Amperometric Screen-Printed Galactose Biosensor for Cell Toxicity Applications

Prosper Kanyong, Gareth Hughes, Roy M. Pemberton, Simon K. Jackson and John P. Hart

Center for Research in Biosciences, Faculty of Health and Life Sciences, University of the West of England, Bristol, United Kingdom

ABSTRACT

This paper reports the development and application of a biosensor for the amperometric determination of galactose in the presence of human hepatocellular carcinoma cells with and without a hepatotoxic agent. The biosensor was fabricated by drop-coating 1.5% cellulose acetate on a 3 x 3 mm screen-printed carbon electrode followed by depositing 2 U of galactose oxidase. The electrodes dimensions were reduced to 3 x 0.5 mm before measurements. Hepatocellular carcinoma cells were utilized for in vitro toxicity testing by evaluating the effect of paracetamol on galactose uptake. The amperometric responses to galactose indicated that the inhibition of uptake was directly proportional to the concentration of paracetamol following 24h of exposure to the hepatocellular carcinoma cells. These results demonstrate that the fabricated biosensor may be used for the real-time monitoring of cell metabolism and toxicity.

ARTICLE HISTORY

Received 19 May 2015
Accepted 02 July 2015

KEYWORDS

Amperometry; biosensor; screen-printed electrode

Introduction

There is considerable interest in using cell-based assays, that is, using live cells, for screening the toxicity of chemical compounds, particularly in the pharmaceutical industry. A variety of techniques are employed for measuring cell proliferation, morphology, viability, cytotoxicity, and motility (Keese and Giaever 1994; Nabhan 2003; Riss, O’Brien, and Morvec 2003). Whereas cell viability assays generally involve measuring the number of live cells, cytotoxicity assays tend to evaluate the number of dead cells. Both types of cell-based assays are routinely used for drug discovery using high-throughput screening, environmental assessment of relevant chemical compounds, and as biosensors for cellular behavior analysis (Ehret et al. 1997; White 2000; Shuileabhain et al. 2004). In 2003, Nabhan discovered that over 30% of prospective candidate drugs fail toxic verification. Consequently, cytotoxicity testing in cell types has become one of the fundamental tools for drug discovery (White 2000; Riss, O’Brien, and Morvec 2003). Hepatotoxicity is a major concern in testing as most toxic effects observed are by pharmaceuticals that are metabolized by the liver; thus liver cells have been utilized by many drug discovery and development laboratories (Anderson et al. 1996; A. P. Li et al. 1999; Ni et al. 2001; Valentin et al. 2001).
Cultured hepatocytes are routinely used to evaluate the toxicity of chemical compounds (Groneberg, Grosse-Siestrup, and Fischer 2002; Gomez-Lechon, Castell, and Donato 2007; Horii and Yamada 2007). Hepatocellular carcinoma cells have been used to screen complex mixtures, in addition to screening individual chemical compounds for potential hepatotoxic effects. Assays using these cells have been used successfully to provide data for determining potential mechanisms of liver toxicity (Kelly and Sussman 2000; Flynn and Ferguson 2008; Z. Li and Chan 2009; Prot et al. 2011).

Hepatotoxicity screening methods include the adenosine triphosphate assay, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay, and the neutral red dye uptake assay (A. P. Li et al. 1999; Ni et al. 2001; Valentin et al. 2001; Groneberg, Grosse-Siestrup, and Fischer 2002). However, nearly all of such traditional biochemical techniques are laborious, time consuming, and involve complex procedures with multiple reagents. For in vitro experiments, these techniques are incapable of providing quantitative data without affecting the target cells. This makes it extraordinarily difficult to monitor continuous toxic effects or to analyze real-time changes in cell viability. Consequently, numerous research studies have focused on developing alternative methods that allow for continuous monitoring of toxic effects (Wegener, Janshoff, and Galla 1998; Wolf et al. 1998; Wegener et al. 1999; Sohn et al. 2000; Phelps and Depaola 2000; Wegener, Keese, and Giaever 2000; Xiao et al. 2002; Xiao and Luong 2003).

An alternative electrochemical approach, based on amperometric measurements using fabricated electrodes, may provide the ideal method for detecting hepatotoxicity with multiple reagents. The possibility of obtaining useful metabolic data from cell-based assays in conjunction with amperometry has been recently investigated by our group (Pemberton et al. 2009, 2010). In these studies, electrochemical microband biosensors incorporating a relevant enzyme were used to continuously and selectively monitor glucose or lactate. The biosensors maintained steady-state current responses in quiescent solution and operated in culture medium for batch-type analysis to monitor the depletion of glucose by hepatocytes in a cell density-dependent manner (Pemberton et al. 2011). The ability to sustain amperometric current responses over several hours provides the possibility of utilizing the biosensors for real-time toxicity monitoring.

Numerous methods for the fabrication of electrodes have been developed and led to the manufacture of miniature, reproducible, and inexpensive devices. Screen-printing has been widely employed (Wring and Hart 1992) to manufacture electrodes for developing biosensors and is simple, rapid, low-cost, and allows for the preparation of various electrode geometries (Wring and Hart 1992). In the present paper, galactose biosensors were prepared using a water-based carbon ink formulation. The formulation and the first part of the procedure have been described previously for the fabrication of macroelectrode galactose biosensors for use in serum analysis (Kanyong et al. 2013). The present paper describes a modified procedure for the fabrication of microband galactose biosensors. The ability to obtain steady-state responses in quiescent phosphate buffer was investigated using these biosensors. The operation of the galactose biosensor was also investigated in culture medium. Further studies, utilizing the galactose biosensor, were conducted to detect the uptake of galactose by the mammalian hepatocellular carcinoma liver cell line at 37°C, over a period of 24 h. The effect of paracetamol, a model hepatotoxic compound, on galactose uptake by hepatocellular carcinoma cells was investigated. The results of this study and potential application in toxicity monitoring are discussed.
Materials and methods

Chemical and reagents

All chemicals including acetone, sodium chloride, high-glucose (4.5 g/L)-supplemented Dulbecco’s Modified Eagle’s medium (DMEM, D5796-500ML), penicillin, and streptomycin sulfate were of analytical grade, purchased from Sigma Aldrich, Dorset, UK. The 50 mM phosphate buffer was prepared by combining appropriate amounts of trisodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dehydrate, and disodium hydrogen orthophosphate dehydrate to yield the desired pH. Cellulose acetate, D- (+)-galactose, and galactose oxidase were obtained from Sigma Aldrich, Dorset, UK. Fetal bovine serum was purchased from Biosera, East Sussex, UK and stored in small aliquots at -22°C. White gloss finish paint and brush were purchased from B&Q, Avon, UK. Cellulose acetate was dissolved in acetone following up to 2 min sonication.

Hepatocellular carcinoma cell culture

The hepatoma cell line, HepG2, (obtained from the European Collection of Animal Cell Cultures) was cultured as a monolayer in 75 cm² flasks in a 5% CO₂-in-air atmosphere at 37°C with an initial density of 10⁵ cells/mL in 15 mL of medium. The medium was high-glucose Dulbecco’s Modified Eagle’s medium containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (pen + strep). When the cells were confluent, known concentrations of galactose and paracetamol were added to the flasks, which were incubated for an additional 24 h.

Apparatus and measurements

All electrochemical measurements were conducted with a three-electrode system, consisting of a cobalt phthalocyanine screen printed working electrode (GEM code: C40511D8), Ag/AgCl reference electrode (GEM Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK), and a separate Pt wire counter electrode. The area of the working electrode was defined using insulating tape (RS, Northants, UK) to define a 3 × 3 mm square area.

The working and reference electrodes were connected to the potentiostat with gold clips. An Autolab Electrochemical Analyzer with General-Purpose Electrochemical Software (Version 4.9, The Netherlands) was used for the acquisition and control, with a 10 mL cell inside a water jacket for electrochemical measurements. Data were further analyzed with Microsoft Excel. Weighing was carried out with a Precisa precision balance 262SMA-FR purchased from Milton Keynes, Buckinghamshire, UK. Measurement and monitoring of pH was conducted with a Fisherbrand Hydrus 400 pH Meter (Orion Research Inc., USA). Sonications were performed with a Devon FS100 sonicating water bath (Ultrasonics, Hove, Sussex, UK). The temperature was controlled with a thermostated water bath (Thermo Scientific HAAKE DC10-P5/U unit). The dimensions of the valox card were measured using a TESA micrometer obtained from Radio Spares, Switzerland.
**Biosensor preparation**

**Immobilization of reagents on the screen printed electrode**

The base unmodified cobalt phthalocyanine screen-printed electrode transducer was prepared using a water-based ink and the sensors were screen-printed in groups of six on valox as previously described by Crouch et al. (2005). The cobalt phthalocyanine screen printed electrode was modified by drop-coating 5 μL of 1% cellulose acetate in acetone directly onto the exposed 9.0 mm² working electrode and allowed to dry. The electrodes were then coated with 5 μL of galactose oxidase containing the appropriate number of units and left to air-dry. Dummy biosensors were prepared using the same procedure, except that the enzyme was substituted for the same mass of bovine serum albumin (BSA), as described previously (Kanyong et al. 2013).

**Microband working electrode area**

After drying, the side of a 18 × 500 μm valox card was carefully vertically fixed on the working area of the electrode with an adhesive. White gloss finish paint was then drop-coated on the working surface to cover the enzyme layer to each side of the vertically fixed valox card and allowed to dry. Once dry, the valox card was carefully removed to obtain a 3 mm × 500 μm working electrode area. A schematic of the final biosensor is shown in Figure 1. The galactose biosensors were stored at 4°C in a vacuum desiccator containing silica gel until use. The same procedure was used to obtain dummy biosensors, by using the same mass of bovine serum albumin as that of the enzyme; any responses resulting from these devices were subtracted from the biosensor response.

**Calibration by amperometry**

Calibration plots for the galactose biosensor were obtained by immersing the electrode in 10 mL of 50 mM phosphate buffer containing 50 mM NaCl or 10 mL Dulbecco’s Modified Eagle’s medium for subsequent measurements. A potential of +0.5 V vs. Ag/AgCl was applied to the working electrode and the system was allowed for the current response to stabilize. Small additions of galactose standards were made using a micropipette, allowing for stabilization between additions. Calibration plots were constructed by plotting current response versus the cell galactose concentration. The mean responses of three replicates were calculated.

**Evaluation of the toxicity of paracetamol on galactose uptake by hepatocellular carcinoma cells**

The 10 mM galactose and 5 μM, 50 μM, 500 μM, or 1000 μM concentrations of paracetamol were incubated with 15 mL of Dulbecco’s Modified Eagle’s medium containing hepatocellular

![Figure 1. Schematic of the galactose biosensor.](image-url)
carcinoma cells for 24 h. Initially each culture medium contained 10 mM galactose. After the 24-h incubation period, the current responses due to remaining galactose were measured by amperometry using the galactose and dummy biosensors in quiescent solution. Any responses resulting from the dummy electrode devices were subtracted from the biosensor response. Consequently, the uptake of galactose following the 24-h incubation period was estimated. The current responses obtained at 8,000 s were used to describe the uptake of galactose by the cells as well as the inhibition of galactose uptake by paracetamol.

**Results and discussion**

**Reaction sequences for the galactose biosensor**

The response for galactose occurs as a result of the enzymatic and electrocatalytic processes. Hydrogen peroxide is generated during the enzymatic conversion of galactose to D-galacto-hexodialdose in the presence of galactose oxidase and oxygen. Hydrogen peroxide was oxidized at the cobalt phthalocyanine screen printed electrode, which constitutes the analytical signal. This response is directly proportional to the original concentration of galactose.

**Calibration**

The effects of enzyme loading (1.2 to 3 U), temperature (25 to 40 °C), pH (5.0 to 10.0), and ionic buffer strength (0.025 to 0.45 mM) on the analytical performance of the biosensor have previously been published (Kanyong et al. 2013). The optimal biosensor performance occurred with a loading of 2 U of galactose oxidase at 35°C with 50 mM phosphate buffer (pH 7.0). Calibration studies were performed with galactose from 1.98 to 9.52 mM in phosphate buffer under quiescent conditions. A fresh biosensor was used with each measurement which was performed in triplicate for each concentration.

Figure 2 shows that each galactose addition elicited a rapid current response. A calibration study was conducted from 1.98 to 18.18 mM; the linear dynamic range was between 1.98 and 9.52 mM. The limit of detection based on a signal-to-noise ratio of three was 0.2 mM. The biosensor response exhibited a sensitivity of 7.267 μA mM⁻¹ cm⁻² with a linear range up to 9.52 mM and a precision based on the coefficient of variation (CV) for replicate biosensors of 1.3% in buffer. These performance characteristics demonstrate that the galactose biosensor is able to give concentration dependent, steady-state current responses.

Consequently, the biosensor performance was evaluated in Dulbecco’s Modified Eagle’s medium. The biosensor exhibited a sensitivity of 3.067 μA mM⁻¹ cm⁻² with a linear dynamic range up to 9.52 mM and a coefficient of variation for replicate biosensors of 1.2%. Clearly, the sensitivity of the galactose biosensor in culture medium was reduced by approximately 50% compared to in buffer. However, the performance in culture medium was considered to be satisfactory for end-point measurements of galactose in culture medium.

**Toxicity of paracetamol on galactose uptake by hepatocellular carcinoma cells**

The biosensor’s ability to determine galactose uptake in cell culture was examined using supernatants from hepatocellular carcinoma cells. The cells were incubated in 15 mL of culture media containing 10 mM galactose with various concentrations of paracetamol
for 24 h. It is well known that cells use glucose as their primary source of energy. It is also worth noting that the depletion of glucose by healthy cells occurs most rapidly during the first 4 h of growth and continues up to approximately 10 h (Pemberton et al. 2011). In the absence of glucose, it is expected that the cells would begin to deplete the 10 mM galactose that was added to the culture medium. Figure 3 shows the complete galactose uptake by the cellular system.
hepatocellular carcinoma cells in the absence of paracetamol. The current of 90 nA for 10 mM galactose was reduced to baseline levels after the 24-h period, indicating that all galactose was consumed by the cells. An estimate of galactose consumption over 24 h, based on initial cell numbers present, indicates uptake of 150 micromoles by $1.5 \times 10^6$ cells, or 100 pmol of galactose per cell. In the presence of paracetamol, galactose current responses were reduced, indicating the inhibition of cell uptake. Paracetamol toxicity appeared to be dose-dependent as shown in Figure 3. In the presence of paracetamol at 1000, 500, 50, and 5 μM, galactose uptake was inhibited by 99, 60, 20, and 12%, respectively. Similar inhibition of glucose uptake was observed in the presence of 1000 μM paracetamol by Pemberton et al. (2011) using a glucose biosensor.

Conclusions

An amperometric galactose biosensor was developed and characterized in phosphate buffer and culture medium. In the latter, the galactose biosensor provided a sensitivity of 7.2 μA mM$^{-1}$ cm$^{-2}$, a linear range up to 9.52 mM, and a coefficient of variation of 1.2%. This performance was considered satisfactory in evaluating the effect of paracetamol, a model hepatotoxic compound, on galactose uptake by hepatocellular carcinoma cells. Paracetamol toxicity appeared to be dose-dependent and these findings correlated with inhibition profiles observed by Xu, Ma, and Purcell (2003) for glucose uptake in the presence of 1000 μM paracetamol. These results suggest that the amperometric galactose biosensor may be used for real-time monitoring of galactose metabolism. In the future, an array system incorporating screen-printed microelectrode biosensors for a variety of biomarkers may be employed for mammalian cell toxicity testing. The latter has the additional potential for the replacement or reduction of animal testing.

Acknowledgment

Gwent Electronic Materials are thanked for supplying the screen printed electrodes.

Funding

The authors thank the University of the West of England, Bristol for funding.

References

Anderson, K., L. Yin, C. Macdonald, and M. H. Grant. 1996. Immortalized hepatocytes as in vitro model systems for toxicity: The comparative toxicity of menadione in immortalized cells, primary cultures of hepatocytes and HTC hepatoma cells. *Toxicology in Vitro* 10: 721–27. doi:10.1016/s0887-2333(96)00059-8

Crouch, E., D. C. Cowell, S. Hoskins, W. P. Pittson, and J. P. Hart. 2005. A novel, disposable, screen-printed amperometric biosensor for glucose in serum fabricated using a water-based carbon ink. *Biosensors and Bioelectronics* 21: 712–18. doi:10.1016/j.bios.2005.01.003

Ehret, R., W. Baumann, M. Brischwein, A. Schwinde, K. Stegbaauer, and B. Wolf. 1997. Monitoring of cellular behavior by impedance measurements on interdigitated electrode structures. *Biosensors and Bioelectronics* 12: 29–41. doi:10.1016/0956-5663(96)89087-7

Flynn, T. J., and M. S. Ferguson. 2008. Multiendpoint mechanistic profiling of hepatotoxicants in HepG2/C3A human hepatoma cells and novel statistical approaches for development of a prediction model for acute hepatotoxicity. *Toxicology in Vitro* 22: 1618–31. doi:10.1016/j.tiv.2008.04.016
Groneberg, D. A., C. Grosse-Siestrup, and A. Fischer. 2002. In vitro models to study hepatotoxicity. *Toxicologic Pathology* 30: 394–99. doi:10.1080/01926230202929972

Gomez-Lechon, M. J., J. V. Castell, and M. T. Donato. 2007. Hepatocytes—the choice to investigate drug metabolism and toxicity in man: In vitro variability as a reflection of in vivo. *Chemico-Biological Interactions* 168: 30–50. doi:10.1016/j.cbi.2006.10.013

Horii, I., and H. Yamada. 2007. In vitro hepatotoxicity testing in the early phase of drug discovery. *Alternatives to Animal Testing and Experimentation* 14: 437–41.

Kanyong, P., R. M. Pemberton, S. K. Jackson, and J. P. Hart. 2013. Development of an amperometric screen-printed galactose biosensor for serum analysis. *Analytical Biochemistry* 435: 114–19. doi:10.1016/j.ab.2013.01.006

Keese, C. R., and I. Giaever. 1994. A biosensor that monitors cell morphology with electrical fields. *IEEE Engineering in Medicine and Biology Magazine* 13: 402–08. doi:10.1109/51.294012

Kelly, J. H., and N. L. Sussman. 2000. A fluorescent cell-based assay for cytochrome P-450 isozyme 1A2 induction and inhibition. *Journal of Biomolecular Screening* 5: 249–54. doi:10.1089/108705700416119

Li, A. P., C. Lu, J. A. Brent, C. Pham, A. Fackett, C. E. Ruegg, and P. M. Siber. 1999. Cryopreserved human hepatocytes; characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chemico-Biological Interactions* 121: 17–35. doi:10.1016/s0009-2797(99)00088-5

Li, Z., and C. Chan. 2009. Systems biology for identifying liver cytotoxicity pathways. *BMC Proceedings* 3 (Suppl 2):S2. doi:10.1186/1753-6561-3-s2-s2

Nabhan, J. D. A. 2003. Cell-based assays; commercial opportunities, legal trends and technology analyses. D & MD Report #9116, Wellesley, Massachusetts: D & MD Publication.

Ni, R., M. A. Leo, J. Zhao, and C. S. Lieber. 2001. Toxicity of β-carotene and its exacerbation by acetyldehyde in HepG2 cells. *Alcohol and Alcoholism* 36: 281–85. doi:10.1093/alcalc/36.4.281

Pemberton, R. M., F. J. Rawson, J. Xu, R. Pittson, G. A. Drago, J. Griffiths, S. K. Jackson, and J. P. Hart. 2010. Application of screen-printed microband biosensors incorporated with cells to monitor metabolic effects of potential environmental toxins. *Microchimica Acta* 170: 321–30. doi:10.1007/s00604-010-0326-0

Pemberton, R. M., J. Xu, R. Pittson, N. Biddle, G. A. Drago, J. Griffiths, S. K. Jackson, and J. P. Hart. 2009. Application of screen-printed microband biosensors to end-point measurements of glucose and cell numbers in HepG2 cell culture. *Analytical Biochemistry* 285: 334–41. doi:10.1016/j.ab.2008.10.037

Pemberton, R. M., J. Xu, R. Pittson, G. A. Drago, J. Griffiths, S. K. Jackson, and J. P. Hart. 2011. A screen-printed microband glucose biosensor system for real-time monitoring of toxicity in cell culture. *Biosensors and Bioelectronics* 26: 2448–53. doi:10.1016/j.bios.2010.10.030

Prot, J. M., C. Aninat, L. Griscom, F. Razan, C. Brochot, C. G. Guillouzo, C. Legallais, A. Corlu, and E. Leclerc. 2011. Improvement of HepG2/C3a cell functions in a microfluidic biochip. *Biotechnology and Bioengineering* 108: 1704–15. doi:10.1002/bit.23104

Phelps, J. E., and N. Depaola. 2000. Spatial variations in endothelial barrier function in disturbed flows in vitro. *American Journal of Physiology. Heart and Circulatory Physiology* 278: 469–76.

Riss, T., M. O’Brien, and R. Morvec. 2003. Choosing the right cell-based assay for your research. *Cell Notes* 6: 6–12.

Shuileabhain, S. N., C. Mothersill, D. Sheehan, N. M. O’Brien, J. O’Halloran, F. N. A. M. Van Pelt, and M. Davoren. 2004. In vitro cytotoxicity testing of three zinc metal salts using established fish cell lines. *Toxicology in Vitro* 18: 365–76. doi:10.1016/j.tiv.2003.10.006

Sohn, L. L., O. A. Saleh, G. R. Facer, A. J. Beavis, R. S. Allan, and D. A. Notterman. 2000. Capacitance cytometry: Measuring biological cells one by one. *Proceedings of the National Academy of Sciences of the United States of America* 97: 1687–90. doi:10.1073/pnas.200361297

Valentin, I., M. Phillippe, J.-C. Lhuguenot, and M.-C. Chagnon. 2001. Uridine uptake inhibition as a cytotoxicity test for a human hepatoma cell line (HepG2 cells): Comparison with the neural red assay. *Toxicology* 158: 127–39. doi:10.1016/s0300-483x(00)00372-3

Wegener, J., A. Janshoff, and H.-J. Galla. 1998. Cell adhesion monitoring using a quartz crystal microbalance: Comparative analysis of different mammalian cell lines. *European Biophysics Journal* 28: 26–37. doi:10.1007/s002490050180
Wegener, J., C. R. Keese, and I. Giaever. 2000. Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. *Experimental Cell Research* 259: 158–66. doi:10.1006/excr.2000.4919

Wegener, J., S. Zink, P. Rosen, and H. J. Galla. 1999. Use of electrochemical impedance measurements to monitor β-adrenergic stimulation of bovine aortic endothelial cells. *Pflügers Archiv: European Journal of Physiology* 437: 925–34. doi:10.1007/s004240050864

White, R. E. 2000. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. *Annual Review of Pharmacology and Toxicology* 40: 133–57. doi:10.1146/annurev.pharmtox.40.1.133

Wolf, B., M. Brischwein, W. Baumann, R. Ehret, and M. Kraus. 1998. Monitoring of cellular signaling and metabolism with modular sensor-technique: The physiocontrol-microsystem (PCM). *Biosensors and Bioelectronics* 13: 501–09. doi:10.1016/s0956-5663(97)00136-x

Wring, S. A., and J. P. Hart. 1992. Chemically modified screen-printed carbon electrodes. *Analyst* 117: 1281–86. doi:10.1039/an9921701281

Xiao, C., B. Lachance, G. Sunahara, and J. H. T. Luong. 2002. Assessment of cytotoxicity using electric cell-substrate impedance sensing: Concentrations and time response function approach. *Analytical Chemistry* 74: 5748–53. doi:10.1021/ac025848f

Xiao, C., and J. H. T. Luong. 2003. On-line monitoring of cell growth and cytotoxicity using electric cell-substrate impedance sensing (ECIS). *Biotechnology Progress* 19: 1000–05. doi:10.1021/bp025733x

Xu, J., M. Ma, and W. M. Purcell. 2003. Characterization of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatotoxicity studies. I. Glucose metabolism and enzyme release as cytotoxic markers. *Toxicology and Applied Pharmacology* 189: 100–11. doi:10.1016/s0041-008x(03)00089-9