ELSEVIER

Journal of Chromatography A, 691 (1995) 29–35

JOURNAL OF
CHROMATOGRAPHY A

Microdialysis sampling coupled on-line to fast microbore liquid chromatography

Anqing Chen, Craig E. Lunte*

Department of Chemistry and Center for Bioanalytical Research, University of Kansas, Lawrence, KS 66045, USA

Abstract

A system which combines intravenous microdialysis sampling on-line with fast microbore liquid chromatographic analysis is described. Using a 14 mm × 1 mm I.D. ODS column, caffeine, theobromine and paraxanthine could be resolved in under 1 min and acetaminophen and its two main metabolites, the glucuronide and sulfate conjugates, could be resolved in 30 s. This fast separation allowed on-line microdialysis sampling to be conducted with a 1-min sampling interval. The system was evaluated by monitoring the pharmacokinetics following intravenous administration of acetaminophen and caffeine.

1. Introduction

Microdialysis sampling has been shown to be a powerful technique for pharmacokinetic investigations [1–5]. Liquid chromatography is commonly used for the analysis of microdialysis samples. 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. For chromatographic analysis, the dialysate is collected over some fixed time interval to provide the required sample volume. 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 often employed with microdialysis sampling [1,2,4,6,7].

A difficulty encountered with microdialysis sampling is collection and injection of the small sample volumes. Using microbore chromatographic systems samples of 1 μl or less are common. With such small volumes loss from evaporation and sample transfer are major limitations requiring sophisticated sample collectors and autosamplers. 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 system also eliminates the problems associated with evaporation and sample transfer. On-line coupling of microdialysis sampling with microbore liquid chromatographic analysis has previously been reported [6–8]. The major limitation of these systems is that the temporal resolution of the sampling system is a function of the analysis time of the chromatographic system. The next sample cannot be injected until the previous one has

* Corresponding author.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00634-2

been completely eluted as no provision for sample storage is possible. This report describes the coupling of microdialysis sampling to fast microbore liquid chromatography to provide analysis times of less than 1 min and therefore 1-min temporal resolution for the pharmacokinetic experiment. This on-line system provides a near real-time response to multiple chemical species in an awake, freely moving experimental animal.

2. Experimental

2.1. Materials

Caffeine, paraxanthine, theobromine, theophylline and acetaminophen (APAP) were purchased from Sigma (St. Louis, MO, USA). Acetaminophen-4-O-sulfate (APAP-S) was prepared by the procedure of Feigenbaum and Neuberg [9]. Acetaminophen-4-O-glucuronide (APAP-G) was isolated from human urine. HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were reagent grade or better and were used as received.

2.2. Microdialysis system

Microdialysis sampling was performed using a CMA/100 microinjection pump from Bioanalytical Systems (BAS)/Carnegie Medicine (West Lafayette, IN, USA). The microdialysis pump was connected to a flexible cannula-style microdialysis probe which was inserted into the jugular vein of a rat. The microdialysis probe was constructed from dialysis tubing of regenerated cellulose with an inner diameter of 220 μm, a wall thickness of 22 μm and a molecular mass cutoff of 15 000 as described previously [4]. All connections from the pump to the microdialysis probe and from the microdialysis probe outlet to the injection valve were made with 0.1 mm I.D. FEP (Teflon) tubing. The perfusion solution was a Ringer's solution consisting of 155 mM NaCl, 5.5 mM KCl and 2.3 mM $CaCl_2$. A perfusion rate of 1.5 μl/min was used for all experiments. The experimental animal was housed in an awake animal containment system as described previously [4]. A two-channel liquid swivel was positioned in the flow path to provide a liquid connection that allowed free movement of the animal without tangling of the tubing.

2.3. Chromatographic system

The HPLC system consisted of a PM-60 LC pump (BAS, West Lafayette, IN, USA), a SPD-6A UV absorbance detector (Shimadzu, Columbia, MD, USA), and an electrically actuated HPLC injection valve with a 0.5-μl internal sample loop (Model E90; Valco Instruments, Houston, TX, USA). The injector switching rate and injection time were controlled by a personal computer. The dialysate was flushed directly into the injection valve. A schematic of the complete on-line microdialysis sampling–microbore chromatography analysis system is shown in Fig. 1.

Separation was achieved using a 14 mm × 1

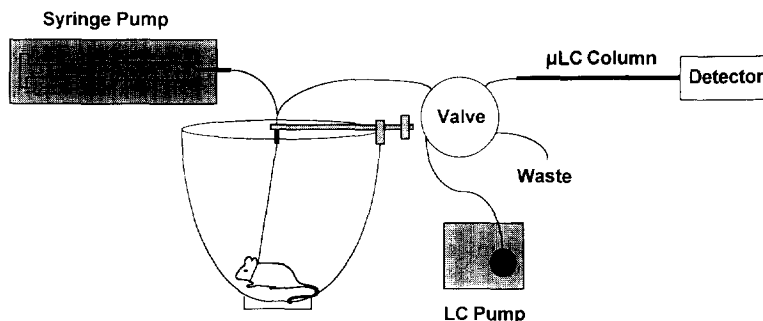

Fig. 1. Schematic diagram of the on-line microdialysis sampling–fast microbore chromatographic analysis system.

mm microbore BAS Sep-Stik column packed with 3-μm ODS material. The mobile phase for APAP and its metabolites was 0.05 *M* ammonium acetate buffer, pH 5.0. For caffeine and its metabolites the mobile phase was 0.05 *M* ammonium phosphate buffer, pH 2.5, with 5% (v/v) acetonitrile. A flow-rate of 200 μl/min was used for all experiments. For detection of APAP and its two metabolites the UV detector was operated at 250 nm. For detection of caffeine and its two metabolites the UV detector was operated at 273 nm.

2.4. *In vitro experiments*

For characterization of the on-line injection and chromatographic analysis system, the sample was directly introduced into the injection valve in a continuous manner. This was accomplished by directly connecting the microperfusion pump to the injection valve with the microdialysis probe replaced by a liquid switch. For these experiments the perfusion fluid was a Ringer's solution containing the desired concentration of APAP and its metabolites or caffeine and its metabolites. Up to three syringes can be controlled by a single microinfusion pump and therefore a blank Ringer's solution and two different concentrations of the analytes could be tested without stopping the system.

The response of the on-line microdialysis sampling–microbore chromatographic analysis system was also evaluated in the microdialysis sampling mode. In this case the microdialysis probe was placed in a beaker containing a stirred Ringer's solution. Concentration steps were achieved by quickly moving the microdialysis probe to beakers containing various concentrations of the analytes.

2.5. *Pharmacokinetic experiments*

Male Sprague-Dawley rates weighing between 300 and 400 g were used. The rat was anesthetized using a mixture of ketamine and xylazine (140 mg/kg ketamine, 12 mg/kg xylazine) given as intramuscular injection. The animal's body temperature was maintained with a heating pad beneath its body during surgery. The microdialysis probe and dosing cannula were implanted into the right jugular vein as previously described [4]. Animals were allowed 24 h to recover from surgery prior to a pharmacokinetic experiment. Microdialysis sampling was initiated at least 1 h prior to dosing the animal. For both APAP and caffeine, a 30 mg/kg dose of the drug in 1 ml of Ringer's solution was administered intravenously (i.v.). Microdialysis sampling was continued until the parent compound was no longer detectable in the dialysate, typically 2 h for APAP and 6 h for caffeine.

3. Results and discussion

3.1. *System characterization*

Using the system in the direct on-line injection mode the separations were optimized and the linearity and precision were determined. For APAP and its metabolites the elution order was APAP-G at 10 s, APAP-S at 19 s and APAP at 29 s, with resolutions of 2.43 and 1.59, respectively (Fig. 2). The response for all three compounds was linear from 0.3 to 20 μg/ml (Table

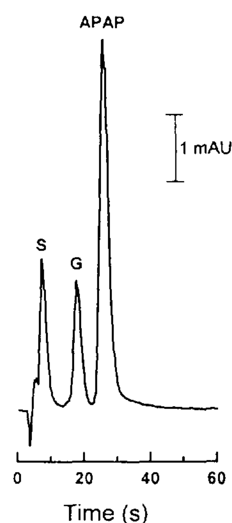

Fig. 2. Chromatogram of direct on-line injection of APAP and metabolites standard. Peaks: APAP = acetaminophen; S = acetaminophen-4-O-sulfate; G = acetaminophen-4-O-glucuronide.

Table 1
Linearity and detection limits

Compound	Range (μg/ml)	Slope (mAU μg^{-1} ml^{-1})	Intercept (AU)	r	Detection limit (μg/ml)[a]
APAP	0.3–20	0.0797	−0.023	0.9994	1.25
APAP-G	0.3–20	0.540	−0.035	0.9998	0.3
APAP-S	0.3–20	0.107	−0.026	0.9992	0.3
Caffeine	0.3–25	0.278	−0.001	0.9997	0.3
Theobromine	0.3–25	0.956	0.035	0.9993	0.3
Paraxanthine	0.3–25	0.831	−0.004	0.9998	0.3

[a] At $S/N = 3$.

1). For caffeine and its metabolites the elution order was theobromine at 13 s, paraxanthine at 21 s and caffeine at 46 s, with resolutions of 1.37 and 3.94, respectively (Fig. 3). Again the response for all three compounds was linear from 0.3 to 25 μg/ml (Table 1). The precision of multiple injections was about 2% R.S.D. in all cases, whether by direct injection or following microdialysis sampling. The precision appears slightly worse for microdialysis sampling relative to direct injection although in no case is the difference statistically significant.

The system response time was then evaluated by making step changes to the sample concentration. The response time was evaluated using both direct on-line injection and on-line microdialysis sampling. Typical responses to step changes in APAP concentration are shown in Fig. 4. There is no difference in the response to direct sample injection and on-line microdialysis sampling. Even with a 1-min sampling interval

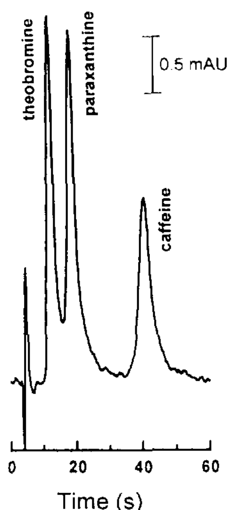

Fig. 3. Chromatogram of direct on-line injection of caffeine and metabolites standard.

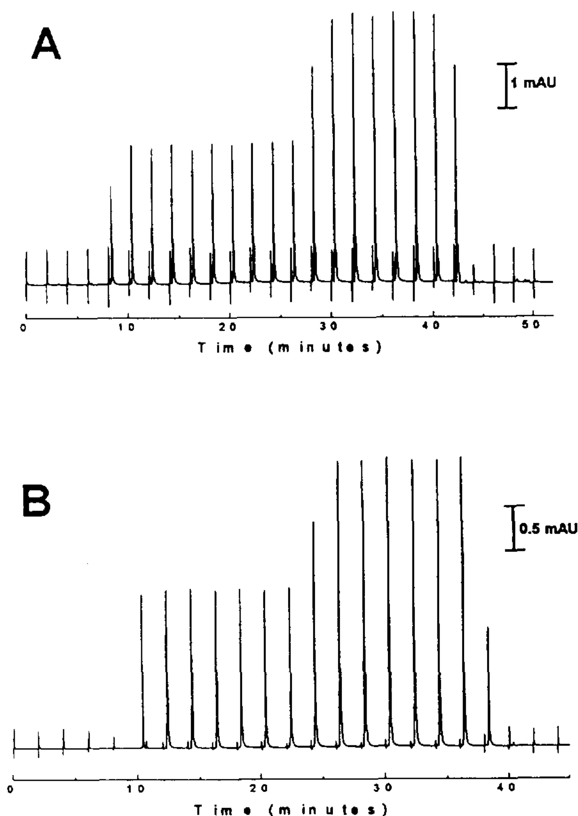

Fig. 4. System response to a step change in sample concentration of APAP. (A) Direct on-line injection; (B) on-line microdialysis sampling.

the microdialysis response can accurately monitor the sample concentration. It can be seen in Fig. 2 that the first sample following a step change in concentration does not give the new sample concentration. This is because the dialysate which is analyzed is of finite volume and reflects the time-averaged concentration of the sample over the sampling interval. For a step change in concentration, the sampling interval rarely begins just at the step and therefore the first dialysate represents some time at the initial concentration and some time at the final concentration. Fortunately, real processes are seldom instantaneous and this phenomena therefore does not result in a distortion of the response. Sampling intervals as short as 30 s have shown no distortion of the response due to slow sampling through the microdialysis probe. These results are in agreement with those recently reported by Newton and Justice [7] and of Hogan et al. [10].

3.2. Intravenous sampling

The performance of the on-line microdialysis sampling–fast microbore LC analysis system was initially evaluated in vivo using APAP. Representative chromatograms of a dialysate prior to dosing and after administration of 30 mg/kg APAP i.v. are shown in Fig. 5. The blank is clean except for a void peak due to endogenous unretained hydrophilic compounds. APAP and APAP-S are well resolved in 30 s in the dialysate following dosing with APAP. Unfortunately, APAP-G is very near the void which limits its quantification. The resulting pharmacokinetic curve for APAP is shown in Fig. 6. The results are plotted as a bar graph because each dialysate sample represents the time-averaged concentration in vivo over the sampling interval. This system provided for near real-time monitoring of the plasma concentrations of unbound APAP and APAP-S with a 1-min temporal resolution. Microdialysis samples the unbound fraction of the drug in plasma as proteins, and therefore protein-bound drugs, are excluded by the membrane. The pharmacokinetic curve shown in Fig. 6 is for free APAP. These results can be converted to total drug concentrations if the degree of protein binding is known. Alternatively, the in vivo binding can be determined by simultaneously withdrawing a whole blood sample from which to determine the total drug concentration.

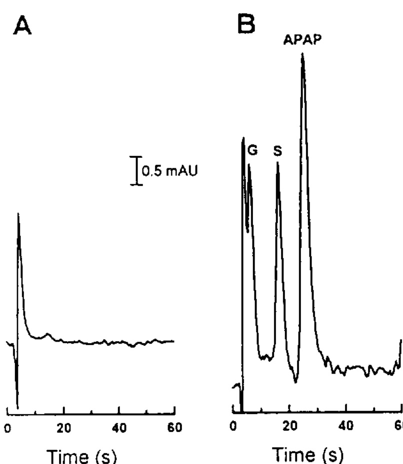

Fig. 5. Chromatograms of on-line intravenous dialysates. (A) Prior to administration; (B) 40 min after administration of 30 mg/kg APAP i.v.

The pharmacokinetics of caffeine were also examined using the on-line microdialysis sampling–fast microbore LC analysis system. Representative chromatograms of a dialysate prior to

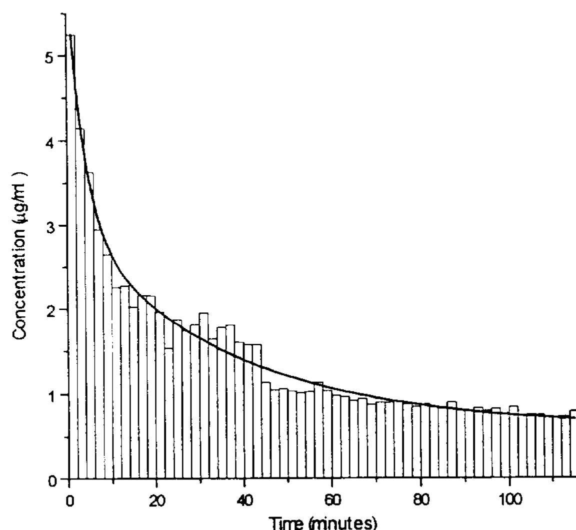

Fig. 6. Pharmacokinetic curve for APAP generated by intravenous microdialysis sampling coupled on-line to fast microbore HPLC.

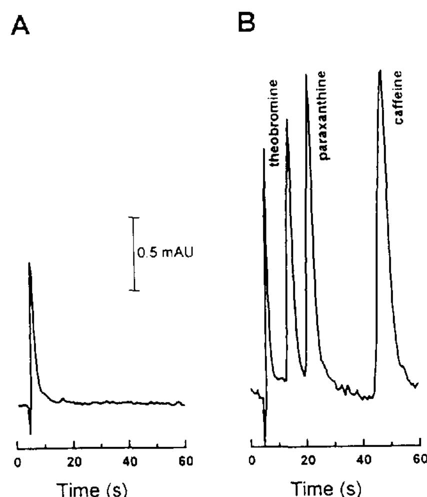

Fig. 7. Chromatograms of on-line intravenous dialysates. (A) Prior to administration; (B) 70 min after administration of 30 mg/kg caffeine i.v.

dosing and after administration of 30 mg/kg caffeine i.v. are shown in Fig. 7. In this case, caffeine and its two major metabolites, theobromine and paraxanthine, are all resolved from each other and the void in 50 s. The chromatographic results from a pharmacokinetic experiment using caffeine are shown in Fig. 8. The inset shows the individual chromatograms which constitute the curve in more detail. As can be

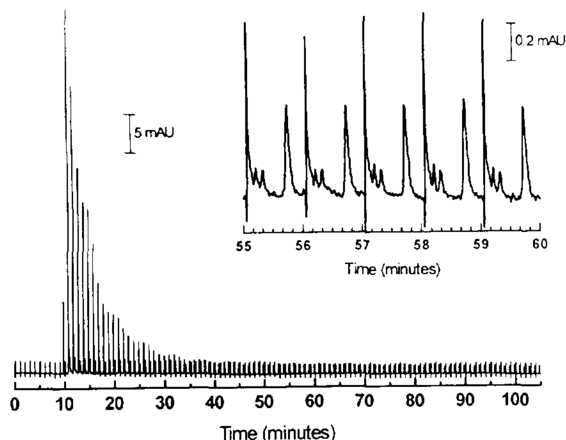

Fig. 8. Detector output from the on-line microdialysis sampling–fast microbore HPLC system following the plasma concentration of caffeine and its main metabolites. Caffeine was administered i.v. (30 mg/kg) at 10 min. The inset shows an expanded scale of the output from 55 to 60 min.

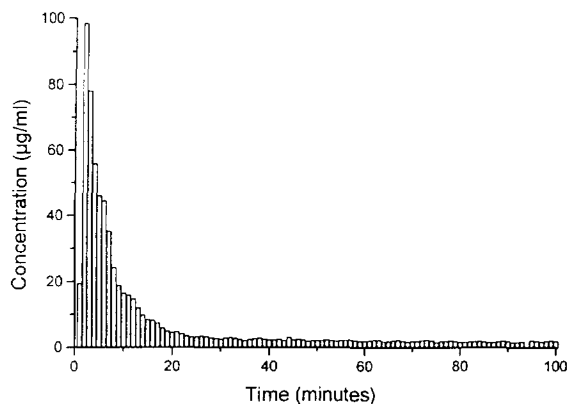

Fig. 9. Pharmacokinetic curve for caffeine generated by intravenous microdialysis sampling coupled on-line to fast microbore HPLC.

seen, the metabolites as well as caffeine can be detected in these dialysates. The resulting pharmacokinetic curve for caffeine is shown in Fig. 9.

4. Conclusions

Microdialysis sampling coupled on-line to fast microbore LC analysis provides near real-time monitoring of the in vivo concentration of multiple analytes with temporal resolutions of less than 1 min. The response time of the system is less than 1 min and a function of the sample volume needed for chromatographic analysis. This provides the possibility of building feedback into the system such that data from the current experiment can be used to modify that experiment. It may ultimately be possible to use an on-line sampling–analysis system such as this to provide therapeutic drug monitoring of multiple drugs and metabolites simultaneously.

Acknowledgements

The authors acknowledge the financial support of the National Institutes of Health through grant R01-GM44990 and the Center for Bioanalytical Research at the University of Kansas.

References

[1] K.L. Steele, D.O. Scott and C.E. Lunte, *Anal. Chim. Acta*, 246 (1991) 181–186.

[2] D.O. Scott, L.R. Sorensen, K.L. Steele, D.L Puckett and C.E. Lunte, *Pharm. Res.*, 8 (1991) 389–392.

[3] P. Lönnroth, J. Carlsten, L. Johnson and U. Smith, *J. Chromatogr.*, 568 (1991) 419–425.

[4] M. Telting-Diaz, D.O. Scott and C.E. Lunte, *Anal. Chem.*, 64 (1992) 806–810.

[5] P. Sjoberg, I.-M. Olofsson and T. Lundqvist, *Pharm. Res.*, 9 (1992) 1592–1598.

[6] S.A. Wages, W.H. Church and J.B. Justice, Jr., *Anal. Chem.*, 58 (1986) 1649–1656.

[7] A.P. Newton and J.B. Justice, Jr., *Anal. Chem.*, 66 (1994) 1468–1472.

[8] K.L. Steele, *M.S. Thesis*, University of Kansas, Lawrence, KS, 1993.

[9] J. Feigenbaum and C.A. Neuberg, *J. Am. Chem. Soc.*, 63 (1941) 3529–3533.

[10] B. Hogan, S.M. Lunte, J.F. Stobaugh and C.E. Lunte, *Anal. Chem.*, 66 (1994) 596–602.