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Microdialysis-perfusion sampling for the investigation of phenol metabolism

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Abstract: *In vivo* methods provide several advantages for the study of metabolism relative to the commonly used *in vitro* techniques. The integrity of the organism and actual physiological conditions are maintained to reflect more accurately the processes occurring on exposure to a xenobiotic compound. Experimental precision is improved because each animal serves as its own control and can be used to generate a complete pharmacokinetic experiment. This may result in the added benefit that fewer experimental animals will be needed for a metabolic investigation using *in vivo* techniques. The technique of microdialysis perfusion was characterized for the *in vivo* study of the hepatic metabolism of phenol and conjugation by glutathione. In this study, *in vivo* experiments were conducted by implanting a microdialysis probe into the intact, in-place liver of a killed rat. These results were compared to *in vitro* experiments using liver homogenate and liver-microsomal protein. Substantial differences were observed between the *in situ* experiments and those performed *in vitro*.

Keywords: *Microdialysis perfusion; LC with electrochemical detection; phenol metabolism; cytochrome P-450; glutathione.*

Introduction

Established methods for measuring xenobiotic metabolism typically depend on either collection of products in biological fluids and excretions or use of *in vitro* tissue preparations [1]. Determining serum, urine and fecal metabolites provides a view of overall metabolic profile in the organism but little insight into the individual steps leading to the end products. *In vitro* tissue incubations can provide information about early metabolic steps but can be perturbed by the necessity of artificially providing components of the system lost during sample preparation. In many cases, compartmentation of the cells is also lost during sample preparation, leading to systems having little resemblance to the *in vivo* case. In addition, no insight into interaction between organs can be obtained from experiments on isolated organs.

A promising technique for the *in vivo* study of hepatic metabolism involves microdialysis perfusion. This technique has found great utility for the *in vivo* study of neurotransmitters [2–5]. Microdialysis perfusion is performed by implanting a small dialysis probe into the experimental animal. This probe consists of a length of

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microdialysis tubing through which solution is slowly pumped. Small molecules diffuse into the probe because of concentration differences between the extracellular fluid and the perfusion medium. The perfusion medium is collected and analysed to determine what compounds were present in the extracellular fluid during dialysis perfusion. The size of molecules sampled is determined by the molecular weight cutoff of the dialysis tubing used in the probe. Alternatively, by incorporating test (substrate) compounds in the perfusion medium, these compounds can be delivered to the test organ in a very controlled manner. In this regard the dialysis probe acts in a manner analogous to a blood vessel, that is substrate is delivered and metabolites are collected.

This report describes the application of microdialysis perfusion to the study of the hepatic metabolism of phenol. The microdialysis sampling technique is coupled to chromatographic analysis with both electrochemical and UV absorbance detection. Liquid chromatography with electrochemical detection (LCEC) has been used previously to study the hepatic metabolism of phenol [6, 7]. LCEC has also been used to study the phase II metabolic process of conjugation with glutathione [8]. In this paper the characteristics of microdialysis perfusion which are important to metabolic studies are discussed. Comparison of investigations of the metabolism of phenol in an intact, in-place liver by microdialysis to *in vitro* studies are also described.

Experimental

Materials

HPLC grade sodium octyl sulphate was obtained from Eastman Kodak (Rochester, NY). Glutathione, glutathione disulphide, hydroquinone, phenol and NADPH were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade or better and were used as received.

Microdialysis apparatus

Microdialysis perfusion was performed using a CMA/100 microinjection pump from Bioanalytical Systems, Inc./Carnegie Medicin (West Lafayette, IN). Perfusion was through a small microdialysis probe which was implanted in the tissue of interest. Perfusion flow rates of 2–20 μl min^{-1} were used as listed in the text. The microdialysis probes were constructed in-house. Samples were collected in 1.5-ml microcentrifuge tubes and injected into the chromatographic system directly.

The microdialysis probes were constructed using the "loop" design [9]. The dialysis tubing was purchased from Spectrum Medical Industries (Los Angeles, CA). The tubing was regenerated cellulose with an inner diameter of 150 μm, a wall thickness of 9 μm, and a molecular weight cutoff of 9000. The ends of a short piece of dialysis tubing were inserted into 26 gauge stainless steel cannulas and sealed with "hot melt" glue (Fig. 1). The size of the probe could be varied by using different lengths of dialysis tubing. In these experiments, the length of the exposed tubing was approximately 0.5 cm. The type of sealant between the dialysis tubing and stainless steel tubing was found to be critical. Acrylate based adhesives entered the stainless steel tubing through capillary action and sealed the probe. Silicon based sealants did not provide leakproof seals. Probes which did not leak could be made using polyamide epoxy. However, it was found that an electroactive compound with the same retention time as hydroquinone was leached from the epoxy. This contaminant was significantly diminished by curing at 100°C for 24 h, but could not be completely eliminated. "Hot melt" glue (Ridlen/AAI Adhesives, Dallas,

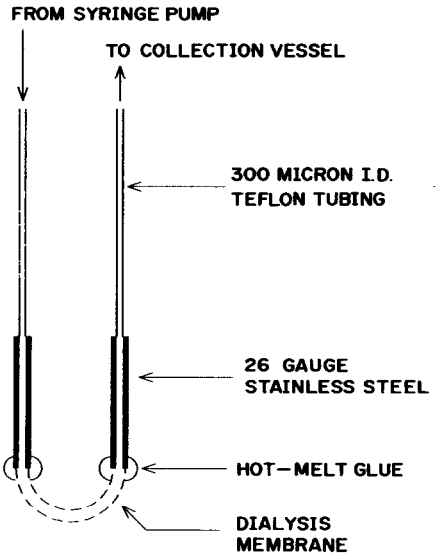

Figure 1
Diagram of the microdialysis probe.

TX), a wax-based adhesive, was found to be simple to use and provided excellent probes. Probes could be constructed in approximately 15 min with excellent mechanical stability and no leaking problems. No electroactive or UV-absorbing contaminants have been observed from the "hot melt" glue.

Prior to use, the dialysis tubing was activated by pretreatment with 0.1 M NaOH. The tubing was primed with a solution of isopropyl alcohol and 20% (v/v) glycerol for 5 min. The tubing was then hydrolyzed for 2 min with 0.1 M methanolic NaOH. A neutralizing solution containing 1% (v/v) acetic acid and 20% (v/v) glycerol in isopropyl alcohol was pumped through the probe for 5 min. The probe was finally perfused with water for 30 min. This pretreatment procedure increased the recovery of phenol by a factor of three compared with untreated dialysis tubing.

Chromatographic system

The liquid chromatographic system was a BAS LC-400 Chromatographic System equipped with a LC-4B dual-electrode amperometric detector from Bioanalytical Systems, Inc. (West Lafayette, IN) and a SPD-6AV variable wavelength UV–Vis absorbance detector from Shimadzu (Columbia, MD). A sample loop of 20 μl was used in all experiments. A Biophase 5 μm ODS (25 cm × 4.6 mm) column was used for the separation of phenol and hydroquinone. The mobile phase was ammonium phosphate (pH 2.5, 0.05 M) ≃ acetonitrile (90:10, v/v). Dual glassy carbon electrodes in the series configuration were used for detection of hydroquinone. The upstream electrode was operated at +0.7 V versus Ag/AgCl and the downstream electrode was operated at −0.2 V. Phenol was detected using the absorbance detector at 250 nm. A Hypersil 5 μm ODS (15 cm × 4.6 mm) column was used to separate glutathione and glutathione disulphide. A mobile phase of sodium phosphate (pH 3.0, 0.05 M) with octyl sodium sulphate (1.5 mM) ≃ methanol (97.5:2.5, v/v) was used. Dual Au/Hg amalgam electrodes in the series configuration were used for detection. The upstream electrode was operated at −1.0 V versus Ag/AgCl and the downstream electrode was operated at +0.15 V. A flow rate of 1.0 ml min^{-1} was used in all experiments.

Dialysis probe characterization

Recovery studies were performed by placing a dialysis probe in an external solution containing known amounts of phenol, hydroquinone, glutathione, or glutathione disulphide and determining the amount collected in the dialysate after perfusion. Delivery studies were performed by placing a dialysis probe in a blank external solution, perfusing with known concentrations of phenol and hydroquinone, and determining the amount of analyte remaining in the dialysate after perfusion. For both types of experiments the external solution and perfusate were both 0.1 M phosphate buffer at pH 7.2 and perfusion rates of 2, 5, 10, 15, and 20 μl min^{-1} were used. Analyte concentrations were selected to bracket the expected extracellular concentrations of the analytes in rat liver. Each probe was characterized prior to use in metabolism studies. In addition, the probe was recharacterized after the experiment to insure that no changes had occurred during experimentation.

Metabolism studies

Approximately one-month-old Sprague–Dawley rats were fasted, in a metabolism cage, for 24 h prior to use. For *in vitro* experiments, the rats were killed, their livers removed and homogenized in KCl–phosphate buffer, pH 7.2 (5 ml buffer per g liver). Microsomes were prepared by standard methods [10]. For metabolism studies, microsomes were reconstituted at 4 mg ml^{-1} in KCl–phosphate buffer, pH 7.2, containing 2 mM NADPH. Liver homogenate was used directly with no added NADPH.

For the whole liver experiments, rats were used immediately after being killed. A small incision was made to expose the liver. A small puncture was made in the liver and the microdialysis probe inserted. The liver was covered with a thin layer of phosphate buffer and a moist towel was placed over the incision to prevent drying of the tissue. Sampling was carried out using probes which had been previously characterized *in vitro*. In all experiments, buffer alone was perfused for at least 30 min or until steady-state levels were observed by chromatographic analysis of the perfusion sample. After steady-state levels were achieved, perfusion with a 10 mM phenol solution was begun and samples were collected at 10 min intervals. A perfusion rate of 5 μl min^{-1} was used in all experiments. The samples were analysed for metabolites by liquid chromatography using dual-electrode amperometric detection while UV absorbance detection was used to determine the amount of phenol being delivered to the liver.

Results and Discussion

Dialysis probe characterization

Recovery can be expressed as either the relative recovery, which refers to the concentration of analyte collected relative to the concentration in the sample, or as absolute recovery, which refers to the mass of analyte collected per unit time. At slow flow rates, equilibrium is reached between the external and internal solutions so that the concentration of the collected solution equals that in the sample giving a high relative recovery. However, at such low flow rates very little sample is collected per unit time and the absolute recovery is therefore low. As the flow rate is increased, equilibrium is no longer achieved and the relative recovery decreases. The absolute recovery, on the other hand, increases, reaches a maximum and then decreases. The maxima in the absolute recovery corresponds to the onset of osmotic flow out of the probe due to the high flow rate. This is illustrated for the recovery of hydroquinone in Fig. 2.

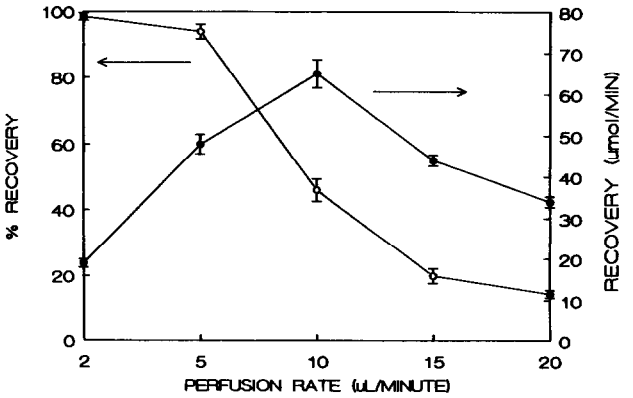

Figure 2
Absolute —●—● and relative —○—○ recovery of hydroquinone.

These results illustrate the interdependency of sample volume, collection time, and recovery. As most detectors for liquid chromatography are concentration sensitive, the higher the relative recovery the fewer demands placed on the detector. However, sufficient sample must be collected for analysis and the sampling time must be short enough to follow transient signals in the system. Using the chromatographic systems currently available in this laboratory, samples of at least 20 μl were needed. For these preliminary investigations, a perfusion rate of 5 μl min^{-1} was chosen as a compromise between recovery and sampling rate.

The recovery is also dependent upon the identity of the analyte. Figure 3 shows the recoveries of several compounds that are encountered in metabolism studies of phenol. As can be seen, the recoveries of glutathione and glutathione disulphide are much lower than the recoveries of hydroquinone and phenol. The size of the molecule alone cannot explain these results, because glutathione disulphide is approximately twice the size of glutathione, yet has a higher recovery. Recovery may also depend on the charge on the

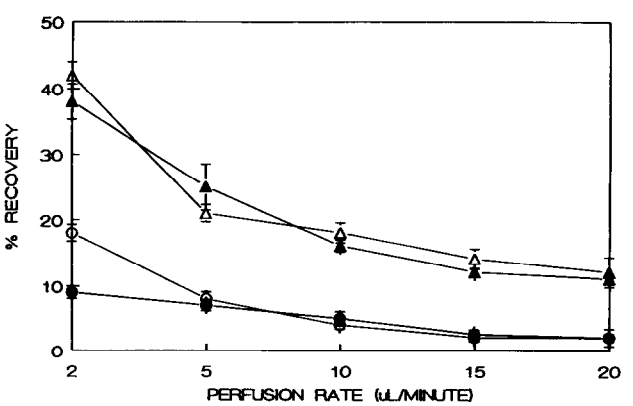

Figure 3
Dependence of recovery on perfusion rate. Symbols: —△—△— phenol (10 mM), —▲—▲— hydroquinone (10 mM), —○—○— glutathione disulphide (5 mM), —●—●— glutathione (5 mM).

molecule; glutathione and glutathione disulphide are both zwiterionic, while phenol and hydroquinone are neutral. This indicates that the nature of the dialysis membrane may play an important role in recovery and may be exploited to impart selectivity to the method. In these studies, the recoveries determined during the characterization procedure were used to calculate extracellular concentrations during the metabolism studies.

The relative delivery was determined as a function of the perfusion rate. The flow rate dependence of delivery was similar to the dependence of recovery on flow rate; that is: delivery increased as flow rate decreased (Fig. 4). Delivery can be determined during the actual metabolism experiment as opposed to recovery which must be based on pre- and post-experiment calibrations. Starting with a known concentration of the compound to be delivered in the perfusate, the amount remaining following perfusion can be determined directly and the difference is the amount that was delivered to the tissue.

Comparison of microdialysis in intact liver with in vitro *experiments*

The *in vitro* systems studied were microsomal incubations and liver homogenate incubations. Microdialysis experiments were performed in dead animals. The animal was killed prior to the experiment to simplify the experimental protocol while developing the microdialysis technique. Because the probe is to be placed in soft tissue, metabolism studies will have to employ anaesthetized animals. Previous microdialysis studies on freely moving animals have relied on the ability to fix the probe to the animal's skull, a luxury not afforded here. The use of anaesthetized animals for metabolism studies requires the use of an anaesthetic which will not interfere with the metabolism of the test compound. To date no suitable anaesthetic has yet been identified. These studies are therefore intended to evaluate microdialysis sampling as a technique to study metabolism *in vivo*. Having established the validity of microdialysis with these initial experiments, the issue of precise *in vivo* protocols will be addressed subsequently.

A 10-mM phenol solution was continuously perfused through the tissue sample at 5 μl min^{-1} during the course of the experiment. By determining the difference between the initial phenol concentration in the perfusion solution and the concentration remaining after perfusion, the rate of phenol delivery was calculated. The rate of delivery to

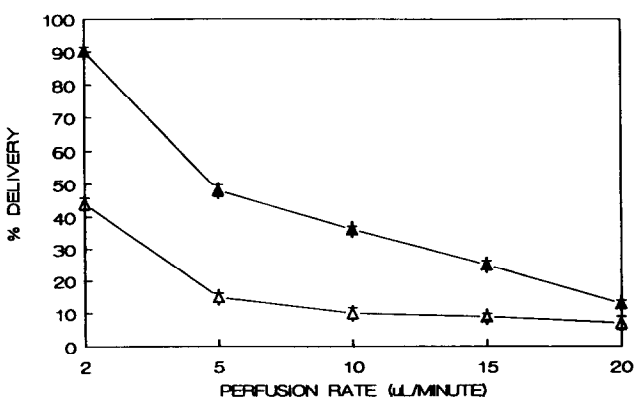

Figure 4
Dependence of delivery on perfusion rate. Symbols: —▲—▲— hydroquinone (100 μM), —△—△— phenol (1 mM).

microsomal and liver homogenate samples was found to be 15 pmol min^{-1} while the rate of delivery to whole liver was 7 pmol min^{-1}. This rate remained constant for the length of all experiments performed (up to 10 h).

A typical chromatogram of the dialysate of a microsomal preparation perfused with phenol is shown in Fig. 5A. The large void peak is due to NADPH, which interferes with the direct determination of hydroquinone. However, hydroquinone can be readily detected at the downstream electrode because it is chemically reversible while the oxidation of NADPH is irreversible.

A chromatogram of the dialysate of liver homogenate perfused with phenol is shown in Fig. 5B. The NADPH peak in these samples was not as large as in the microsomal incubations because no NADPH was added. However, the hydroquinone peak was still difficult to detect directly and the response at the downstream electrode was therefore used. A second metabolite was detected in the liver homogenate samples, and was identified as the glutathione conjugate of hydroquinone. Identification was based on

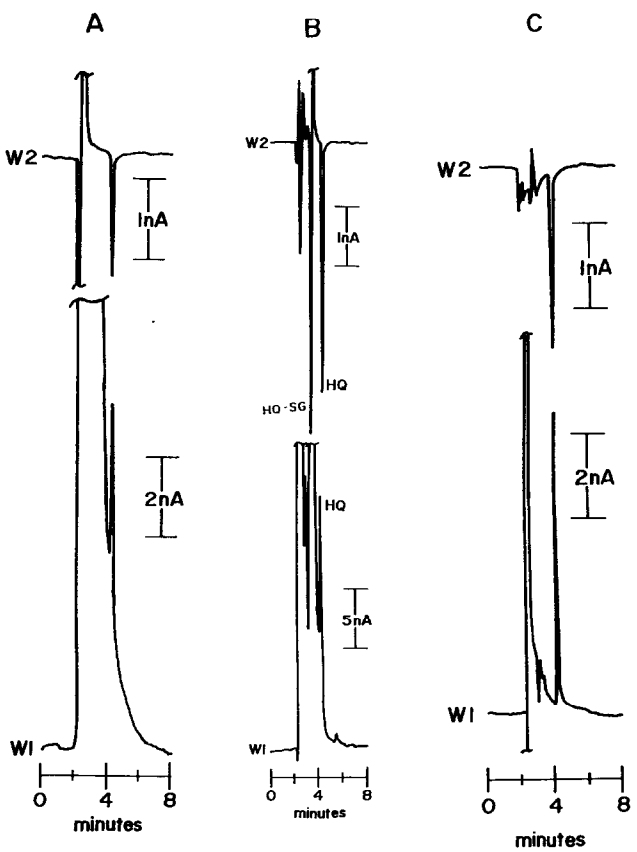

Figure 5
Chromatograms of hydroquinone, in (A) microsomes, (B) liver homogenate and (C) *in situ* whole liver, during perfusion with 10 mM phenol. Chromatographic conditions: mobile phase, 0.1 M ammonium phosphate buffer, pH 2.5 with 20% (v/v) acetonitrile; flow rate, 1 ml min^{-1}; column, Biophase II ODS 5 μm (250 × 4.3 mm). Detection conditions: W1, upstream electrode operated at +0.7 V vs Ag/AgCl; W2, downstream electrode operated at 0.0 V. Peak identities: HQ, hydroquinone; HQ–SG, hydroquinone–glutathione conjugate.

comparison of chromatographic retention times and voltammetry of the metabolite and synthetically prepared standard hydroquinone–glutathione conjugate.

A chromatogram of the dialysate of an *in situ* whole liver (i.e. the liver was in place but the animal was dead) during perfusion with phenol is shown in Fig. 5C. This chromatogram is much simpler than either the microsomal or liver homogenate chromatograms. In particular, the large NADPH peak is no longer present. Initial dialysate samples do contain NADPH, likely from cell damage during insertion of the microdialysis probe, but this NADPH disappears quickly. The fact that an efflux of NADPH into the extracellular fluid does not occur after the initial release indicates that cellular integrity is maintained after the initial insult. Hydroquinone can readily be detected directly in the dialysate from whole liver samples. No hydroquinone–glutathione conjugate was detected in the extracellular fluid. During continuous perfusion of the liver with phenol, a steady-state concentration of hydroquinone in the extracellular fluid was quickly reached (Fig. 6). This steady state was maintained for several hours.

To investigate the reason for the absence of the hydroquinone–glutathione conjugate, perfusion studies were performed in which glutathione and glutathione disulphide were determined in addition to hydroquinone. When the microdialysis probe was first implanted in the liver, a large concentration of glutathione was detected that decreased in a manner similar to NADPH. This again appears to be due to cell damage caused by the surgical incision and the insertion of the dialysis probe. Unlike NADPH, whose concentration dropped to undetectable levels in the extracellular fluid, the concentration

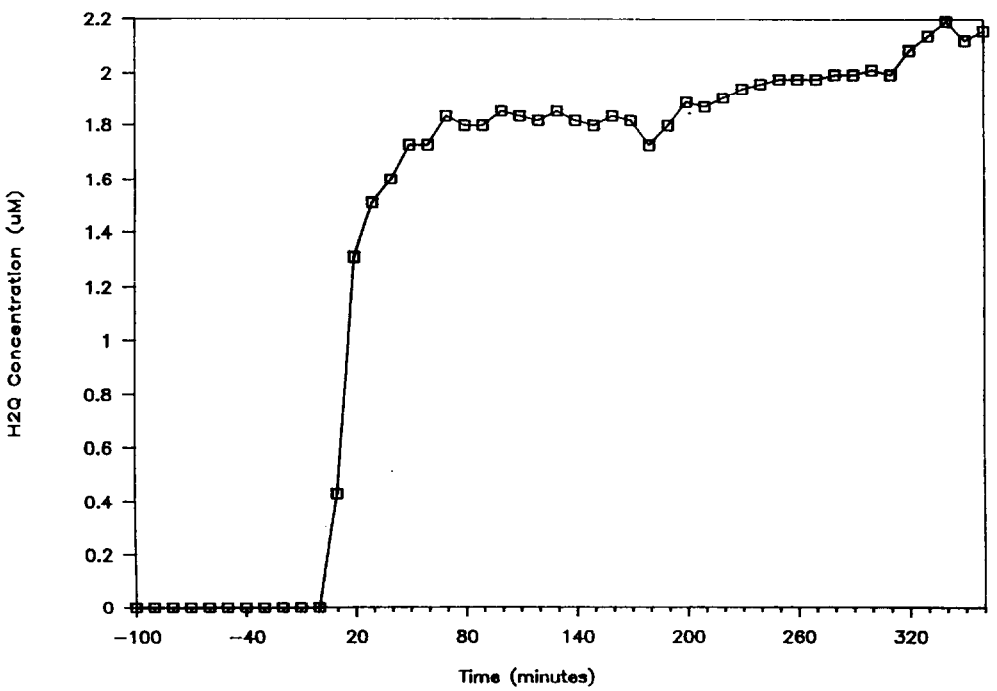

Figure 6
Time course of hydroquinone formation in whole liver perfused with phenol.

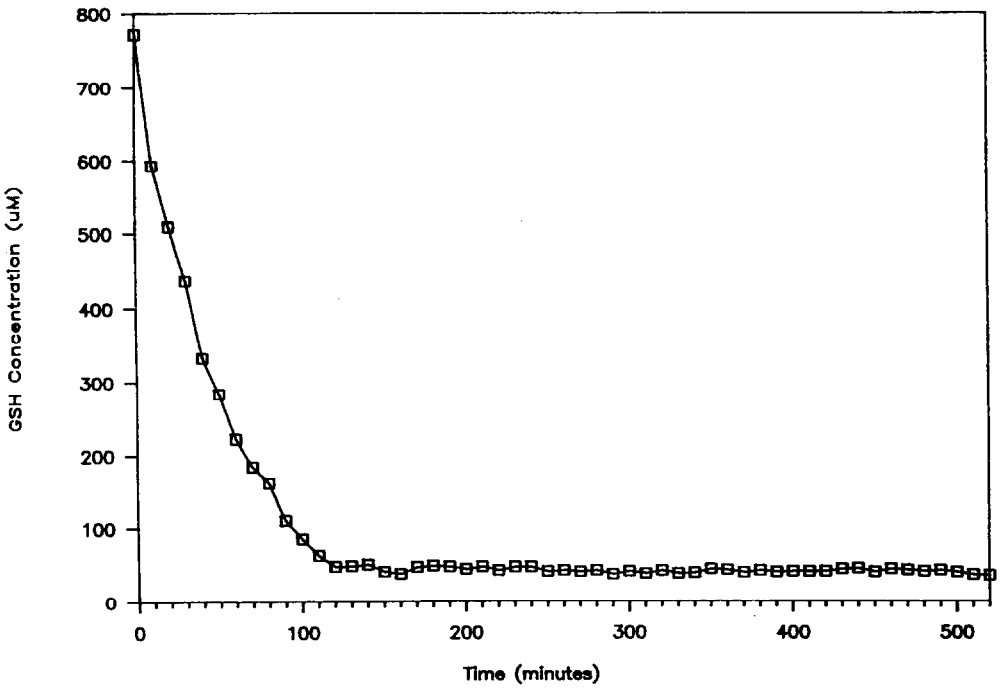

Figure 7
Extracellular concentration of glutathione during microdialysis perfusion of buffer.

of glutathione decreased to a constant level that was maintained for several hours (Fig. 7). This would indicate that glutathione is constantly being excreted from the liver cells into the extracellular fluid to maintain a constant concentration. No glutathione disulphide was detected in the liver sample.

When the liver was perfused with phenol, the glutathione concentration decreased as the hydroquinone concentration increased (Fig. 8) and eventually both reached steady state. This indicated that hydroquinone/benzoquinone had reacted with glutathione and depleted the hepatic pool. After the perfusion experiment, a small tissue sample around the dialysis probe was biopsied. When this tissue was homogenized and analysed, both hydroquinone and glutathione–hydroquinone conjugate were detected. The glutathione conjugate must have been eliminated from the liver by active transport in the bile and therefore it was not detected in the extracellular fluid. This result indicates that microdialysis is suitable not only for determining what metabolites are formed but in studying their transport mechanisms.

No glutathione disulphide could be detected during perfusion with phenol. If glutathione was reducing benzoquinone instead of undergoing 1,4-Michael addition, disulphide should be detected. That glutathione disulphide is not detected would indicate that glutathione is not serving as a reducing agent but only as a nucleophile. When similar experiments were conducted using liver homogenate instead of *in situ* whole liver, some glutathione disulphide could be detected leading to an ambiguous interpretation. Based on the *in situ* experiments, however, it seems this disulphide is the result of air oxidation of glutathione and not from the metabolism of phenol.

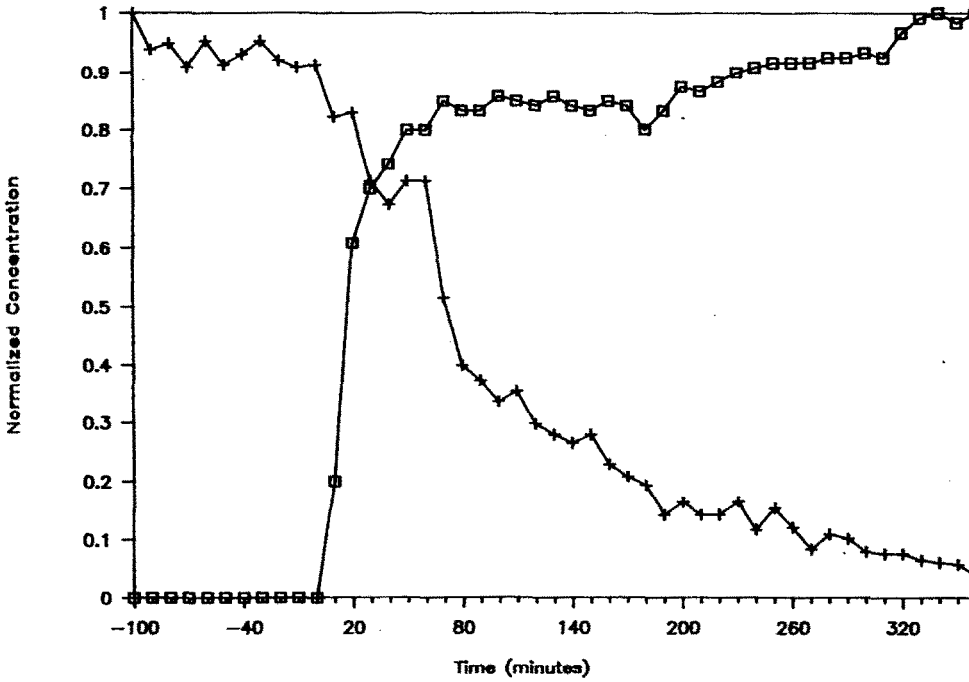

Figure 8
Time course of hydroquinone formation and glutathione depletion in whole liver perfused with phenol. Data are normalized to the steady-state concentration of 2.2 μM hydroquinone and the basal concentration of 40 μM glutathione. Symbols: —□—□— hydroquinone, −+−+− glutathione.

Conclusions

In vivo microdialysis perfusion offers a promising technique for the study of hepatic metabolism. Because the integrity of the biological system is maintained and endogenous levels of cofactors are used, such experiments should more accurately reflect actual metabolic reactions from exposure to xenobiotics. Indeed, even with this preliminary investigation, differences were observed between the *in vivo* and *in vitro* experiments. The *in vivo* microdialysis technique also offers the experimental advantage that the animal serves as its own control. Each animal can also be used to acquire the entire time course for pharmacokinetic studies instead of using an animal per time point as is currently necessary. This will result in a tremendous reduction in the number of experimental animals required, in addition to an increase in the precision of the data.

Before microdialysis perfusion can be a routine technique several fundamental issues must still be addressed. It is critical that an improved understanding of the interaction of the analyte with the membrane be achieved. The most severe limitation of microdialysis at this point is the uncertainty in calibration for tissue studies. Calibration is based on the recovery determined in some artificial medium and may differ from the recovery in the actual experimental tissue. This is not a limitation if only changes in concentration are of interest as was the case in these experiments. If the actual tissue concentration is required, a reliable calibration technique for tissue samples must be developed.

Alternatively, a larger dialysis probe can be employed in some instances, which provides 100% relative recovery and therefore does not require calibration. Finally, for *in vivo* studies a suitable anaesthetic must be identified. Even more desirable would be a reliable method of securely implanting the dialysis probe in soft tissue.

These preliminary studies illustrate the potential of microdialysis sampling for studying xenobiotic metabolism. For each experimental animal, basal levels of endogenous compounds can be established, and their change in response to the exposure to xenobiotics monitored. The metabolic profile of a compound can be determined by coupling microdialysis to a series of analytical techniques. Transport mechanisms can be investigated by consideration of the site of sampling. Finally, using multiple microdialysis probes and using probes to both sample and dose provides the ability to design unlimited numbers of exquisite experimental procedures.

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