, uricase (type 1), and glutamate dehydrogenase (type 1), Sigma Chemical Co., St. Louis, Mo.; pregnandiol, Koch-light Laboratories, Colnbrook, Bucks., U.K.; NADH, Boehringer Corp., London.

Glutamate dehydrogenase activity was assayed at 30°C by continuous monitoring of the decrease in absorbancy of NADH at 340 nm using a Unicam SP 1800 equipped with a chart recorder and a thermostatted cuvette holder. Analytical reaction mixtures contained 130 μmol imidazole buffer, pH 7.3, 40 μmol α-ketoglutarate, 160 μmol ammonium acetate, 0.42 μmol NADH, 90 μmol EDTA, 10 μg enzyme, and 50–100 μl of ethanol containing steroid or urine extract in a final volume of 3 ml. Control reactions, which were identical to the above except for the inclusion of ethanol alone, were carried out at the start and finish of each set of analyses as a check on the stability of the enzyme [3]. Non-enzymic breakdown of NADH was allowed for by using blanks containing all components of the reaction mixture except enzyme with each assay. A fresh enzyme preparation was used for each set of assays by dissolving an aliquot of the crystalline suspension in cold 0.05 M potassium phosphate buffer, pH 7.0.

Oestrogens were extracted from filtered 24 hr urine samples by a modification [4] of Nelson's method [5], following acid hydrolysis of conjugated steroids. 5 ml of urine and 1 ml of conc. HCl in a tightly

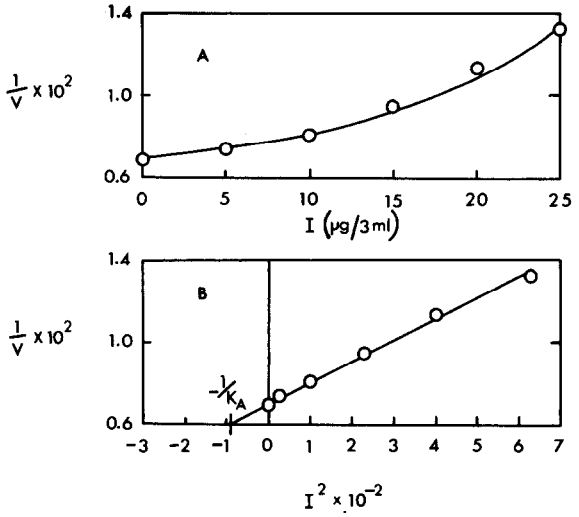

Fig. 1. Modulation of glutamate dehydrogenase activity by oestradiol-17β. The reciprocal of the reaction velocity (V) was plotted against A) inhibitor (I) concentration and B) against the square of the inhibitor concentration.

capped tube were placed in a boiling water bath for 30 min and then cooled. The pH was adjusted to 14 with satd. NaOH and the mixture was extracted for 15 sec with 10 ml of $CHCl_3$. 100 mg of phenolphthalein in ethanol was added to the mixture before the pH step as an internal standard for oestriol determination by gas chromatography. The pH of the aqueous phase from above was adjusted to 1 with conc. HCl and oestrogens were extracted by washing twice with 15 ml of $CHCl_3$ for 1 min. The combined $CHCl_3$ extracts were filtered through anhydrous sodium sulphate and the chloroform evaporated at 60°C under a stream of N_2. The residue was dissolved in 0.5 ml ethanol and aliquots used for total oestrogen determination by the enzymic method. For gas chromatographic estimation of oestriol, the residue was dissolved in 1 ml of acetic anhydride and mixed with 0.1 ml of conc. HCl. The tubes were capped and placed in an oven at 110°C for 20 min. Excess reagent was evaporated under a stream of N_2 at 60°C. The precipitate was dissolved in 0.1 ml ethanol and 2 μl aliquots used for oestriol measurement [4].

Standard solutions for total oestrogen determination by the enzymic method contained oestriol, oestrone and oestradiol-17β in the ratio 29:1.4:0.5 – the ratio found in pregnancy urine at term [6]. Purification of conjugated urinary oestrogens prior to hydrolysis and extraction was effected by gel filtration. 5 ml samples of urine were applied to a column (15 X 5 cm) of Sephadex G-25, pre-equilibrated with 0.1 M NaCl. In the presence of salt, conjugated oestrogens bind to the gel whereas other hormones do not, and may then be eluted with distilled water [7]. Uric acid was removed from urine by treatment with uricase (EC 1.7.3.3). 5 ml samples were incubated at 30°C with 5 ml of 0.05 M Tris-HCl buffer, pH 8.5, containing 0.1 mM EDTA and 0.015 i.u. of uricase until the decrease in A_{295} ceased.

Table 1
Concentration of steroid giving 50% inhibition of glutamate dehydrogenase activity.

Hormone	K_A (μM)
Oestradiol-17β	11.5
Oestrone	295.5
Oestriol	316.5
Pregnanediol	28
Progesterone	153

3. Results and discussion

The inhibition of glutamate dehydrogenase activity by oestradiol-17β is illustrated in fig. 1. The intercept on the abcissa (fig. 1B) gives the negative reciprocal of K_A, the concentration of inhibitor required to give 50% inhibition [2]. The K_A values for those steroids found in relatively large amounts in pregnancy urine are listed in table 1.

Table 2
Enzymic measurement of total oestrogens in pregnancy urine.

Sample	Total volume (ml/24 hr)	Week of pregnancy	Total oestrogens (mg/24 hr) Enzyme method	Oestriol (GLC) (mg/24 hr)
1	1060	36	245.9	29.5
2	1760	39	242.4	30.1
3	1040	39	146.4	42.0
4	1140	35	123.6	14.8

Standard curves, total oestrogen and oestriol determinations were obtained as described in Methods.

Table 3
Inhibition of glutamate dehydrogenase activity by steroids and oxypurines with and without extraction.

Compound	(μg/3 ml assay)	% of Control activity	
		Direct assay	After extraction
Pregnanediol	18	91.3	100.8
Progesterone	150	70.6	102.9
Uric acid	528	82.0	86.4
Total oestrogens	30.9	–	91.0
Uric acid + total oestrogens	528 + 30.9	–	66.1

Control activity is set at 100. Standard total oestrogens, extraction procedure and assays were as described in Methods.

Douville and Warren [2], who have obtained results similar to those shown in fig. 1, reasoned that 2 moles of steroid must bind for complete inactivation of 1 equivalent of catalytic site, but did not rule out allosterism as an alternative explanation. Since we wished to measure total urinary oestrogens, the latter possibility represented a potential problem. However, all combinations of the three major oestrogens acted additively rather than synergistically in inhibiting enzyme activity.

Oestrogens were extracted from urine following hydrolysis of their conjugates. Total oestrogens were estimated enzymically, and oestriol by gas–liquid chromatography. Initially there was wide disparity between the values obtained by the two methods (table 2). Since oestriol is by far the most abundant of urinary oestrogens during pregnancy [6], it followed that factors other than oestrogen contributed to inhibition of enzyme activity, thereby giving rise to erroneously high values. Moreover, since the disparity between the values obtained by the two methods ranged from 3- to 10-fold, these interfering factors must vary in amount depending on the sample.

Synergistic inhibition of enzyme activity by oestrogens has been ruled out as the cause of this disparity. Neither progesterone nor pregnanediol, both of which inhibit glutamate dehydrogenase, came through the extraction procedure (table 3) and were eliminated as "contributory factors".

Of the oxypurines present in urine, only uric acid inhibited enzyme activity at *in vivo* concentrations (table 3).

Table 4
Summary of procedures involved in the enzymic estimation of total urinary oestrogens.

Procedure	Enzymic method	GLC
	Total oestrogens (mg/24 hr)	Oestriol (mg/24 hr)
Hydrolysis and extraction	171	33
Uricase treatment, hydrolysis and extraction	98	–
Gel filtration, hydrolysis and extraction	102	–
Uricase treatment gel filtration, hydrolysis and extraction	50	–

Moreover, uric acid came through the extraction procedure (as judged by UV spectral studies and uricase treatment of extracts of standard solutions of uric acid) and appeared to act synergistically with oestrogens in inhibiting glutamate dehydrogenase activity. It is possible that uric acid may bind to that site on the enzyme at which the modulators ADP and GTP are bound [8] since all are purine derivatives. This site is distinct from that which binds oestrogens (R.A. Yeates, personal communication) and may explain the observed synergistic inhibition of activity by uric acid and oestradiol-17β.

Treatment of urine samples with uricase significantly reduced the disparity between the values obtained by the two methods (table 4). This disparity was also reduced on preliminary purification of conjugated oestrogens by gel filtration prior to hydrolysis and extraction (table 4). The enzymatically determined value for total oestrogens (50 mg/24 hr), obtained on combining the above procedures, is in good agreement with that found for oestriol alone (33 mg/24 hr) using gas chromatography.

The technical procedures involved in removing interfering substances render this particular enzymic method impractical for routine determination of urinary oestrogens. The exercise does demonstrate, however, that clinical assays of metabolites based on their ability to modulate enzyme activity, are feasible.

Should the compound to be measured not modulate any enzyme activity, the above approach should still feasible by judicious choice of a coupled enzyme system.

References

[1] H.U. Bergmeyer and G. Michal, (1973), Abstr. Commun. Special Meet. Fed. Eur. Biochem. Soc. 9.

[2] A.W. Douville and J.C. Warren, Biochemistry 7 (1968) 4052–4059.

[3] G. Ellis and D.M. Goldberg, Clin. Chim. Acta 39 (1972) 472–474.

[4] D.W. Nugent and P.F. Fottrell, Clin. Chim. Acta, in press.

[5] G.A. Nelson, Am. J. Obstet. Gynaecol. 106 (1970) 649–655.

[6] Documenta Geigy Scientific Tables, 6th. ed. pp. 493–497.

[7] "Sephadex in Clinical Analysis", p. 5. Literature supplied by Pharmacia, Uppsala, Sweden.

[8] C. Frieden, J. Biol. Chem. 240 (1965) 2028–2035.