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Automated analytical systems for drug development studies. V. A system for enzyme kinetic studies[1]

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Abstract

Two similar automated analytical systems using liquid chromatography (LC) and microdialysis as an on-line sampling technique were applied to studies of enzyme kinetics. 2′,3′,5′-Triacetyl-6-azauridine (azaribine) with porcine liver esterase (PLE) and *N*-acetylphenylalanyl-3,5-diiodotyrosine (AcFY′) with pepsin were used as model compounds. The microdialysis sampling technique permitted the rapid separation of low molecular weight analytes from macromolecules, thus simultaneously achieving clean-up of the samples and quenching of the reaction. The combination of rapid LC analysis and microdialysis sampling provided selectivity and automation. The systems are rugged and give reproducible results in agreement with those from manual sampling methods.

Keywords: Acetylphenylalanyl-3,5-diiodotyrosine; Automation; Enzyme kinetics; Microdialysis; Porcine liver esterase; Reversed-phase chromatography; 2′,3′,5′-triacetyl-6-azauridine

1. Introduction

Previous papers in this series have described the application in drug development of automated systems coupling HPLC analysis with microdialysis sampling [1–4]. Here these studies were extended for the first time to the measurement of the kinetics of enzyme catalysis. The main advantage of microdialysis in this study lies in its ability to separate rapidly low molecular weight analytes from macromolecules, thus simultaneously achieving clean-up of the samples and quenching of the reaction. Two model systems were employed. One system involves the hydrolysis of 2′,3′,5′-triacetyl-6-azauridine (azaribine, Fig. 1) by porcine liver

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esterase, a model of liver metabolism of this prodrug. Previously a related system has been developed to determine the kinetics of the non-enzymic reaction of azaribine [1]. A second system involves the hydrolysis of a dipeptide, *N*-acetylphenylalanyl-3,5-diiodotyrosine (AcFY′), by the acid protease pepsin. AcFY′ is a dipeptide substrate of pepsin [5].

2. Experimental

2.1. Materials

2′,3′,5′-Triacetyl-6-azauridine (azaribine, Fig. 1) was a gift from Dr. William Drell, U.R. Laboratories, La Jolla, CA, USA. Pepsin (EC 3.4.23.1), porcine liver esterase (PLE, EC 3.1.1.1), *N*-acetylphenylalanyl-3,5-diiodotyrosine (AcFY′), *N*-acetylphenylalanine (AcF), 3,5-diiodotyrosine (Y′), *p*-nitrophenyl acetate (PNPA), *p*-nitrophenol (PNP, spectrophotometric grade), trifluoroacetic acid (TFA) and sodium hydroxide were obtained from Sigma Chemical (St. Louis, MO, USA). All water was deionized with a Nanopure II Deionization System from Barnstead/Thermolyne (Boston, MA, USA). Sodium dihydrogenphosphate, disodium monohydrogenphosphate, sodium chloride and citric acid were of reagent grade and acetonitrile (ACN) and methanol (MeOH) were of HPLC grade. All were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Apparatus

Two automated analytical systems were used. System I [Fig. 2(a)], consisting of a thermostated reaction vessel connected to an autotitrator, a microdialysis sampling system and a liquid chromatograph, was applied to the investigation of in vitro metabolism (ester hydrolysis) of azaribine by PLE. A 50 ml volume of drug solution was placed in a 100 ml customized water-jacketed reaction vessel (Cat. No. 5340-972, ACE Glass, Vineland, NJ, USA), thermostated ($\pm$ 0.2°C) using a Fisher Scientific (Pittsburgh, PA, USA) Model 801 Isotemp constant-temperature circulator. A Metrohm (Herisau, Switzerland) Model 632 pH meter was used with a Model 655 Dosimat and Model 614 Impulsomat in stat-mode to maintain the pH of the reaction mixture. The sampling system consisted of a BAS (West Lafayette, IN, USA) DL-5 microdialysis probe (30 000 Da molecular weight cutoff) mounted radially across the reaction vessel (Fig. 3) and a Harvard Apparatus (South Natick, MA, USA) Model 44 infusion pump for continuous perfusion of the probe. The dialysate was perfused into a 2 μl injection loop on a Valco Instrument (Houston, TX, USA) Model EF-60 on-line injector. The injector was equipped with an electrical actuator for rapid sampling and was controlled by the signal from the infusion pump. The LC system consisted of a Beckman (Fullerton, CA, USA) Model 126 programmble solvent module and Model 168 diode-array detector module, both of which were controlled through an IBM (Armonk, NY, USA) PS/2 Model 56SX computer with Beckman System Gold Chromatography Software, connected to an Epson (Torrance, CA, USA) LQ-570 printer. A 30 × 4.6 mm i.d. (3 μm) ODS Hypersil column from Keystone (Bellefonte, PA, USA) with a 10 × 4.6 mm i.d. (5 μm) ODS Hypersil guard column from Sigma was used in the LC system.

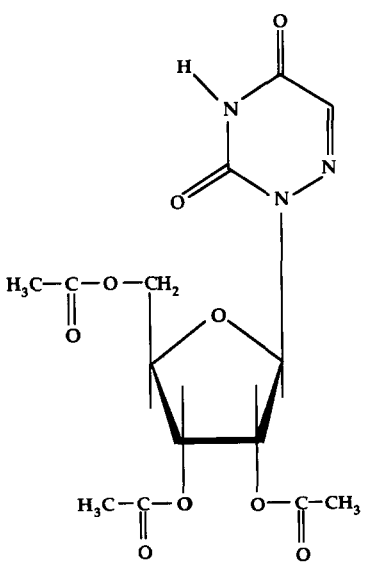

Fig. 1. Structure of 2′,3′,5′-triacetyl-6-azauridine.

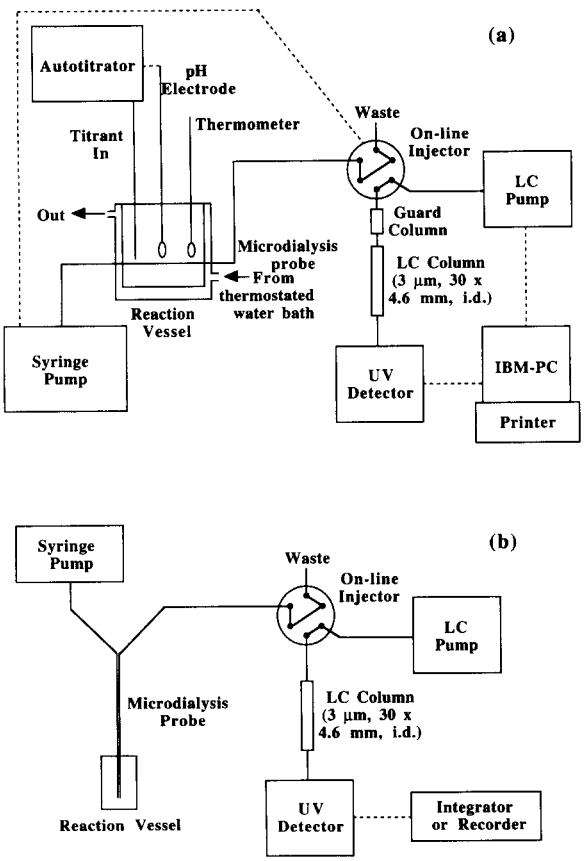

Fig. 2. Diagrams of the automated system I (a) for azaribine metabolism studies and automated system II (b) for the AcFY′ enzymatic hydrolysis studies.

System II [Fig. 2(b)] was used to study the enzymatic hydrolysis of AcFY′ by pepsin. The system consisted of a Shimadzu (Kyoto, Japan) LC-6A liquid chromatograph pump, a Valco Instrument Model EF-60 on-line injector with a 5 μl infection loop, a Keystone ODS 30 × 4.6 mm i.d. (3 μm) column, a Beckman Model 153 analytical UV/Vis detector equipped with a 254 nm filter and an 8 μl flow cell, a Kipp-Zonen (Delft, Netherlands) Model BD40 single-pen chart recorder with event marking capability, a BAS Model A Razel syringe pump to deliver 4.8 μl min^{-1} of perfusate and a BAS CMA 12 microdialysis probe with a 4 mm membrane at the tip of a 14 mm cannula. The molecular weight cutoff of the membrane was 20 000 Da. Peak response was measured manually using peak heights only.

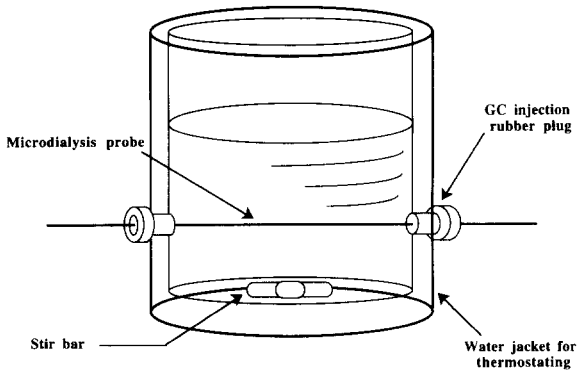

Fig. 3. Diagram of the DL-5 probe mounted radially across the customized, water-jacketed reaction vessel. The inlet and outlet of the reaction vessel for mounting the microdialysis probe were sealed with gas chromatography (GC) injection rubber plugs. Adapted from J.M. Ault, PhD Dissertation in Pharmaceutical Chemistry, University of Kansas (1994).

2.3. HPLC assays

Two HPLC assays were used for the studies. To monitor its hydrolysis, azaribine was eluted at 30 s with MeOH–phosphate buffer (50 mM, pH 6.3) (34:66, v/v) at a 2.5 ml min^{-1} flow rate and detected by UV spectrophotometry at 254 nm. All of the metabolites were eluted at the solvent front. For AcFY′ hydrolysis studies, the loss of AcFY′ and the appearance of Y′ were monitored at 254 nm with mobile phase consisting of ACN–citrate

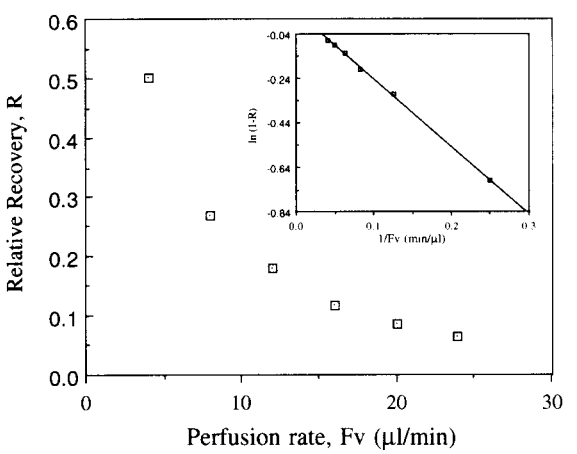

Fig. 4. Effect of perfusion rate on relative recovery of azaribine with a DL-5 probe. Inset: linear relationship between $\ln(1-R)$, where R is the relative recovery, and reciprocal perfusion rate ($1/Fv$). Error bars are smaller than the symbols.

buffer (50 mM, pH 2.3) (31:69, v/v) at a flow rate of 2.0 ml min^{-1}. The retention times of AcFY′ and Y′ were 76 and 44 s, respectively. AcF was eluted at the solvent front.

2.4. Buffer solutions

Phosphate buffer (pH 6.79, 25 mM NaH_2PO_4 and 25 mM Na_2HPO_4) was used in all azaribine studies with sodium chloride added to adjust the ionic strength to 0.50. Citrate buffer, prepared by dissolving 0.05 mol of citric acid in 1000 ml of water and adjusting the pH to 4.3 with NaOH, was used in all AcFY′ studies.

2.5. Enzyme solutions

Partially purified PLE (Sigma, Cat. No. E2884) was used in the studies of azaribine. A 3 ml aliquot of the esterase suspension [in 3.2 M $(NH_4)_2SO_4$ solution, ca. 40 mg ml^{-1}] was diluted to 15 ml with 50 mM phosphate buffer (pH 6.79). After it had been centrifuged at 1000*g* for 5 min, the supernatant was saved and stored in a refrigerator at 1°C. The stability of the enzyme and the activity of each enzyme preparation were assessed by the method of Zhou et al. [6]. Under two different sets of conditions (7.2 mg ml^{-1} protein at 0°C and 0.92 μg ml^{-1} protein at room temperature), PLE was stable for at least 2 days, with one preparation (7.4 mg ml^{-1}, 0°C) maintaining its activity for 48 days.

Crystallized and lyophilized pepsin (from porcine stomach mucosa; Sigma, Cat. No. P-7012) was purchased for the studies of AcFY′ hydrolysis. Pepsin solution was prepared by dissolving pepsin in 50 mM citrate buffer (pH 4.3). The concentration of active pepsin (ca. 0.1 mM) was titrated with a pepstatin solution of known concentration [7].

2.6. Substrate solutions

Azaribine (50 mg) was weighed and dissolved in 500 ml of MeOH–phosphate buffer (50 mM, pH 6.79) (4:96, v/v) immediately before the kinetic

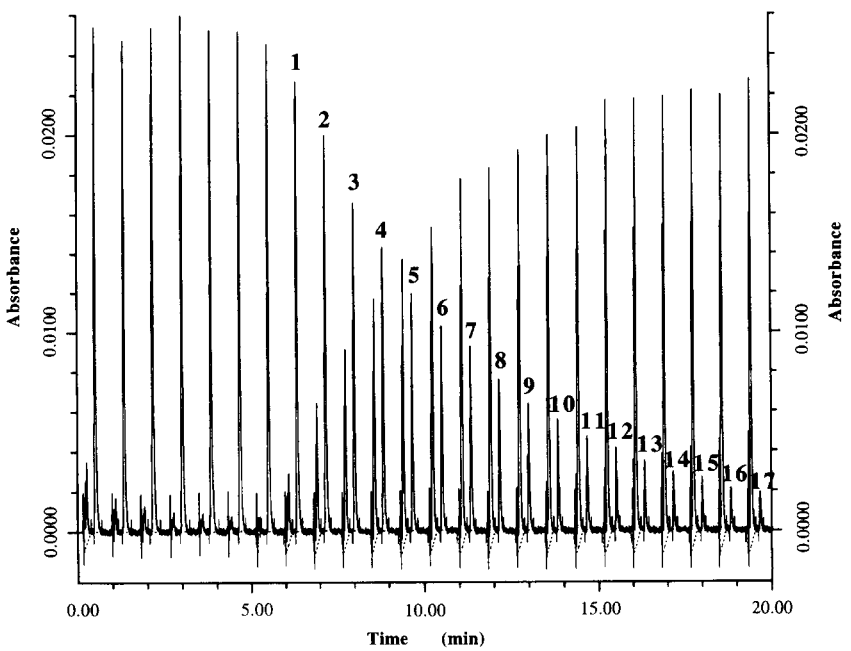

Fig. 5. Kinetic run showing the metabolism of azaribine by PLE at 30.0°C and pH 7.50. Stationary phase: ODS Hypersil column (3 μm, 30 mm × 4.6 mm i.d.) and guard column (5 μm, 10 mm × 4 mm i.d.). Mobile phase: MeOH–phosphate buffer (50 mM, pH 6.3) (34:66, v/v). PLE was added to the reaction medium at the fifth injection.

Table 1
Reproducibility of the automated analytical system and comparison of the results between manual and automated sampling

Method	Average k_{obs} (min^{-1})	Total enzyme activity (unit)	$10^5\ k_{obs}/A_T$ mean ± SD ($min^{-1}\ unit^{-1}$)	RSD (%)
Automated[a]	0.189	1947	9.71 ± 0.39	4.0
Manual[b]	0.236	2541	9.39 ± 0.55	5.8

[a]Substrate concentration ($n = 7$): 220 μM.
[b]Substrate concentration ($n = 2$): 216 μM.

experiments. AcFY′ stock solution was prepared by dissolving AcFY′ in 50 mM citrate buffer (pH 4.3).

2.7. Recovery of the microdialysis probes

The relative recovery of the microdialysis probe was determined before each kinetic run to calibrate and insure the integrity of the microdialysis probe. A separate set of experiments on relative recovery were also performed to study the system behavior with the DL-5 probe. The relative recovery of the microdialysis probe is defined by

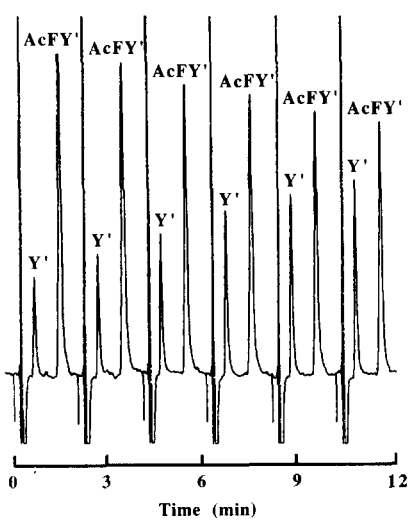

Fig. 6. Kinetic run showing the enzymatic hydrolysis of AcFY′ by pepsin in 50 mM citrate buffer (pH 4.3) at room temperature. Stationary phase: ODS Hypersil column (3 μm, 30 mm × 4.6 mm i.d.). Mobile phase: ACN–citrate buffer (50 mM, pH 2.3) (31:69, v/v).

$$R = \frac{C_{in}}{C_{out}} \quad (1)$$

where C_{in} and C_{out} are the concentrations of the analyte in the microdialysis medium and in the external solution, respectively.

2.8. Kinetic experiments

2.8.1. Automated system I

A 50 ml aliquot of the azaribine solution was incubated in the water-jacketed reaction vessel to a pre-set value of temperature. The pH of the solution was adjusted to 7.50 by adding a small amount (ca. 1.2 ml) of MeOH–sodium hydroxide solution (0.5 M) (4:96, v/v). The reactions were initiated by adding 1 ml of the PLE solution to the reaction medium. The reaction medium was kept homogeneous by stirring with a magnetic stirring bar. The pH of the reaction medium was maintained at 7.50 throughout the kinetic run using a pH-stat. A 5-cm DL-5 microdialysis probe was immersed in the reaction medium for sampling (Fig. 3). Unless mentioned otherwise, the microdialysis infusion pump was set at 4 μl min^{-1} and the probe was perfused with 1 M phosphate buffer (pH 6.4). The samples were collected every 45 s and injected on-line every 50 s (5 s at the injection position). The computer which controlled the LC pump and detector was set up to collect the data for the entire experiment and a printout of the results and chromatogram was obtained at the end of the run. Pseudo-first-order rate constants (k_{obs}) were measured by fitting the data by least-squares linear regression to the following equation:

$$\ln\left(\frac{D}{D_0}\right) = -k_{obs}t \quad (2)$$

where D and D_0 are the concentrations of the analyte at time t and time 0, respectively.

2.8.2. Manual sampling for azaribine metabolism studies

The same experimental procedures as above were adopted except that, instead of sampling by microdialysis, a 50 μl Hamilton (Reno, NV, USA) microliter syringe was used to take the sample and injected it directly into the HPLC system every 2 min.

2.8.3. Automated system II

A 2.50 ml AcFY′ solution of known concentration in 50 mM citrate buffer was placed in a 3.0 ml flat-bottomed polyethylene vial. A 3 × 10 mm magnetic stirring bar was placed in the vial to provide continuous mixing. The microdialysis probe was placed in the solution and was perfused with 50 mM citrate buffer (pH 4.3). After 15 min of equilibration time had elapsed, multiple injections were made into the HPLC system to establish that the system was stable. Freshly prepared pepsin solution was then added to the vial to start the enzymic reaction. The concentration of pepsin was sufficiently high that the reaction mixture was not diluted more than 5%. The final concentration of active pepsin in the reaction medium was ca. 10 μM. Samples were injected into the HPLC system every 2 min beginning 1 min after the addition of pepsin. The reaction was monitored until the reaction was ca. 40% complete. The kinetic runs were carried out at room temperature (24 ± 0.5°C).

2.8.4. Manual sampling for AcFY′ enzymatic hydrolysis studies

Enzyme was equilibrated with 50 mM citrate buffer at room temperature for 10 min. The reaction was started by adding the AcFY′ solution to the reaction vessel. The reaction was quenched by addition of an equal volume of 10% TFA 5 min after the reaction was started. The initial reaction rate was linear up to 20% of reaction. The sample was centrifuged to precipate protein and the supernatant was injected into the HPLC system. The rate was calculated by dividing measured product concentration by the time of reaction.

3. Results

3.1. Linearity and reproducibility of the HPLC assay

3.1.1. HPLC assay for azaribine metabolism studies

The linearity of the chromatographic system was determined over the range 11–320 μM by manual injection of solutions of azaribine in phosphate buffer (pH 6.79). Each solution was injected in duplicate. The mean peak area (A_P) of azaribine was related to the concentration injected by the equation

$$A_P = (9.79 \pm 0.04 \times 10^{-3})[\text{azaribine}] + (1.39 \pm 0.72 \times 10^{-2}) \quad (n = 6; r^2 = 1.000) \quad (3)$$

The intercept was less than 0.5% of the peak area at the highest standard concentration (319.7 μM). The reproducibility of the assay was determined at the lowest and highest concentration levels in the linearity study. The relative standard deviation (RSD) for the peak area at 319.7 μM ($n = 6$) and 11.63 ($n = 7$) was 0.2% and 2.8%, respectively.

3.1.2. HPLC assay for AcFY′ enzymatic hydrolysis studies

The linearity of the HPLC assay for the pepsin-catalyzed reaction was determined over the range of 0.2–1.0 mM for both AcFY′ and Y′ by manual injection. Each concentration solution was injected at least four times. The mean peak heights (H) of both AcFY′ and Y′ were related to their concentration by the following equations:

For Y′ with direct manual injection:

$$H = (10.41 \pm 0.15)[\text{Y}'] + (0.31 \pm 0.10) \quad (n = 5; r^2 = 0.9993) \quad (4)$$

For AcFY′ with direct manual injection:

$$H = (10.47 \pm 0.09)[\text{AcFY}'] + (0.11 \pm 0.06) \quad (n = 5; r^2 = 0.9998) \quad (5)$$

The intercept was less than 3.0% of the peak height at the highest standard concentration (1.007 mM) for Y′ and 1.2% of the peak height at

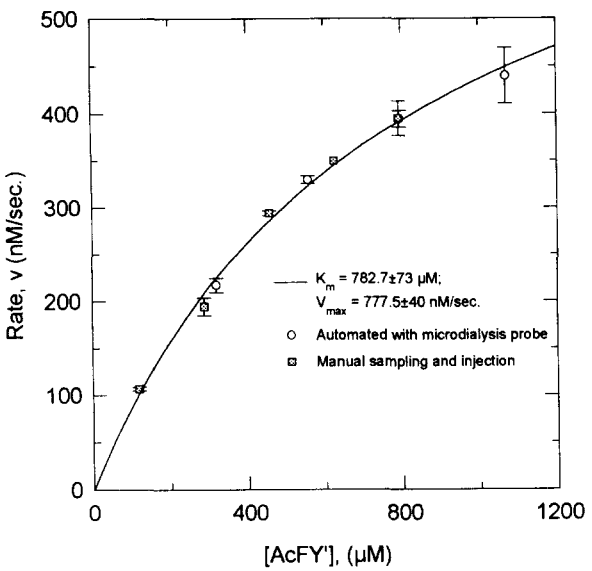

Fig. 7. Michaelis–Menten plot of initial rates of hydrolysis of AcFY′ measured by automated microdialysis sampling (open circles) and by manual sampling (filled squares). All data are fitted to the same function.

the highest standard concentration (0.965 mM) for AcFY′. The reproducibility of the assay for both compounds was examined at the lowest and highest concentration levels in the linearity study. The RSD for the peak height at each concentration injected ($n = 4$) was less than 2.3%.

3.2. Recovery and probe durability

3.2.1. DL-5 probe

The effect of perfusion rate on the relative recovery of azaribine with the DL-5 probe at 30°C is shown in Fig. 4. The relative recovery decreased with increasing prefusion rate, consistent with previous results [2] with different analytes and perfusate. A plot of $\ln(1 - R)$ versus $1/Fv$ gave a straight line (Fig. 4, inset) according to the following linear relationship [8]:

$$\ln(1 - R) = -\frac{PeS}{Fv} \quad (6)$$

where Pe is the effective solute permeability and S is the microdialysis membrane surface area. The excellent fit ($r^2 = 1.000$) of the data to Eq. (6) indicated that the system behaved well and the relative recovery of the system can be predicted from the perfusion rate. The PeS values (μl min^{-1}) for azaribine (mean ± SE; $n = 6$) with the DL-5 probe obtained in this study was 3.038 ± 0.025.

The durability of the DL-5 probe was studied by determining the relative recovery of the probe before each kinetic run. The concern was that, since enzyme was present in the reaction solution, it might be able to digest or clog the microdialysis membrane, hence changing the recovery of the probe. It was oberved that the relative recoveries (mean ± SD) of a single DL-5 probe for azaribine at 4 and 24 μl min^{-1} perfusion rates over 1 month were 0.642 ± 0.018 ($n = 17$) and 0.1065 ± 0.0034 ($n = 18$), respectively, revealing that the system was rugged.

3.2.2. CMA12 probe

Since a single-speed infusion pump was used, the relationship between recovery and perfusion rate was not investigated for AcFY′. Studies of the CMA12 probe with azaribine (data not shown) indicated that it was a well behaved system fitting Eq. (6) well. The durability of the CMA12 probe was studied by measuring the consistency of peak height before the kinetic runs. The ratio of the peak height to AcFY′ (mean ± SE, in cm mM^{-1}) remained within the limits of 14.5 ± 1.2 over 4 days of observation.

3.3. Reproducibility of the automated analytical system and comparison of the results between manual and automated sampling

3.3.1. Azaribine studies

A typical chromatogram of a kinetic run is shown in Fig. 5. The azaribine concentration decreased exponentially with time. The reproducibility of the automated analytical system was studied by repetition of kinetic experiments at low substrate concentration (220 μM). Table 1 shows that the precision of the automated analytical system was acceptable (RSD = 4%). The average k_{obs}/A_T (A_T = total enzyme activity in the reaction medium, in enzyme units) obtained by the automated sampling gave a value of (9.7 ± 0.4) × 10^{-5} min^{-1} unit^{-1}, which compares very favorably with the value of (9.4 ± 0.6) × 10^{-5} min^{-1} unit^{-1} measured by manual sampling.

Table 2
AcFY′ hydrolysis rate as a function of AcFY′ concentration

Automated sampling ($n = 4$)		Manual sampling ($n = 2$)	
[AcFY′] (μM)	V (mean ± SD) (nM s^{-1})	[AcFY′] (μM)	V (mean ± SD) (nM s^{-1})
		118	106.5 ± 2.0
319	217.2 ± 7.6	287	194.5 ± 9.2
557	329.7 ± 4.0	456	294.2 ± 2.5
796	394.5 ± 8.8	625	350.0 ± 0.0
1070	439.7 ± 29	793	395.0 ± 18

3.3.2. AcFY′ enzymatic hydrolysis studies

A typical chromatogram for the automated analytical system is shown in Fig. 6. Fig. 7. shows the initial rate of pepsin-catalyzed hydrolysis of AcFY′ as a function of AcFY′ concentration. Data from both manual and automated experiments are included. The Michaelis–Menten equation is obeyed with $K_m = 783 \pm 73$ μM and $V_{max} = 778 \pm 40$ μM s^{-1} ($k_{cat} = 78 \pm 4$ s^{-1}). As can be seen from Fig. 7 and Table 2, the automated and manual systems behave equally well, and the data are indistinguishable (no previous studies for AcFY′ at this pH have been reported).

4. Discussion

The HPLC assays used for azaribine and AcFY′ are linear and reproducible. The DL-5 and CMA-12 probes give adequate and reproducible recovery. They are also adequately durable and behave as theoretically predicted. The two automated systems gave rates and rate constants which were in agreement with manual sampling results.

First-order kinetics were unexpectedly observed with azaribine hydrolysis by PLE over a wide range of the substrate concentrations, with the result that the evaluation of k_{cat} and kM was not possible. This behavior has implications for the enzyme mechanism of this substrate with PLE, which are the subject of a report in preparation.

5. Conclusion

Automated systems coupling HPLC and on-line microdialysis sampling constitute a convenient and effective method for enzyme kinetic studies. The extension to inhibitor characterization and screening and drug metabolism in pharmaceutical research and development will be apparent.

Acknowledgements

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