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Microdialysis sampling coupled to on-line microbore liquid chromatography for pharmacokinetic studies

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Abstract: Microdialysis sampling has been coupled on-line to microbore liquid chromatography for pharmacokinetic investigations in awake, freely-moving animals. Valve systems are described which provide continuous collection of the microdialysis sample for injection into the chromatographic system. One valve system consisted of two six-port valves used in tandem. The other valve system consisted of an eight-port valve with two sample loops. The eight-port valve system provided better precision than the dual six-port valve system yet worse than an autosampler. However, when used for dialysis samples, the precision of the various injection methods was equivalent. The on-line system was capable of rapidly following changes in the dialysate concentration and provided near real-time analysis of the plasma concentration of analytes. The system was evaluated by monitoring the pharmacokinetics of acetaminophen in awake, freely-moving rats.

Keywords: *Microdialysis; liquid chromatography; pharmacokinetic studies.*

Introduction

Microdialysis sampling has been shown to be a powerful technique for pharmacokinetic investigations [1–5]. Both tissue and fluid compartments can be continuously sampled in awake, freely-moving animals. Microdialysis sampling is most commonly coupled to liquid chromatographic analysis. The microdialysis sample is protein-free allowing direct injection into the chromatographic system. However, while microdialysis sampling is a continuous sampling method, liquid chromatography requires discrete samples. Therefore, when these two techniques are coupled, the dialysate is collected over some fixed time interval to provide the sample for chromatographic analysis. The high temporal resolution of microdialysis is, therefore, lost and becomes dependent upon the sample requirements of the chromatographic system. To minimize the sample volume required and, therefore, increase the temporal resolution of the experiment, microbore chromatographic systems are commonly employed with microdialysis sampling. Collection of dialysate over a fixed interval prior to analysis also changes the nature of the dialysis sample. Where the dialysate in the dialysis system represents an instantaneous picture of the *in vivo* system, the collected dialysate represents the time-averaged concentration over the sampling interval. To maintain the continuity provided by microdialysis sampling, all of the dialysate must be collected and analysed. This requirement is particularly important for pharmacokinetic investigations where several of the calculated parameters depend on the area of the concentration–time curve. If the collection requirement is met, the area-under-the-curve (AUC) is simply the sum of the individual dialysis samples as these each are the integral over the collection time interval. However, if some of the dialysate is not collected, then an approximation technique, such as the trapezoidal rule, must be used to estimate the AUC.

That microdialysis samples are protein-free provides the potential for direct coupling of the microdialysis system to the chromatographic system because no sample clean-up is necessary. Such an on-line injection system consisting of a six-port injection valve has previously been reported [6, 7]. While this system is useful for many experiments, it is not suitable for use with microbore LC for pharmacokinetic investigations. Since it employs only a single

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sample loop, while the sample in this loop is being injected into the chromatographic system the dialysate is being directed to waste. At a typical flow rate of 1 ml min^{-1} for conventional liquid chromatography and using a 5-μl sample loop, little sample is lost as only 3 s are required to flush the loop with 10 volumes of mobile phase. However, using a typical microbore chromatography flow rate of 50 μl min^{-1}, 1 min is required to flush a 5-μl loop with 10 loop volumes. Since the dialysis sample is continuously varying, the sample which is lost is not represented by any other portion of the dialysate. It is, therefore, important for an on-line injection system for pharmacokinetic investigations that all of the dialysate be injected into the chromatographic system. This can be accomplished by using two sample loops instead of only one. In this way, one loop is being filled with dialysate while the other is being injected into the chromatograph. The role of the loops then reverses so that at no time is the dialysate diverted to waste. Such a dual-loop system can be constructed in two ways, either using two six-port valves in combination, each with a single sample loop, or using an eight-port valve with two sample loops. The plumbing of these on-line valve systems and their evaluation for microdialysis sampling is described in this report.

Experimental

Reagents

Acetaminophen (APAP) was purchased from the Sigma Chemical Co. (St Louis, MO, USA). Acetaminophen-4-*O*-sulphate, APAP-S, was prepared by the procedure of Feigenbaum and Neuberg [8]. Acetaminophen-4-*O*-glucuronide, APAP-G, was isolated from human urine collected after an oral dose of APAP. LC-grade acetonitrile was obtained from Fisher (Fair Lawn, NJ, USA). All other chemicals were reagent grade or better and used as received.

Dialysis system

Microdialysis probes of the flexible cannula design were constructed of fused silica tubing (75-μm i.d., 147-μm o.d.) with a 4 mm length of regenerated cellulose dialysis fibre (232-μm i.d., 250-μm o.d., 5000 MW cut off). The fabrication of these probes has been described previously [4]. The inlet of the microdialysis probe was connected with FEP tubing to a Hamilton syringe mounted on a CMA/100 microinjection pump through a liquid swivel, all from BAS (Bioanalytical Systems, Inc., West Lafayette, IN, USA). The microdialysis probe outlet was connected to the injection valve of the chromatographic system with FEP tubing. The microinjection pump delivered the perfusion medium at a flow rate of 1.00 μl min^{-1} for all experiments. The experimental animal was housed in an awake animal system from BAS as described previously [4]. A schematic of the on-line microdialysis sample system is shown in Fig. 1.

Chromatographic system

The chromatographic system consisted of a BAS PM-60 pump and a Shimadzu SPD-2AM UV–Vis spectrophotometric detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Separation of APAP was achieved with a BAS SepStik microbore column (100 mm × 1 mm i.d.) packed with 5 μm ODS stationary phase. The mobile phase was acetonitrile–ammonium phosphate buffer (pH 2.5, 0.05 M; 7.93 v/v) at a flow rate of 100 μl min^{-1}. Detection was at a wavelength of 250 nm. A Datajet Integrator (Spectra-

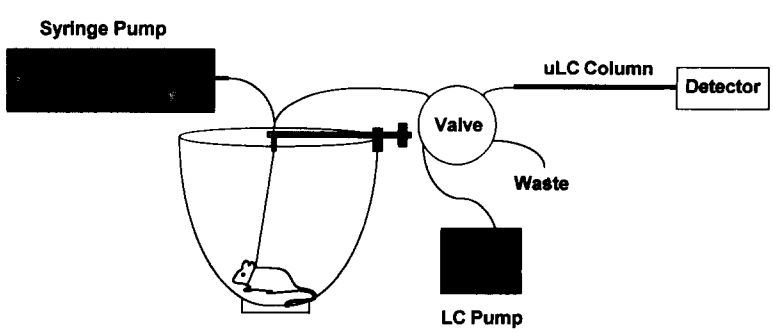

Figure 1
Schematic diagram of the on-line microdialysis sampling microbore liquid chromatography analysis system.

Physics, San Jose, CA, USA) connected to a WINner/386 workstation was used to obtain the chromatographic data.

On-line valves

The initial design of the on-line injector consisted of two six-port injection valves (7067-005 enrichment injectors, Rheodyne Inc., Cotati, CA, USA) with 7-μl sample loops and a Rheodyne 7064 pneumatically actuated controller. The injection volume consists not only of the sample loop volume but also the volume of the rotor groove. The Rheodyne 7064 controller switches the valve in one direction as it comes from the manufacturer. This results in different grooves of the rotor being used on subsequent injections. Variation in rotor groove volume was found to be the limiting factor in injection volume precision. This was corrected by modification to the controller and careful plumbing of the valve system so that a single groove in each rotor was used in each of the injection positions. The ultimate plumbing scheme of the dual six-port injection valve system is shown in Fig. 2. In Position A the injection volume consists of groove c and loop 1 while groove b and loop 2 are filling with the next dialysate sample. In Position B the injection volume consists of groove b and loop 2, while groove c and loop 1 are filling with the next dialysate sample. Groove a, groove A and the connecting tubing on valve 1 are always filled with mobile phase, while groove 2, groove 3, and the connecting tubing on valve 2 are always filled with waste. When the valve is used in an underfill mode (i.e. the sample volume is less than the sample loop volume), this waste is mobile phase displaced as the sample loop fills with dialysate. In the underfill mode all of the dialysate is injected into the chromatograph, none goes to waste.

To overcome the complexity of the dual six-port injection valve system and yet maintain continuous dialysate collection, an eight-port injection valve was evaluated. The eight-port valve was a two-position electrically actuated valve from Valco Instruments Co. (Houston, TX, USA) with 7-μl sample loops. This valve was capable of employing two sample loops that alternated between the dialysate flow path and the chromatographic flow path. The plumbing of this system is shown in Fig. 3.

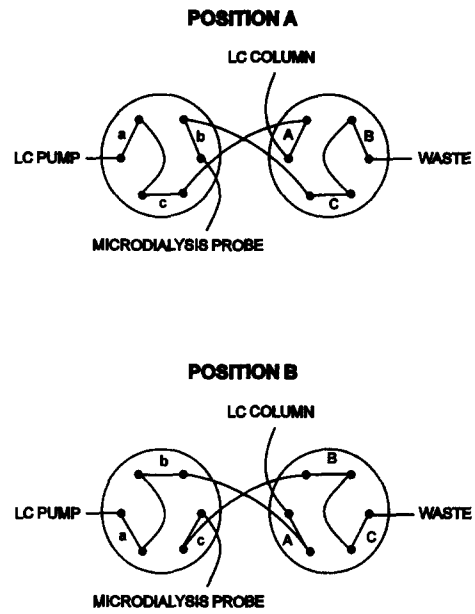

Figure 2
Schematic diagram of the dual six-port on-line injection valve system.

Sample concentration step

The two on-line systems were evaluated for response time to a step change in the sample concentration. This evaluation was performed in two ways. Firstly, the on-line injector was connected directly to the perfusion pump through a liquid switch which allowed essentially instantaneous change between three different perfusion solutions. Three perfusion

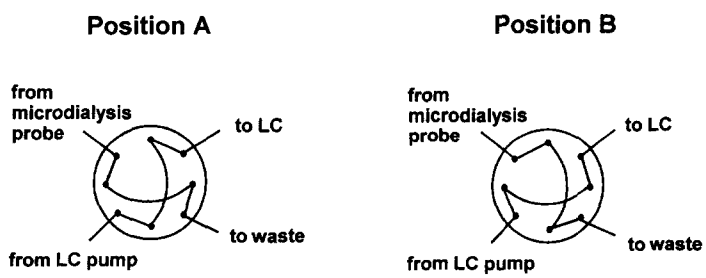

Figure 3
Schematic diagram of the eight-port on-line injection valve.

mediums were used, a Ringer's blank, Ringer's containing 100 μg ml^{-1} APAP, and Ringer's containing 200 μg ml^{-1} APAP. Initially, the blank solution was directed to the on-line injection system and at timed intervals the solution was changed to the two APAP solutions causing a step change in the sample APAP concentration. This allowed evaluation of the system response without the time constant of the dialysis sampling. Secondly, a single perfusion solution of Ringer's solution was used and the liquid switch was replaced by a microdialysis probe. This probe was sequentially moved from a beaker containing a Ringer's blank to a beaker containing 500 μg ml^{-1} APAP, and finally to a beaker containing 1000 μg ml^{-1} APAP. This allowed evaluation of the time response of the complete sampling and analysis system. For both experiments a 2 min sampling interval and a perfusion flow rate of 1 μl min^{-1} were used.

Pharmacokinetic experiments

Four- to 5-month old Sprague–Dawley rats weighing *ca* 400 g were used. Rats were anaesthetized with the inhalation anesthetic isoflurane during surgery. A microdialysis probe and a cannula were implanted into the right jugular vein as previously described [8]. The animals were allowed to recover for at least 2 h prior to the start of sampling. Microdialysis samples were collected for at least 1 h prior to dosing. On-line injections were made at 5-min intervals for all pharmacokinetic experiments. No interferences were observed in these blanks. A 10 mg kg^{-1} dose of APAP was administered in 500 μl saline solution by injection into the cannula in the right jugular vein. Sampling was continued until APAP was no longer detectable in the microdialysis samples (*ca* 0.1 μg ml^{-1}).

Results and Discussion

Evaluation of on-line injector precision

The two on-line injector systems were compared to manual injection and autosampler injection for reproducibility. Evaluation was made using both a directly injected standard APAP solution and a collected dialysate sample. The relative standard deviations of the analyte peak area for the four injections methods are listed in Table 1. When considering direct injection of a standard solution, both manual injection and an autosampler were more precise than either of the on-line systems. The eight-port valve was only slightly less precise while the precision of the dual six-port system was considerably less than the other methods. However, when the sample was a dialysate the difference between the eight-port on-line system and the off-line system is not significant. Under these conditions even the dual six-port valve operates acceptably. This is because dialysis sampling introduces roughly 1–2% to the deviation which masks the differences in the injection method.

Evaluation of on-line system response time

The results from the step change in sample concentration were equivalent whether the sample was directly injected or collected by microdialysis. The response of the systems to a step change in sample concentration using microdialysis sampling are shown in Fig. 4 for both the dual six-port valve system and the eight-port valve. In the case of the dual six-port valve system the forward response time is essentially instantaneous on the sampling time scale. It must be remembered that dialysis sampling is continuous and that the resulting sample reflects the average (or integral) of the sample concentration over the sampling interval. Therefore, the first sample after a step

Table 1
Precision (% RSD) of various modes of injection*

	Volume injected (μl)	Dual six-port valve	Eight-port valve	Autosampler	Manual
Direct injection	0.5	7.5	1.4	0.5	0.6
	1.0	2.1	1.4	0.6	1.4
	2.0	0.6	0.8	0.4	0.9
Dialysate injection	0.5	6.7	3.1	2.6	3.9
	1.0	3.2	1.1	2.3	6.6
	2.0	3.9	1.6	2.0	7.2

*n = 5 in all cases.

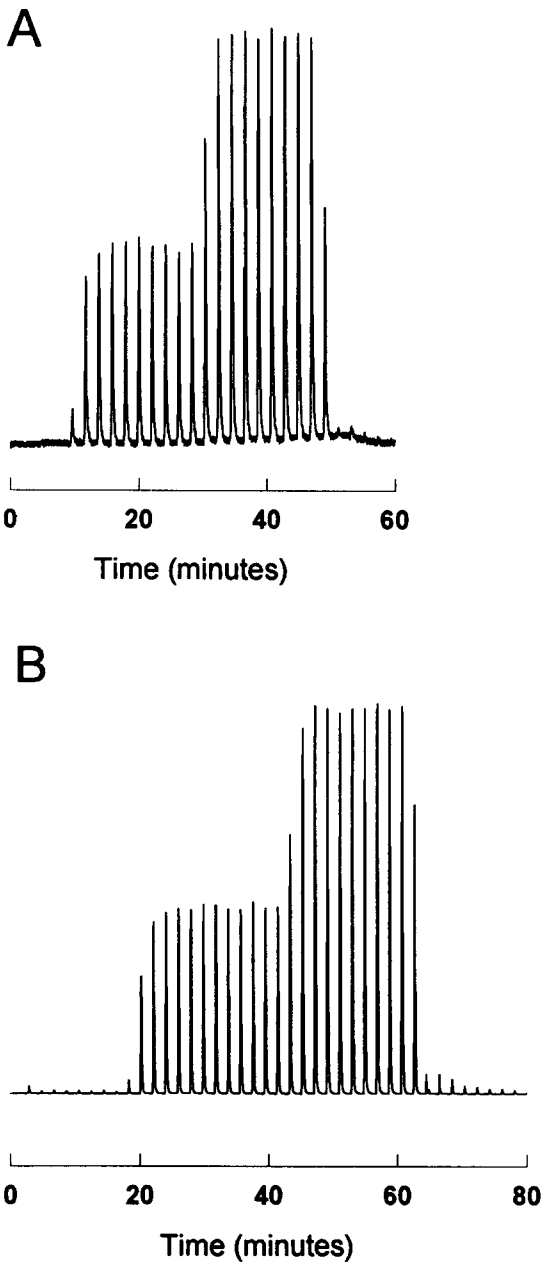

Figure 4
System response to a step change in sample concentration. (A) Dual six-port valve system; (B) eight-port valve system.

change in concentration contains a portion that reflects the initial concentration and a portion that reflects the final concentration. The analytical result is then the time weighted average of the two concentrations. Varying the injection interval from 0.5 to 5 min results in identical step function responses where the first injection is between the two step concentrations. Fortunately, biological processes are not typically discontinuous but rather continuously varying, therefore, the integrating nature of this sampling regime accurately reflects the kinetics of the process. Obviously, shorter injections intervals result in better temporal resolution for the experiment.

System delay time

Step changes in sample concentration were not immediately reflected in the analytical system response. A delay occurs because the dialysate must be transferred from the microdialysis probe to the chromatographic injector. The FEP tubing connecting the microdialysis probe to the injection valve had a length of 70 cm with a volume of 8.4 μl (0.12 μl cm^{-1}). In addition, the liquid swivel had a dead-volume of 1.4 μl. This results in a 10 min delay between the *in vivo* event and injection of the relevant dialysate sample. This delay volume was experimentally verified by directly connecting the FEP tubing to a UV detector and measuring the delay for a step change in sample concentration. The observed value was in agreement with that calculated from the tubing length and swivel dead-volume. With a 5 min analysis time, the detector output represents events occurring in the experimental animal 15 min previously.

On-line pharmacokinetic analysis

This on-line microdialysis sampling system was demonstrated by following the pharmacokinetics of APAP following a 10 mg kg^{-1} i.v. dose in an awake, freely-moving rat. No interferences were observed in the dialysate blanks prior to dosing (Fig. 5a). A typical plasma dialysate following dosing of APAP is shown in Fig. 5(b). The two major APAP metabolites, APAP-S and APAP-G, as well as APAP are readily detected. The clearance of APAP could be directly observed from the chromatographic detector output as seen in Fig. 6. A pharmacokinetic curve can be constructed by converting the peak area results into concentration values and plotting these as a bar graph.

Conclusions

On-line microdialysis sampling coupled to chromatographic analysis provides near-real-time monitoring of multiple *in vivo* compounds. The precision of the on-line system is comparable to off-line analysis for microdialysis samples. The response time of the system is fast relative to the analysis time.

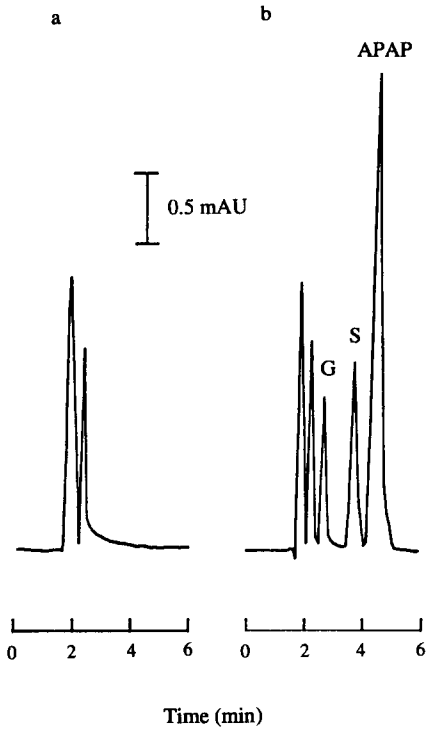

Figure 5
Chromatograms of typical blood dialysate samples. (a) Sample prior to dosing with APAP; (b) sample collected 30 min after a 10 mg kg^{-1} i.v. dose of APAP. Peak identities are: APAP, acetaminophen; G, acetaminophen-4-*O*-glucuronide; S, acetaminophen-4-*O*-sulphate.

Better temporal resolution for the experiment could be achieved through a shorter analysis time. The use of a two sample loop injection system allows all of the dialysate to be injected into the chromatographic system even at microbore chromatography flow rates. Finally, the eight-port valve provides a simpler system with higher precision relative to the dual six-port valve system.

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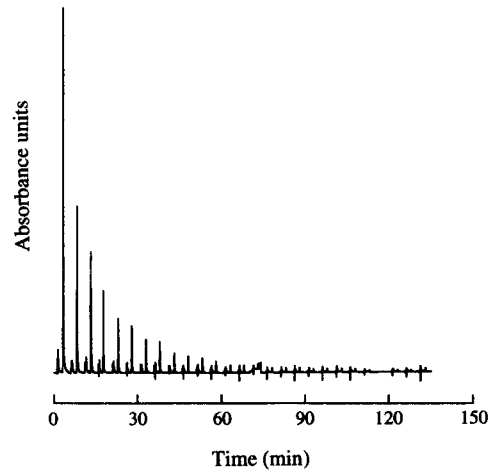

Figure 6
Chromatograms resulting from on-line intravenous microdialysis sampling coupled to microbore liquid chromatography following a 10 mg kg^{-1} i.v. dose of APAP to an awake, freely-moving rat.

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