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Toxicity order of cholanic acids using an immobilised cell biosensor[1]

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Abstract

There is considerable published evidence of the use of cells of various species to evaluate the toxicity of numerous compounds, many of pharmaceutical interest. The coupling of cell colonies with a suitable transduction device has led to the development in recent years of toxicity biosensors based on the alteration of a process or a cell metabolic function by the toxic substance under examination.

A biosensor based on immobilised yeast cells (*Saccharomyces cerevisiae*) has been developed recently in this department for the purpose of performing a rapid toxicity test in aqueous environmental matrices. This biosensor has now been used in the toxicity screening of a number of sodium salts of conjugated and free cholanic acids. The "toxicity degree" scale, which was found by placing in decreasing order the values of the slopes of the straight lines obtained by quantifying changes in the behaviour of the respirometric curve, plotted before and after incubation, using known concentrations of cholanic acid sodium salts, was: deoxycholic acid > chenodeoxycholic acid > ursodeoxycholic acid > cholic acid, for free cholanic acids; and glycodeoxycholic acid > glycochenodeoxycholic acid > glycocholic acid, for glycocholanic acids. These values are in good agreement with published toxicity data obtained in vitro. This sensor can thus be considered to provide a valid instrument for the preliminary evaluation of the toxicity of organic compounds or drugs.

Keywords: Toxicity; Cholanic acids; Yeast cell biosensor

1. Introduction

The determination of the toxicity of an active principle is a pharmacological problem that is usually time-consuming; it also requires special laboratory equipment. However, the growing sensitivity to the indiscriminate use of animals in toxicity tests means that it is becoming increasingly difficult to use classical drug-testing methods. This has boosted interest in developing methods that do not use animals but use cell cultures or micro-organisms whose functional and/or metabolic alterations can be monitored rapidly and easily [1].

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A method has been deveoloped recently that is based on a toxicity biosensor obtained by coupling a classical Clark electrode to *Saccharomyces cerevisiae* cells immobilised in an agarised culture medium [2].

Cholanic acid sodium salts are widely used in commercial antilithogenic products [3]; in formulating these medicinal products the degree of toxicity is one of the fundamental criteria in the choice of the active component. Therefore, and because other researchers [4] have recently proposed the use of biosensors that are very different from the type investigated by the authors for toxicity studies on pharmacologically active principles, although they have many points in common with respect to possible applications, it was decided to use the toxicity biosensor developed to compare the toxicity of two series of conjugated and free cholanic acids.

The toxicity scales for these compounds [5,6] obtained in vitro using conventional methods have therefore been compared with results for the sodium cholates tested. Furthermore, in recent years, a substantial number of analytical and bioclinical tests have been carried out in the authors' laboratory in view of their considerable gastroenterological [7] and pharmacological [8] importance.

2. Experimental

2.1. Chemicals and apparatus

The following cholanic acid sodium salts were used: sodium cholate, sodium chenodeoxycholate, sodium deoxycholate, sodium ursodeoxycholate, sodium glycocholate, sodium glycochenodeoxycholate, and sodium glycodeoxycholate, all supplied by Sigma, (St. Louis, MO); yeast extract for microbiology, universal peptone for microbiology M66, D(+)-glucose for microbiology, analytical reagent grade glycine and high purity agar–agar were all supplied by Merck, (Darmstadt, Germany). The nylon net was from Bozzone, (Appiano Gentile, Italy). The cells used were of a wild diploid strain of *Saccharomyces cerevisiae* kindly made availabe by the Rome Botanical Gardens.

The following apparatus was used: a glass measurement cell with thermostatted forced water-circulation jacket, 35 ml capacity; an Orion O_2 model 97-08-99 amperometric oxygen gas diffusion sensor (Clark electrode); an Orion Microprocessor ionalyzer/901 potentiometer; an Amel Model 868 recorder; and a thermostat Julabo V.

2.2. Cell immobilisation in agarised culture medium

The cells were immobilised in an agar gel containing the culture medium ("agarised medium"); the composition of the gel was: yeast extract 1%, peptone 1%, glucose 2% and 1% w/w in agar. The agar gell containing the cells was obtained in the form of a layer by pouring 10 ml of sterile "agarised medium", to which 200 μl of cell suspension had been added, onto a sterile Petri dish, allowing it to gel, and then storing it, closed and sealed, in a refrigerator.

The *Saccharomyces cerevisiae* cell suspension was obtained by allowing cells to grow for 30 h at 37°C with stirring in a 100 ml sterile Erlenmeyer flask containing 50 ml of culture medium.

2.3. Biosensor assembly

A suitably-sized disk (about 8 mm in diameter and about 1 mm thick) containing the entrapped yeast cells was cut out of the "agarised medium" stratified on a Petri dish. The disk was then removed from the dish and placed between a nylon net and the gas-permeable membrane of the Clark electrode; the whole assembly was secured to the head of the electrode by means of an O-ring (Fig. 1).

3. Methods

Measurements were carried out using the biosensor immersed in a thermostatted (25°C) measurement cell containing 15 ml of glycine buffer solution (pH 9.0; 0.15 M) with constant magnetic stirring.

The electrode response was expressed directly in ppm O_2 by the Orion Microprocessor ionalyzer, which was connected to the Amel analog

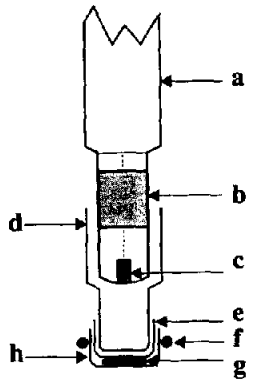

Fig. 1. Biosensor assembly: a, oxygen electrode; b, Ag/AgCl anode; c, Pt cathode; d, cap containing the inner solution (KCl 0.1 M, phosphate buffer 1/15 M and pH 6.6); e, gas-permeable membrane; f, O-ring; g, agarised culture medium with immobilised cells; h, nylon net.

recorder, and could be simultaneously observed as a function of time.

For each measurement two different "respirometric" curves were recorded: one in the presence of the substrate alone (i.e. 0.5% w/w of glucose); and the other in the presence of substrate, but after initial incubation (for 8 min) with the substance, which had been tested for toxicity (i.e. cholanic acids). The results were processed using one of the two following experimental data: (a) the difference in current intensity (δ), which is proportional to the "distance" between the two respirometric curves, at a fixed time (30 min) after the addition of glucose (see Fig. 2); (b) the difference in amplitude of the angles (α-α_0) of the slopes at which the two respirometric curves attain a stationary state, which was measured 25 min after the addition of glucose (see Fig. 2).

These values were then suitably normalised (Fig. 2) in order to increase their reproducibility; the experimental results were found to correlate with the concentration and toxicity of the substance under test [9]. The measurement method used, which is illustrated in Fig. 2, proved to be fast and, at the same time, extremely reliable, even in comparison with the more classical procedures [2,9,10], which also take much longer to carry out.

4. Results and discussion

Two typical calibration curves for deoxycholate and glycodeoxycholate, obtained using the biosensor, are shown in Fig. 3(a) and Fig. 4(a).

In previous research [9] it was shown that it was possible to define a suitable toxicity index "I_{tox}" (measured as Δ_N or α_N). The values of Δ_N and α_N (see Fig. 2), which have been shown to be adequately determined using the biosensor and the procedure described herein, can be used to repre-

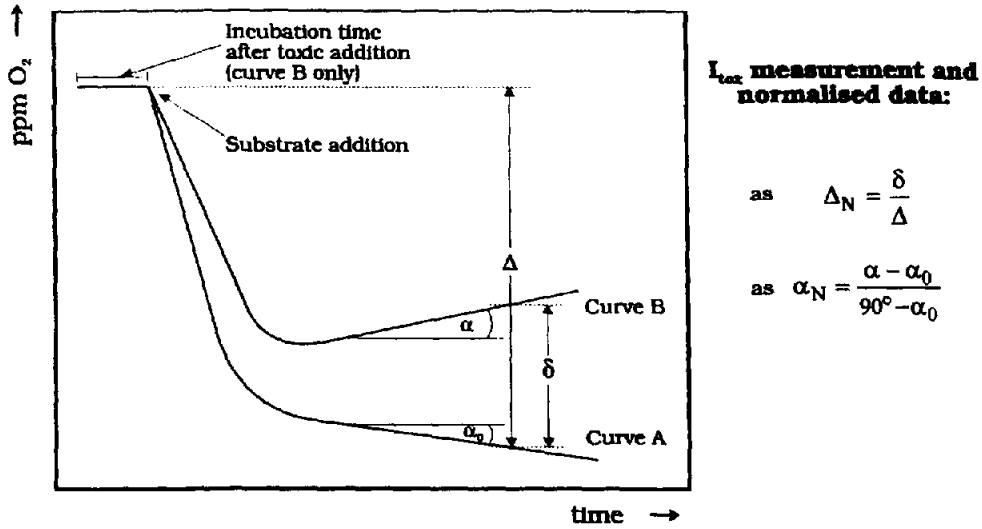

Fig. 2. Measurement method used in this research.

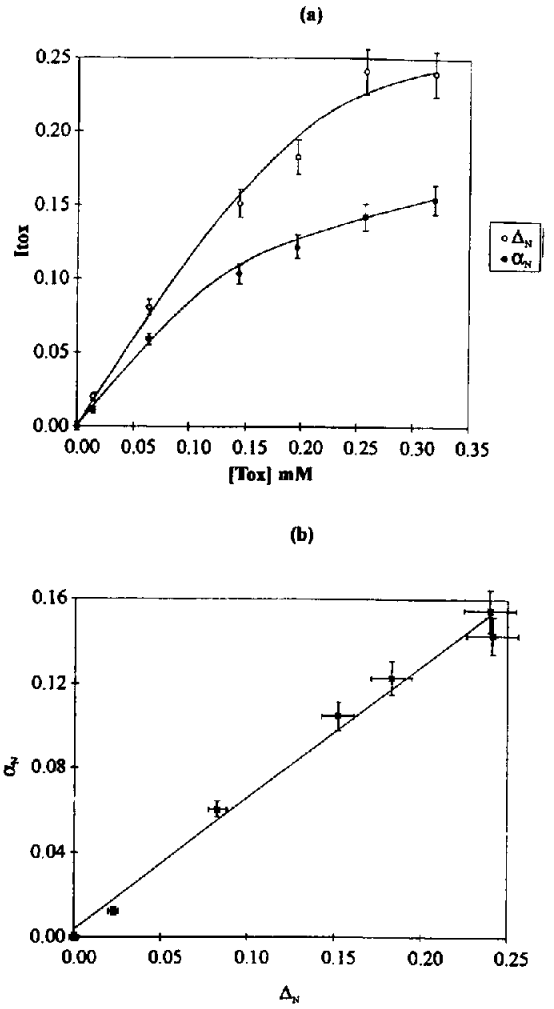

Fig. 3. (a) Plot of toxicity index (calculated as α_N or Δ_N) as a function of the toxic (sodium deoxycholate) concentration, obtained using the biosensor. (b) correlation between α_N and Δ_N values for sodium deoxycholate.

sent a toxicity index that varies between 0 and ∞ in the case of Δ_N, and between 0 and 1, in the case of α_N. Clearly I_{tox}, measured as Δ_N or α_N, is only a conventional index, the significance of which is as follows: $\Delta_N = 0$ or $\alpha_N = 0$, corresponds to the lowest toxicity value, whereas $\Delta_N = \infty$ or $\alpha_N = 1$ corresponds to the highest toxicity value. The numerical values of Δ_N and α_N are of course generally well correlated. In fact, whether it is Δ_N or α_N that is being measured, using the method described (see Fig. 2) the results are in satisfactory mutual agreement, as demonstrated in a previous paper [9] and as can be seen from the correlation between Δ_N and α_N data obtained from the two above-mentioned standard cholanic acid solutions over the concentration range 1.0×10^{-5} M–0.25×10^{-4} M (Fig. 3(b) and Fig. 4(b)).

The main analytical results obtained by applying the method to the determination of the sodium salts of different cholanic acids, both conjugated and free, are summarised in Table 1 and Table 2 respectively.

From the analytical point of view, by observing data for the straight-line equations in Tables 1

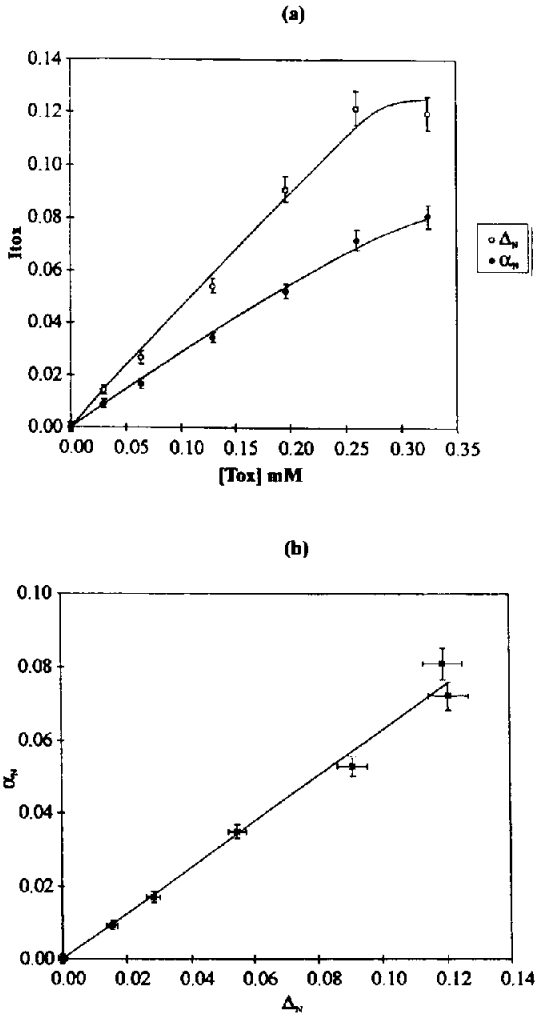

Fig. 4. (a) Plot of toxicity index (calculated as α_N or Δ_N) as a function of the toxic (sodium glycodeoxycholate) concentration, obtained using the biosensor. (b) correlation between α_N and Δ_N values for sodium glycodeoxycholate.

Table 1
Main analytical data for several free cholates obtained using the biosensor

Tested compound (as sodium salt)	Linearity range (mM)	Equation of the straight line (y = a.u.; x = mM)	Precision (%RSD)	Lower detection limit (mM)
The toxicity index I_{tox} (expressed as α_N) as a function of cholate concentration				
Cholate	0.03–0.25	$y = 0.322x + 0.004$	5.0	0.03
Deoxycholate	0.01–0.10	$y = 0.879x + 0.006$	6.3	0.005
Chenodeoxycholate	0.02–0.15	$y = 0.736x - 0.006$	5.8	0.01
Ursodeoxycholate	0.03–0.20	$y = 0.443x - 0.002$	4.5	0.01
The toxicity index I_{tox} (expressed as Δ_N) as a function of cholate concentration				
Cholate	0.03–0.25	$y = 0.256x + 0.100$	5.0	0.03
Deoxycholate	0.01–0.25	$y = 0.963x + 0.105$	6.3	0.005
Chenodeoxycholate	0.02–0.20	$y = 0.818x - 0.050$	5.8	0.01
Ursodeoxycholate	0.03–0.20	$y = 0.346x + 0.003$	4.5	0.01

and 2, the linearity range is seen to be comparable for both free and conjugated cholanic acids; this range spans, in fact, at least one decade (from about 2×10^{-5} M–2×10^{-4} M). For all the measurements performed, the precision, expressed as RSD, was found to be 4.1–6.3% regardless of the compound tested for toxicity. The lower detection limit was found to be of the same order (10^{-5} M) for all the glycocholates tested, as well as for chenodeoxycholate and ursodeoxycholate; it was of the order of 3×10^{-5} M and about 5×10^{-5} M for cholate and deoxycholate respectively.

Analysis of Table 1 shows that for the four free cholanic acids, the system appears to be most sensitive (see the slope values of the calibration curves in respect of "toxicity degree") to deoxycholate, followed by chenodeoxycholate, and ursodeoxycholate; it is least sensitive to cholate. In the case of conjugated cholates (Table 2), the "toxicity degree" is higher for glycodeoxycholate, followed by glycochenodeoxycholate and glycocholate.

Table 3 shows a comparison of two series of conjugated and free cholanic acids arranged in order on the basis of the arbitrary "toxicity degree", evaluated as described above. The same Table also contains a similar series for the same compounds obtained using both classical methods [5,6] of in vitro toxicity measurement and that developed by Roda et al. [4] using a toxicity biosensor based on luminescent marine bacteria. It is significant to note how the series obtained in the present work are in perfect agreement with the corresponding series obtined by other researchers

Table 2
Main analytical data for several conjugated cholates obtained using the biosensor

Tested compound (as sodium salt)	Linearity range (mM)	Equation of the straight line (y = a.u.; x = mM)	Precision (%RSD)	Lower detection limit (mM)
The toxicity index I_{tox} (expressed as α_N) as a function of cholate concentration				
Glycocholate	0.03–0.40	$y = 0.153x - 0.001$	5.2	0.01
Glycodeoxycholate	0.03–0.25	$y = 0.272x + 0.001$	5.2	0.01
Glycochenodeoxycholate	0.03–0.30	$y = 0.180x - 0.005$	4.1	0.01
The toxicity index I_{tox} (expressed as Δ_N) as a function of cholate concentration				
Glycocholate	0.03–0.40	$y = 0.215x + 0.001$	5.2	0.01
Glycodeoxycholate	0.03–0.25	$y = 0.457x + 0.001$	5.2	0.01
Glycochenodeoxycholate	0.03–0.30	$y = 0.395x + 0.008$	4.1	0.01

Table 3
"Toxicity degree" (as the slope value of the straight-line equation), set in decreasing order, for two series of conjugated and free cholanic acids as the sodium salts. Comparison[a] of orders obtained using the biosensor and other methods

Reference	Toxicity order for free cholanic acid sodium salts[b]	Toxicity order for conjugated cholanic acid sodium salts[b]	Measurement method
This paper	DCA > CDCA > UDCA > CA	GDCA > GCDCA > GCA	Determination of the inhibition of *Saccharomyces cerevisiae* cells, after 8 min incubation with the cholate under test
4	DCA > CDCA > UDCA > CA		Determination of the light emitted by sea bacteria after 30 s incubation with the choloate under test
5	DCA > CDCA > UDCA > CA	GCDCA > GCA	Determination of the extent of enzyme release and increase of the absorbance at $\lambda = 280$ nm
6		GDCA > GCA	Determination of the rate of release of the membrane-bound and cytosolic enzymes into the incubation media

[a] This paper shows that free cholates are more toxic than their glycoconjugated forms; similarly Ohta et al. [5] showed that free cholates are more toxic than their glyco- or tauro-conjugated forms.
[b] Abbreviations: DCA, deoxycholic acid; CDCA, Chenodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, Cholic acid; GDCA, Glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GCA, glyocholic acid.

using different methods. In addition, the present results confirm the observation [5] that free cholates are more toxic than their conjugates (e.g. glyconconjugates).

5. Conclusions

At present, the proposed method must be considered as an "up front" test to evaluate the toxicity of a compound and not as an alternative to classical methods such as those based on the extent of enzyme release [6] and the measurement of the absorbance increase at $\lambda = 280$ nm [5], which thus retain their validity as confirmatory and quantitative methods.

Although the "toxicity degree" is expressed in arbitrary terms as well as the toxicity index (I_{tox}), the relative toxicity scales found are in agreement with similar scales reported for the same compounds using both classical and more recently published methods.

In the present authors' opinion, the interest of the proposed method lies in the low cost of the required equipment and in the simplicity and rapidity of measurements. Comparison of the total time of measurement using the proposed system and that using methods based on the extent of enzyme release [6] or on the measurement of the absorbance increase [5], shows that all are of the same order; however, the growth of the *Saccharomyces cerevisiae* cells is faster and easier [2,9] than the preparation of hepatocytes from rats, which is also more complex and requires expert operators [5].

The results confirm the reliability of a comparatively rapid and simple test that rapidly provides toxicity scales for compounds of pharmaceutical interest using a colony of immobilised cells.

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