ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1995) 113–119

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Dual-electrode amperometric detection for the determination of SR4233 and its metabolites with microbore liquid chromatography ☆

Xiao-Zhong Liang, Rita K. Palsmeier [1], Craig E. Lunte *

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

Received for review 30 May 1995

Abstract

3-Amino-1,2,4-benzotriazine-2,4-di-*N*-oxide (SR4233) is a promising new antineoplastic agent based on reductive activation. SR4233 and its major metabolites (SR4317 and SR4330) are all easily reduced at a carbon electrode. Reductive amperometric detection can therefore provide high selectivity and low detection limits with chromatographic analysis and is an ideal approach to detection of SR4233 in microdialysis samples. However, in order to use amperometric detection in the reductive mode, sample deoxygenation is necessary. This is typically done by purging the sample with either argon or nitrogen prior to injection. This approach is not feasible for microdialysis samples because only 5–10 μl is usually available. In this report, a microbore liquid chromatographic method with dual-electrode amperometric detection is described for the determination of SR4233 and its metabolites without predeoxygenation. A dual-electrode amperometric detector was used in the series configuratioin with an upstream potential of −450 mV to reduce SR4233 and its metabolites to a common product and a downstream potential of +400 mV to oxidize this product. Oxygen is only electroactive at the upstream electrode because of its irreversible behavior. This method is compatible with the small sample volumes provided by microdialysis sampling. Linear calibration graphs were obtained up to 55 μM for SR4233, and 140 μM for both SR4317 and SR4330. The detection limits were 70 nM for SR4233, and 50 nM for SR4317 and SR4330. The average intra-day variation over 5 days was 1.8% (SR4233), 1.4% (SR4330), and 1.8% (SR4317), whereas the inter-day variation over 5 days was 14.1% (SR4233), 8.6% (SR4317), and 2.6% (SR4330).

Keywords: SR4233; Benzotriazenes; Dual-electrode LCEC; Reductive amperometric detection; Microbore LC

1. Introduction

Conventional antitumor agents have been successful in treating many types of cancers. However, solid tumors still present a difficult target for both chemotherapy and radiotherapy approaches

☆ Presented at the Sixth International Symposium on Pharmaceutical and Biomedical Analysis, April 1995, St. Louis, MO, USA.

* Corresponding author.

[1] Present address: Sigma Chemical Co., St. Louis, MO 63178, USA.

SSDI 0731-7085(95)01585-X

[1–3]. It is believed that one of the important contributory factors to the resistance of radio- or chemotherapy is the presence of hypoxic cells in solid tumors [1,4]. Recently, there has been a considerable effort toward the development of hypoxic cell specific cytotoxins [5]. There are three classes of compounds which belong to this category: nitroimidazole hypoxic cell sensitizers, quinone bioreductive alkylating agents such as mitomycin C, and benzotriazine-*N*-oxides [6]. 3-Amino-1,2,4-benzotriazine-2,4-di-*N*-oxide (SR4233), which is the lead compound of the benzotriazine-*N*-ozide series, has shown considerable hypoxic cell killing selectivity in both in vitro and in vivo studies [7–9], and is currently in a phase I clinical trial [10].

In recent years, in vivo microdialysis sampling has been used for the study of pharmacokinetics and drug metabolism in blood, muscle, tumor, and other tissues [11–13]. Microdialysis sampling is performed by implanting a semipermeable dialysis membrane at the site of interest. A sampling solution is then slowly perfused through the implanted membrane. One of the advantages of microdialysis sampling is that only low molecular weight species in the extracellular space are collected with minimal disturbance to the subject under study [14]. However, with a typical perfusion rate of 1 μl min^{-1}, only a 5–10 μl microdialysis sample volume is available for analysis. Therefore, a low detection limit analytical method with small sample volume requirements is needed in order to fully utilize microdialysis sampling for the study of pharmacokinetics and drug metabolism.

Two liquid chromatographic methods have been published for the analysis of SR4233 and its two major metabolites, the 1-*N*-oxide (SR4317) and the des-oxo (SR4330) [15,16]. Both methods used analytical scale chromatographic columns (i.e. i.d. $\geq$ 4 mm) with UV absorbance detection. Sample volumes of at least 50 μl were needed to obtain a detection limit of 2 μM (0.4 μg ml^{-1}). These analytical methods were not compatible with microdialysis sampling, and its sample volume and detection limit requirements. In this report, amperometric detection was employed with microbore liquid chromatography to achieve improved detection limits. The use of the dual-electrode series configuration of the detector to eliminate oxygen interferences in microdialysis samples is described.

2. Experimental

2.1. Chemicals and Reagents

3-Amino-1,2,4-benzotriazine-1,4-di-*N*-oxide (SR4233), 3-amino-1,2,4-benzotriazine-1-*N*-oxide (SR4317), and 3-amino-1,2,4-benzotriazine (SR4-330) were gifts from Sterling Drug Company (New York, NY, USA). HPLC grade acetonitrile was obtained from Fisher Scientific (Fairlawn, NJ, USA). Solutions were prepared with water from a Barnstead Nanopure water purification system (Boston, MA, USA). All other chemicals were reagent grade or better and used as received.

2.2. Chromatographic system

Liquid chromatographic analysis was carried out using a reverse-phase microbore system with electrochemical detection. The system consisted of a PM-60 pump, a CMA/200 microsampler, and dual LC-4B amperometric detectors (Bioanalytical Systems, Inc., West Lafayette, IN, USA). Data collection was by an SP400 integrator connected to an IBM-compatible personal computer running WINNER/386 software (Spectra-Physics/SP ThermoSeparations).

The separation conditions were similar to those described by Cahill et al. [15], except that a microbore LC column was used along with appropriate modification to the mobile phase. Separation was achieved on a 3 μm ODS Sep-Stik microbore column (1 mm × 100 mm) from Bioanalytical Systems, Inc. The column was protected with a repackable microbore guard column (1 × 20 mm) from Upchurch Scientific (Oak Harbor, WA, USA). The mobile phase was a 4% acetonitrile aqueous solution with 50 mM sodium phosphate buffer (pH 2.5) and was deoxygenated with a constant argon purging prior to use. The flow rate was 75 μl min^{-1} and the injection volume was 3.5 μl for all samples unless otherwise specified.

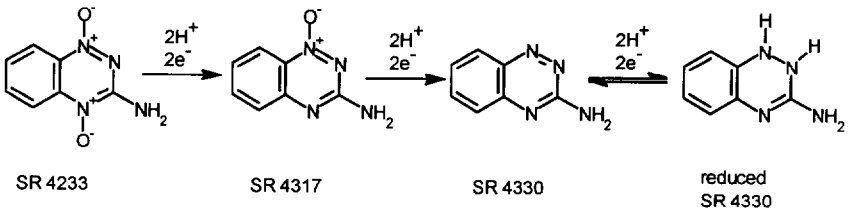

Fig. 1. Mechanism for the electrochemical reduction of SR4233, SR4317, and SR4330.

The dual-electrode thin-layer electrochemical cell was operated in the series configuration using glassy carbon working electrodes. The upstream electrode was operated at −450 mV versus a Ag/AgCl reference electrode and the downstream electrode was operated at +400 mV versus Ag/AgCl. All potentials are reported versus the Ag/AgCl reference electrode. Only the current at the downstream electrode was monitored for detection. The upstream electrode was used to remove the interference from oxygen.

2.3. Microdialysis sampling

Microdialysis sampling was performed using a CMA/100 microinjection pump (Bioanalytical Systems, Inc., West Lafayette, IN, USA) coupled to a linear microdialysis probe. The probe fabrication procedure was described previously [12]. The perfusion fluid was a Ringer's solution consisting of 155 mM NaCl, 5.5 mM KCl, and 2.3 mM $CaCl_2$. A perfusate flow rate of 1 μl min^{-1} with a 5 min collection interval was used. The microdialysis samples from the muscle or tumor of three to four month-old male American Cancer Institute rats were directly injected into the chromatographic system without prior cleanup.

3. Results and discussion

3.1. Microbore column

Microdialysis sampling is a continuous technique which is typically coupled to liquid chromatography as an analytical method. For the study of pharmacokinetics, the required temporal resolution of the experiment, dialysis perfusion rate, and sample volume for analysis are all interdependent. The faster the flow rate of the perfusion solution, the lower the recovery and therefore the lower the concentration of the dialysate obtained for analysis. Larger sample volumes take longer to collect and therefore provide poorer temporal resolution. To maintain sufficient temporal resolution and achieve reasonable recovery, a perfusion rate of 1 μl min^{-1} and collection interval of 5 min were used in this study. These conditions provide a recovery of approximately 45% and 5 μl samples for analysis. Any detection advantage from sample preconcentration is precluded by the small total volume. Sample dilution in the LC column becomes a significant issue with such small sample volumes. To minimize dilution during separation, a microbore column with an internal diameter of 1 mm packed with 3 μm particles was used to minimize band broadening. Injection of a 3.5 μl sample resulted in a peak with a 32.5 μl width.

3.2. Amperometric detection

Detailed electrochemical studies of SR4233 and its major metabolites have been reported [17,18]. All three benzotriazines are reduced to a common product (reduced SR4330) at −450 mV (Fig. 1). SR4233 undergoes a six-electron reduction process at this potential, whereas SR4317 and SR4330 undergo four-electron and two-electron reductions respectively. However, at the potential necessary to reduce the benzotriazines, oxygen is also reduced. The interference of oxygen in the mobile phase can easily be removed by an argon purge of the mobile phase reservoir. Unfortunately, it is not practical to argon purge a 5 μl microdialysis sample. Under the chromatographic

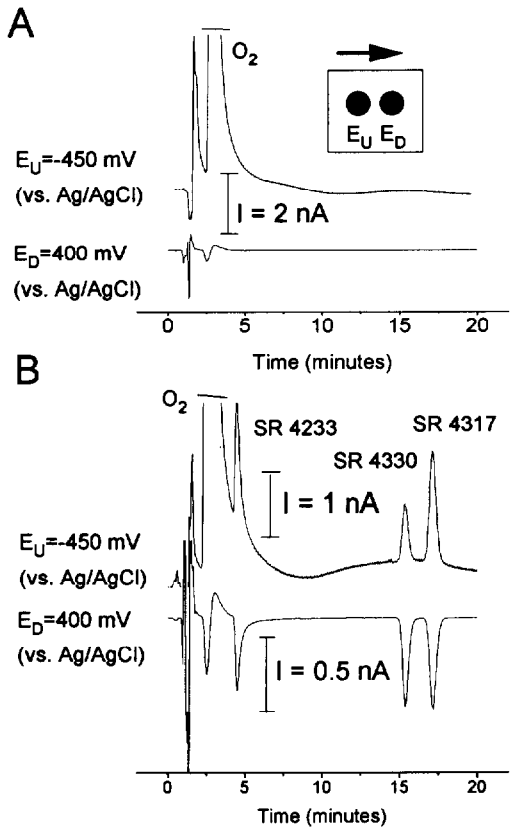

Fig. 2. Dual-electrode chromatograms of (A) blank (mobile phase) and (B) standard mixture (1 μM each compound).

conditions used in this study, oxygen almost co-elutes with SR4233 and significantly interferes with its detection. This oxygen interference can be removed by taking advantage of the electrochemical behavior of the benzotriazines relative to oxygen. Reduced SR4330 can be reversibly oxidized to SR4330 at a potential of +400 mV (Fig. 1), but the reduction products of oxygen are not oxidizable at this potential. A dual-electrode electrochemical cell in the series configuration can therefore be used to eliminate the oxygen interference (Fig. 2). The upstream electrode is operated at a potential of −450 mV in order to reduce SR4233 and its metabolites to reduced SR4330. The direct reduction current is not analytically useful for detection of SR4233 because of the concurrent reduction of oxygen (Fig. 2(A)). The downstream electrode is used to re-oxidize the reduced SR4330 produced at the upstream electrode without interference from oxygen. The collection efficiency (the amount of upstream product detected at the downstream electrode) was 64% under the conditions of these experiments. The current at the downstream electrode is used as the analytical signal.

3.3. Linear range and detection limit

Stock solutions of SR4233, SR4317, and SR4330 were prepared by directly dissolving an appropriate amount of standard in purified water. A series of standards were then prepared with purified water. The detector response was linear up to 55 μM for SR4233 and 140 μM for both SR4317 and SR4330. The smaller linear range is probably due to saturation of the current at the upstream electrode. At this electrode, SR4233 undergoes a six-electron reduction. The current is also higher because less on-column dilution of SR4233 occurs relative to SR4317 or SR4330 owing to its short retention time. The extrapolation of all three calibration graphs passed through zero (Table 1). The detection limits (S/N = 3) were 50 nM for SR4317 and SR4300 and 70 nM for SR4233. These compare very favorably with the previously published detection limits of 2 μM for SR4233 [15]. These parameters provide a suitable working range for the study of the pharmacokinetics of SR4233 in muscle and tumor using

Table 1
Calibration parameters for the benzotriazines

Compound	Linear range (μM)	Sensitivity (nA μM^{-1})	Detection limit (nM)	Correlation (R)
SR4233	0–55	3.07	70	0.9998
SR4317	0–140	1.64	50	0.999
SR4330	0–140	2.23	50	0.9998

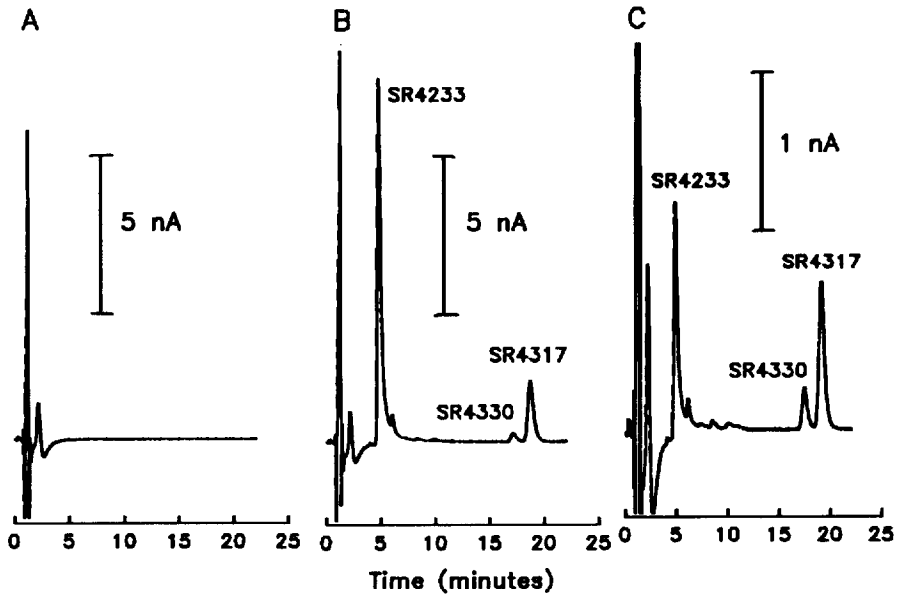

Fig. 3. Chromatograms of blood dialysates. (A) Blank prior to dosing with SR4233; (B) sample from the 5–10 min interval after a 3.0 mg kg^{-1} i.v. dose of SR4233; (C) sample from the 35–40 min interval after dosing.

microdialysis sampling. Chromatograms of blood dialysates from an ACI rat dosed with SR4233 (3.0 mg per kg, i.v.) are shown in Fig. 3. Chromatograms of muscle and tumor dialysates following i.v. dosing of SR4233 are shown in Fig. 4.

3.4. Electrode reproducibility and stability

The system reproducibility was evaluated by analyzing a 1 μM standard solution of each of the benzotriazines. Ten standards were analyzed each day for five days. The results are shown in Table 2, in which the average intra-day variations over five days were 1.8%, 1.4%, and 1.8% for SR4233, SR4300, and SR4317 respectively. These results are somewhat high owing to poor reproducibility on day 1 (Table 2), a result for which we do not have an explanation other than operator inexper-ience. The inter-day variations over the five days were 14.1% (SR4233), 8.6% (SR4317), and 2.6% (SR4330). As expected, the intra-day variation was greater than the inter-day variation for all three compounds. The intra-day variance was particularly high for SR4233. This is partly due to its short retention time and therefore higher sensitivity to slight variations in mobile phase composition. This can be seen in the higher day-to-day variation in retention time for SR4233 relative to the other compounds (Table 2). To correct for the intra-day variation, the system was calibrated each day before the pharmacokinetic experiment by analysis of three standards. The same standard solutions were injected after the experiment to verify the stability of the system.

The stability of the working electrodes depended upon the extent of use. A slight decline in response was noted after several days of constant use with either dialysates or standards. This was compensated for by making injections of standard samples at regular intervals to provide calibration (every 50 injections) and to monitor the detector response. When the detector response had declined to <90% of initial values the working electrodes were repolished.

4. Conclusion

This report demonstrates the utility of microbore liquid chromatography with electrochemical

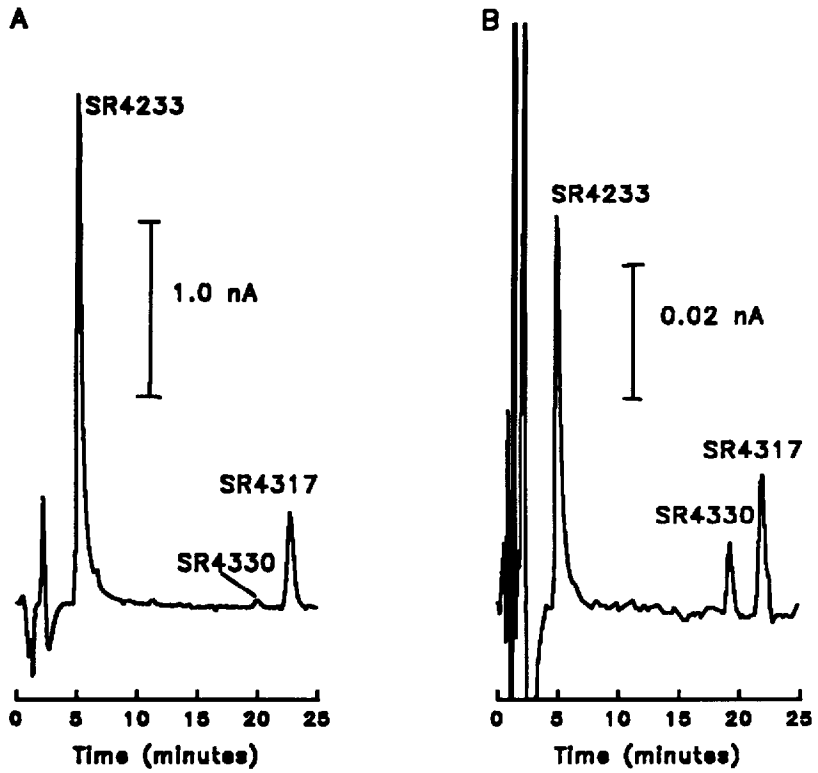

Fig. 4. Chromatograms of tissue dialysates from the 15–20 min interval following a 3.0 mg kg^{-1} i.v. dose of SR4233. (A) Muscle dialysate; (B) tumor dialysate.

Table 2
Reproducibilities for SR4233, SR4317, and SR4330

Compound	Parameter	Day 1	Day 2	Day 3	Day 4	Day 5	Average	SD (%)
SR4233	PKHT	57191	67622	65431	53140	48043	58286	14.1
	SD%	3.7	1.8	1.0	1.6	0.9	1.8	
	RT	2.93	2.79	2.91	3.67	3.76	3.21	14.4
	SD%	0.53	0.35	0.23	1.30	1.63	0.81	
SR4317	PKHT	95773	94993	92639	93342	89648	93279	2.6
	SD%	3.9	0.9	0.5	0.8	0.7	1.4	
	RT	10.53	0.97	10.33	12.89	13.25	11.39	13.6
	SD%	0.44	0.13	0.23	1.11	1.68	0.72	
SR4330	PKHT	80614	90647	88042	77604	73884	82158	8.6
	SD%	3.8	1.6	1.4	1.2	0.8	1.8	
	RT	9.69	9.14	9.49	11.85	12.16	10.47	13.6
	SD%	0.42	0.17	0.18	1.14	1.66	0.71	

PKHT = peak height; RT = retention time

detection for the analysis of microdialysis samples. Interference from oxygen with reductive mode detection can be accomplished using a dual-electrode detector in the series configuration if the analyte is reversibly reduced.

Acknowledgments

Financial support for this work was provided by the NIH through grant R01GM45566 and an NCI training grant.

References

[1] J.E. Moulder and S. Rockwell, Int. J. Radiat. Oncol. Biol. Phys., 10 (1984) 695–712.

[2] I.F. Tannock and P. Guttman, Br. J. Cancer, 43 (1981) 245–248.

[3] J.S. Bedford and J.B. Mitchell, Br. J. Radiol., 47 (1974) 687 696.

[4] G.E. Adams, in A. Breccia, C. Rimondi and C.E. Adams (Eds.), Radiosensitizers of Hypoxic Cells, Elsevier, Amsterdam, 1979, pp. 13–29.

[5] R.M. Sutherland, Cancer Res., 34 (1974) 3501–3503.

[6] K.A. Kennedy, B.A. Teicher, S. Rockwell and A.C. Sartorelli, Biochem. Pharmacol., 29 (1980) 1–80.

[7] E.M. Zeman, J.M. Brown, M.J. Lemmon, V.K. Hirst and W.W. Lee, Int. J. Radiat. Oncol. Biol. Phys., 12 (1986) 1239–1242.

[8] E.M. Zeman and J.M. Brown, Int. J. Radiat. Oncol. Biol. Phys., 16 (1989) 967–971.

[9] C.J. Koch, Cancer Res., 53 (1993) 3992–3997.

[10] J.B. Brown, Br. J. Cancer, 67 (1993) 1163–1170.

[11] D.O. Scott and C.E. Lunte, Pharm. Res., 10 (1993) 335–342.

[12] Rita K. Palsmeier and Craig E. Lunte, Life Sci., 55 (1994) 815–825.

[13] T. Obata, H. Hosokawa and Y. Yamanaka, Comp. Biochem. Physiol. C, 106 (1993) 629–634.

[14] C.E. Lunte, D.O. Scott and P.T. Kissinger, Anal. Chem., 63 (1991) 773A–780A.

[15] A. Cahill, T.C. Jenkins and I.N.H. White, Biochem. Pharmacol., 45 (1993) 321–329.

[16] M.I. Walton and P. Workman, J. Chromatogr., 430 (1988) 429–437.

[17] J.H. Tocher, N.S. Virk and D.I. Edwards, Biochem. Pharmacol., 40 (1990) 1405–1410.

[18] J.H. Tocher, N.S. Virk and D.I. Edwards, Biochem. Pharmacol., 39 (1989) 781–786.