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INTERLABORATORY COMPARISON OF ETHOXYRESOFURIN O-DEETHYLASE (EROD) ACTIVITY

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The interlaboratory comparison of Ethoxyresofurin O-Deethylase (EROD) activity was conducted by the Laboratoire de Toxicologie Marine, Faculté de Médecine, Université de Nice, 06107 Nice Cedex 2, France

1. INTRODUCTION

Studying the effects of pollution on marine organisms has been one of the first activities of MED POL and still represents an important element of the research component. A large number of field studies has now been carried out in which the fish hepatic monoxygenase response has been related to pollution. In particular, ethoxyresorufin O-deethylase (EROD) activity in fish liver is being used increasingly to indicate the presence or the effect of certain organic contaminants.

Measurements of EROD activity have been incorporated into the training workshop of the MED POL programme organized in Nice at the end of September 1992. In the Mediterranean sea, EROD is used by several laboratories in pollution surveys. Despite the fact that this test is widely used there is no consensus on the choice of "standard" conditions which would permit some inter-laboratory comparison of EROD measurements. In fact, to establish the reliability of measuring EROD activity in fish, it is important to test the ability of laboratories to differentiate induced from non-induced fish using current methodology.

The comparison described here is the first step of an intercalibration exercise; this approach concerns sea bass EROD measurements with different conditions as each laboratory has used its own method. The sea bass (*Dicentrarchus labrax*) can be raised in aquaculture farms. This provides the advantage of studying individuals, generally males, of a standardized size and limited genetic polymorphism, thus avoiding a too wide range of variations in the oxidation phenotypes. During this exercise, some scientists have also tried to use one single technique; this experiment can provide some information about differences only due to handling conditions in the different laboratories.

The laboratories which participated in the exercise appear in the Annex.

2. MATERIALS AND METHODS

Thirty fishes were injected intraperitoneally 20 mg BaP/kg body weight, 28 hours prior to killing. Each control fish was given a single intraperitoneal injection of corn oil (n=30). The livers of fishes were dissected out, cut into small pieces and immediately frozen in liquid nitrogen. They were submitted to potter homogenisation at a ratio of 5 ml per g of tissue in buffer Hepes 10 mM, sucrose 250 mM, glycerol 20% (w/v) and PMSF 1 mM, pH 7.4 Glycerol was added as a cryoprotector because the homogenate had to be frozen immediately after mixing. Homogenates were prepared by pooling 30 livers from either control or BaP induced sea bass.

Homogenates provided to participants were sent in dry ice. In laboratory 1, the analysis was also performed after a 24h conservation in dry ice so that to have the same conditions. Scientists measured EROD activities using S9 or microsomal fractions. In general, microsomes have been prepared by performing an ultra-centrifugation of the supernantants (100,000g), however, laboratory 4 used calcium precipitation to obtain microsomal pellets.

The first EROD measurements were made in laboratory 1 on the 17th of October 1994; another measurement was made 5 months after conservation of the homogenates in liquid nitrogen. No loss of activity was observed (see Table 1).

Participants were also given a resorufin standard **b** prepared in laboratory 1; moreover, each laboratory had its own standard **a** and used both of them for estimating EROD activities.

Fluorescence measurements were performed in the different laboratories using several buffers for microsome preparation and EROD assay; nevertheless, one main method was used for measuring EROD on microsomes (laboratory 1,2,3,9 & 10).

The buffers used in this method are:

- for microsome preparation: Hepes (10 mM), sucrose (250 mM), glycerol (20%) and PMSF (1 mM) (pH 7.4)

- for EROD assay: Na_2HPO_4 (35 mM), KH_2PO_4 (7.18 mM) (pH 7.4), NADP (0.25 mM), G-6-P (2.5 mM), G-6-P dehydrogenase (1U/ml), ethoxyresorufin (1.24 µM) and the reaction is stopped by the addition of 2 ml of acetone.

The activity was measured by using a fluorimeter (excitation wavelength 537 nm, emission wavelength 583 nm). Some laboratories have measured EROD activities by using this method as well as their usual techniques, it represents an interesting intercomparison exercise.

3. RESULTS AND DISCUSSION

Specific activities are summarized in 2 Tables, Table 1 for measurements on microsomal pellets and Table 2 for S9 fractions and whole homogenate.

3.1 General remarks on different methods

EROD measurements have been performed by stopping the reaction with acetone (Acet) or by following the products formation in kinetics (Kin). The second method allows scientists to have a mean value resulting from different points of hydroxyresorufin formation at different times of the reaction. With the second method, we can expect to obtain results having smaller standard deviations (from one measure to another) , than activity values resulting from a single measure with the first method.

Nevertheless, the variation percentage (based on the standard deviation) observed for the two ways of measuring EROD activities were not really different. For the first method (using acetone) the percentage of deviation varied from 1 to 30% (n=3 or 4 measures, depending on the laboratory) for EROD measurements on microsomes and from 1 to 5% when performing the assay on S9 fractions. When following ethoxyresorufin dealkylation on kinetics, the percentage varies from 3 to 23% for microsomes and from 5 to 50% for S9.

Table 1

Specific activities (picomoles/minute/mg protein) measured on microsomes.
(a=own standard, b= common standard)

LAB No.	TECHNICAL REMARKS	NI (non -induced fish)		I (induced fish)		INDUCTION FACTOR	
		a	b	a	b	a	b
1	U.C., Cuv + Acet, pH Phosphate: 7.4		6.90±2.40 6.78±3.68*		79.90±1.27 102.53±5.70*		11.58
2	U.C., Cuv + Acet, pH Phosphate: 7.4		8.67		69.0		7.97
3	U.C., Cuv + Acet, pH Phosphate: 7.4		15.88±1.15		165.13±4.7		10.4
9	U.C., Cuv + Acet, pH Phosphate: 7.4	5.99±0.65	7.60±0.33	97.70±13.04	84.33±1.95	16.31	11.1
10	U.C., Cuv + Acet, pH Phosphate: 7.4	1.2±1.5	0.5±0.6	35.3±4.6	15.2±2.0	29.4	30.4
5	U.C., Cuv + Acet, pH	11.31±1.65	10.75±1.78	105.65±2.92	101.15±4.62	9.43	9.39
2	U.C., Cuv + Kin, pH TRIS-Phosphate: 8.0		13.22±2.97		122.95±19.99		9.3
3	U.C., Cuv + Kin, pH TRIS-Phosphate: 8.0		7.22±0.60		99.69±13.09		12.91
4	U.C., Cuv + Kin, pH Phosphate: 7.4	0.94±0.18	0.58±0.02	8.14±1.85	5.41±1.10	8.66	9.26

*EROD measurements on microsomes after a 5-month conservation in liquid nitrogen

Legends: Cuv: cuvette; Acet: Acetone; Kin: kinetics; Micr: microplate; U.C.: ultra-centrifugation; C.P.: calcium precipitation;

Table 2

Specific activities (picomoles/min/mg prot) measured on S9 fractions
(a=own standard, b=common standard)

LAB No.	TECHNICAL REMARKS	NI (non-induced fish)		I (induced fish)		INDUCTION FACTOR	
		a	b	a	b	a	b
2	Cuv + Kin, pH Tris-Phosphate:8.0		3.77±0.43		35.5±3.0		9.42
3	Cuv + Kin, pH Tris-Phosphate:8.0		3.66±0.20		39.44±3.50		10.77
6	Micr + Kin, pH Tris-Phosphate:8.0	1.35±0.35	1.00±0.24	11.56±2.75	9.58±2.29	8.56	9.58
7	Micr + Kin	1.75±0.18	1.50±0.17	13.35±0.92	12.35±0.87	7.63	8.23
8	Micr + Kin, pH Phosphate: 7.8	5.68±1.39	7.21±7.79	61.50±7.30	79.50±9.50	11.02	11.02
8	Cuv + Acet, pH Phosphate: 7.8	9.91±0.08	10.63±0.10	89.97±4.87	96.44±5.22	9.08	9.07
10	Cuv + Acet, pH Phosphate: 7.4	9.2±3.3	6.2±3.1	92.3±10.5	45.8±6.1	10	7.4
11	measures on homogenates	11.48±1.00	7.43±0.01	175.93±17.33	115.23±7.13	15.3	15.5

Note: The results of laboratory no. 11 are on whole homogenates.

Even if the absolute activities varied a lot from one laboratory to another for control or induced samples, the induction factor observed is remarkably similar. The detected induction was not affected by whether the assays utilised S9 fractions or microsomes.

3.2 Resorufin standards

Table 3 shows the percentage of variation between the two EROD values due to the use of different resorufin standards.

Table 3

Percentage variation when using 2 different resorufin standards

Fraction	Laboratory no.	% (NI)	% (I)
Microsomes	4	47.4	40.3
	5	3.9	4.4
	9	23.7	14.7
	10	82.3	79.6
S9	6	29.8	18.7
	7	15.4	7.8
	8	7.0	6.9
		23.7	25.0
	10	39.0	67.3

As, the resorufin batches seem to be very different from one another, it is clear that scientists must, in the future, try to choose the same resorufin. For example, in the case of laboratory 10 an 80% difference is observed between the activities estimated with 2 different standards.

These differences in the "purity" of the standards have been already observed in another interlaboratory comparison exercise of EROD measurements in Dab (*Limanda limanda*) liver (Stagg and Addison, 1995). During this exercise, participants have calculated the molar absorbance of their own resorufin and found differences from 18 to 115% compared to the molar absorbance reported by Klotz *et al.* (1984). Big differences between resorufin solutions have also been observed by Canadian scientists during an interlaboratory comparison of EROD activity in white sucker *(Catostomus commersoni)* (Munkittrick *et al.*, 1993).

3.3 Differences between laboratories using the same technique

Table 4 summarizes the results obtained by different laboratories using the same buffers (microsome preparation, enzymatic assay), the same resorufin and the same technique for EROD measurements.

Table 4

Microsomal specific activities (picomoles/min/mg prot) measured with the same technique (Cuv + Acet, pH Phosphate: 7.4)

Laboratories	NI	I	Induction factor
1	6.90 ± 2.40	79.90 ± 1.27	11.6
2	8.67	69.07	7.9
3	15.88 ± 1.15	165.13 ± 4.70	10.4
9	7.60 ± 0.33	84.33 ± 1.95	11.1
10	0.50 ± 0.60	15.20 ± 2.00	30.4
Mean value	7.91 ± 5.48	82.73 ± 53.73	

The percentage of deviation for non induced activities is 69.3% and for induced activities 64.9%. As, EROD measurements were performed with the same technique and buffers, handling conditions in the laboratories as well as the transport of resorufin standard and liver homogenates must be responsible for this quite large variation.

4. REFERENCES

Klotz, A.V., J.J. Stegeman and C. Walsh (1984, An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurements of cytochrome P-450 monooxygenase activity. *Anal.Biochem.*, 140:138-145

Munkittrick, K.R., M.R. Van Den Heuvel, D.A. Metner, W.L. Lockhart and J.J. Stegeman (1993), Interlaboratory comparison and optimization of hepatic microsomal ethoxyresorufin O-deethylase activity in white sucker (*Catastomus commersoni*) exposed to bleached kraft pulp mill effluent. *Env.Toxicol.Chem.*, 12:1273-1282

Stagg, R. and R.F. Addison (1995), An interlaboratory comparison of measurements of ethoxyresorufin O-deethylase in Dab (*Limanda limanda*) liver. *Mar.Environ.Res.*, 40:93 -108

ANNEX

List of participating Institutions

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