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Journal of Chromatography B, 709 (1998) 35–45

JOURNAL OF CHROMATOGRAPHY B

Simple microbore high-performance liquid chromatographic method for the determination of dopamine and cocaine from a single in vivo brain microdialysis sample

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Received 31 July 1997; received in revised form 8 January 1998; accepted 12 January 1998

Abstract

A microbore chromatographic method for the analysis of both dopamine and cocaine from in vivo brain microdialysis samples is described. To eliminate the need for separate chromatographic systems for each analyte, post-column electrochemical and ultraviolet detection systems were arranged in series. The limit of quantitation for dopamine (5 fmol) was well within range for detecting dialysate concentrations of this neurotransmitter in rats which were in a baseline, drug-free state. The limit of quantitation for cocaine (0.5 pmol) was sufficient to detect brain cocaine levels following the peripheral administration of a low dose of this psychostimulant (5 mg/kg, i.p.). Estimates of dialysate dopamine and cocaine concentrations after 5, 10 and 20 mg/kg cocaine (i.p.) were in agreement with reports which utilized separate HPLC analyses for each analyte. © 1998 Elsevier Science B.V.

Keywords: Dopamine; Cocaine

1. Introduction

In recent decades cocaine has become one of the most abused psychomotor stimulant drugs, and substantial research efforts have been directed at identifying the neural mechanisms which mediate the rewarding and addictive effects of this alkaloid. There is now a large body of evidence which suggests that the reinforcing effects of cocaine are dependent on its ability to enhance synaptic concentrations of the catecholamine neurotransmitter dopamine in the terminal regions of the mesocorticolimbic system of the brain [1,2].

The concurrent analysis of both dopamine and cocaine levels in biological samples is of obvious utility for studies concerned with the short- and long-term consequences of cocaine on dopamine neurotransmission, pharmacokinetic analyses, as well as for research involving the development of drug treatments which may alter the ability of cocaine to elevate synaptic dopamine concentrations. In an effort to characterize the relationship between brain cocaine concentrations and alterations in dopamine neurotransmission, recent studies have employed the in vivo microdialysis technique [3] to sample extracellular brain levels of both of these analytes in rats [4–6]. Once collected, these microdialysis samples are typically split and analyzed by separate high-performance liquid chromatography (HPLC) systems optimized for the detection of dopamine

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0378-4347/98/$19.00
PII S0378-4347(98)00024-3

[HPLC–electrochemical detection (ED)] or cocaine [HPLC–ultraviolet detection (UV)]. The need for separate analytical systems for these analytes creates at least two difficulties. First, in order to collect sufficient sample volumes for these separate assays, relatively fast microdialysis perfusate flow-rates are typically required (>1–2 μl/min). These flow-rates may produce more substantial neurochemical depletions around the microdialysis probe than slower flow-rates [7], and with sufficiently high flow-rates neurochemicals may be sampled from brain regions beyond the anatomical structure of interest. Second, separate analytical systems for the determination of each analyte can be prohibitive with respect to instrumentation cost and maintenance.

To circumvent these problems, we have developed and characterized a simple chromatographic method for the analysis of both dopamine and cocaine from a single, small volume (5 μl) dialysate sample.

2. Experimental

All chemicals for the chromatographic mobile phase and artificial cerebrospinal fluid (aCSF) were of reagent grade and were obtained from Sigma (St. Louis, MO, USA) with the exception of tetrahydrofuran which was from J.T. Baker (Phillipsburg, NJ, USA). The dopamine chromatographic standard was purchased from Sigma. Cocaine HCl (used both as a chromatographic standard and as the pharmacological agent for the in vivo microdialysis experiments) was obtained from the National Institute on Drug Abuse.

2.1. LC instrumentation for dopamine and cocaine determination

Five-μl sample volumes were injected onto a HPLC system equipped with a 1×100 mm column (SepStik® packed with 3 μm C_{18} spheres from BioAnalytical Systems, West Layfayette, IN, USA) over which a mobile phase consisting of a 50 m*M* NaH_2PO_4 buffer with 24% tetrahydrofuran (v/v), 0.2 m*M* Na_2EDTA, 3.7 m*M* 1-decanesulfonic acid and 4.9 m*M* triethylamine (apparent pH of 5.5) was pumped at 23 μl/min using an ISCO Model 500D pulseless syringe pump (Lincoln, NE, USA). The eluent from the column was delivered directly to a standard electrochemical cell containing two glassy carbon working electrodes (EG&G Princeton Applied Research, Model MP1304; Trenton, NJ, USA) arranged in series (see Fig. 1). The first electrode in the flow path was set at +700 mV against an Ag/AgCl reference electrode (Model RE4; BioAnalytical Systems) and was used to reduce the solvent front signal at the second working electrode. The second electrode was set at 0 mV and was used for the measurement of the reduction peak produced by dopamine. The electrode potentials and current analyses were controlled by an EG&G Princeton Applied Research amperometric detector (Model 400). After traversing the electrochemical cell, the eluent was delivered directly to the flow cell of a SpectraPhysics (San Jose, CA, USA) SpectraFocus scanning multiple-wavelength ultraviolet detector for the analysis of cocaine (flow cell absorption pathlength, 3 mm; total cell volume, 1.2 μl). The maximum UV absorbance for cocaine was determined to be at a wavelength of 225 nm (see Section 3.1) and absorbance at this wavelength was used for all quantitative analyses. External calibration curves were generated daily from fresh standard solutions for each analyte, made in an aqueous ascorbic acid solution (0.25 m*M*). The electrochemical signal (dopamine) was recorded by a Kipp and Zonen strip chart recorder (Model BD112) and the UV signal was recorded by a SpectraPhysics SpectraSYSTEM acquisition/analysis system.

2.2. Dose–effect analysis of nucleus accumbens dopamine and cocaine levels using in vivo microdialysis

Male Wistar rats (n=12; Charles River, Holister, CA, USA) weighing 250–275 g were housed in groups of two or three in a humidity- and temperature-controlled (22°C) vivarium on a 12-h light–dark cycle (lights off 1800 h). Animals had ad libitum access to food and water throughout the course of the study. All procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

At least one week prior to the microdialysis experiments the rats were anesthetized with halothane (1–2%) and stereotaxically implanted with

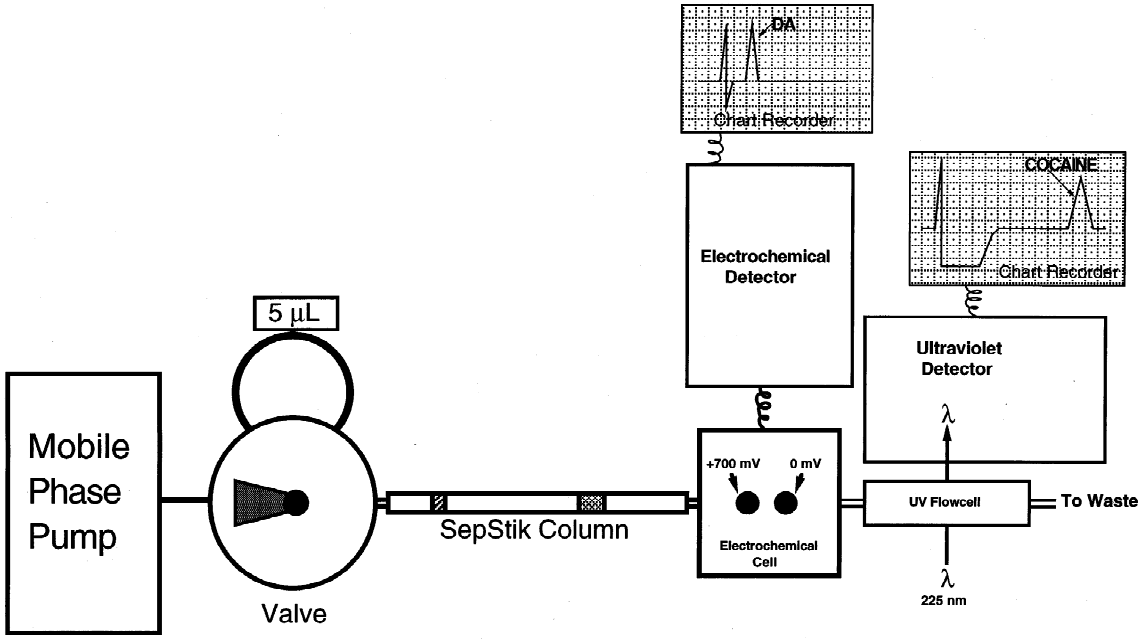

Fig. 1. Diagram of the HPLC and detector configuration.

a sterilized stainless steel microdialysis guide cannula (21 gauge; Plastics One, Roanoke, VA, USA) which terminated at the dorsal surface of the nucleus accumbens (AP, +1.7 mm; ML, ±1.4 mm; V, −6.1; according to the atlas of Paxinos and Watson [8]). The cannula was secured to the skull with sterilized stainless steel screws and methylmethacrylate cement. Stylets cut to the length of the cannula were inserted to prevent cannula blockade and the introduction of foreign particles. Approximately 12 h before the start of the experiment, animals were lightly anesthetized (1–2% halothane) and microdialysis probes were inserted through the guide cannula. Animals regained consciousness and movement within 3 min of probe insertion. Microdialysis probes were constructed using regenerated cellulose membrane (300 μm O.D.; 13 000 Da cut-off; Spectrum Medical Industries, Houston, TX, USA) with a 2 mm active length (the probes were designed to extend 2 mm beyond the tip of the guide cannula) as described elsewhere [9]. The perfusion medium consisted of an aCSF consisting of 145 m*M* NaCl, 2.8 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 0.25 m*M* ascorbic acid and 5.4 m*M* D-glucose (pH 7.2–7.4) and was delivered to the probe using a Harvard Apparatus syringe pump (Model 975; South Natick, MA, USA) via a liquid swivel (Model 375/D/22QE; Instech, Plymouth Meeting, PA, USA) attached to a balance arm above the animals' cage to ensure freedom of movement during the experiment. To minimize disturbance to the brain extracellular environment, the perfusate flow-rate was 0.1 μl/min from the time of probe implantation until 2 h prior to the start of the experiment, at which time the flow-rate was increased to 0.6 μl/min. This latter flow-rate was chosen for the experiments to provide both adequate sample volume for analysis and reasonable time resolution (i.e., 10 min sampling intervals) for monitoring interstitial cocaine concentrations and cocaine-induced alterations in dopamine levels. At the start of the experiment, five baseline samples were collected to provide a reference ''drug-free'' (i.e., baseline) dopamine concentration. Subsequently, each animal was given an intraperitoneal (i.p.) injection of cocaine [either 5 mg/kg (n=4), 10 mg/kg (n=5) or 20 mg/kg (n=3)] and dialysate samples were collected for an additional 120 min. Immediately after collection, dialysate samples were

frozen on dry ice, and then stored at −70°C for later HPLC analysis. At the end of the experiment, the anatomical placement of the microdialysis probes was determined by comparing 50 μm coronal brain sections from each animal with the atlas of Paxinos and Watson [8]. Examination of these slices with a light microscope confirmed that >80% of the active dialysis membrane was located in the nucleus accumbens.

3. Results and discussion

3.1. Characterization of the HPLC separation and the detection of dopamine and cocaine

In developing this method, the primary difficulty was obtaining a separation which adequately resolved DA from the solvent front, without unreasonably extending the elution time for cocaine. Brain microdialysates contain high levels of ascorbic acid and acidic monoamine metabolites, which are present at more than a 1000-fold greater concentration than baseline neurotransmitter concentrations. With mobile phases which elute cocaine within a reasonable amount of time (i.e., in less than 20 min), the elution of ascorbate lasts more than 7 min, making the determination of low concentrations of dopamine (which elutes within the ascorbate front) nearly impossible by standard oxidation. However, once ascorbate is oxidized it is not subsequently reducible whereas, dopamine is both readily oxidized and reduced. Thus the first glassy carbon electrode in the flowpath was used to oxidize electroactive constituents while the second electrode was used to reduce those analytes which undergo reversible electrochemical reactions (i.e., dopamine and the monoamine metabolites) with minimal interference from ascorbate. At the relatively high mobile phase pH employed, the monoamine metabolites eluted very early and did not interfere with the dopamine peak. Using this technique it was possible to detect low concentrations of dopamine with an elution time of approximately 5 min. Representative chromatograms of a 5 n*M* dopamine standard solution and a baseline (i.e., ''cocaine-free'') microdialysate sample from the nucleus accumbens are shown in Fig. 2.

The retention time for cocaine was approximately 25 min under these chromatographic conditions. Cocaine has been reported to be electroactive at the electrode potentials employed in the present method [10]. Because the UV flowcell was positioned after the electrochemical cell, it is possible that some cocaine is degraded as it passes by the active electrochemical electrode, thus potentially altering the UV detection of cocaine. To investigate this possibility, the UV absorption (λ=210–310 nm) of two cocaine standard solutions (0.125 and 4 m*M* cocaine) was examined under conditions of both an active and inactive electrochemical cell. The pres-

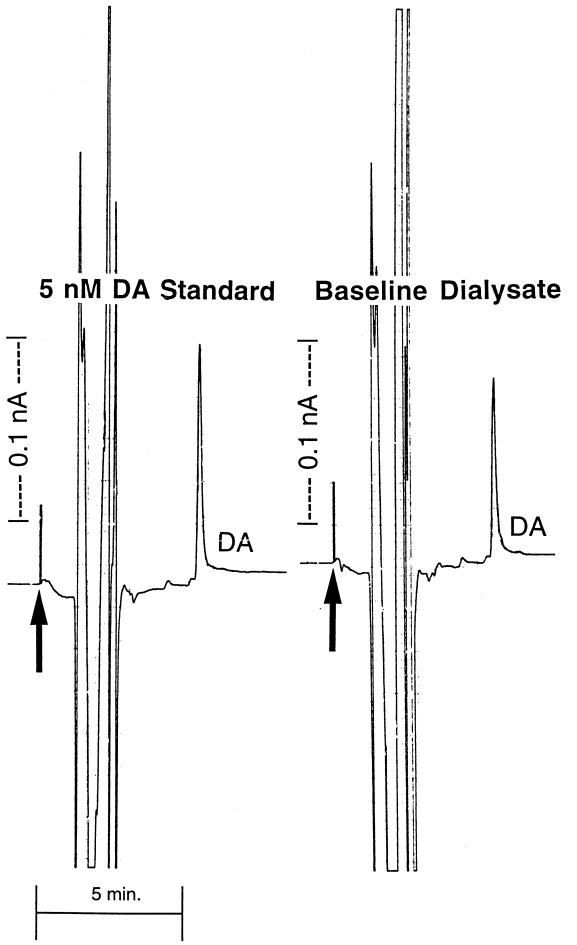

Fig. 2. Representative chromatograms of a 5 n*M* dopamine (DA) standard (left) and a baseline dialysate sample collected from the nucleus accumbens of a rat (right). Both traces were recorded from the second (reducing) glassy carbon electrode.

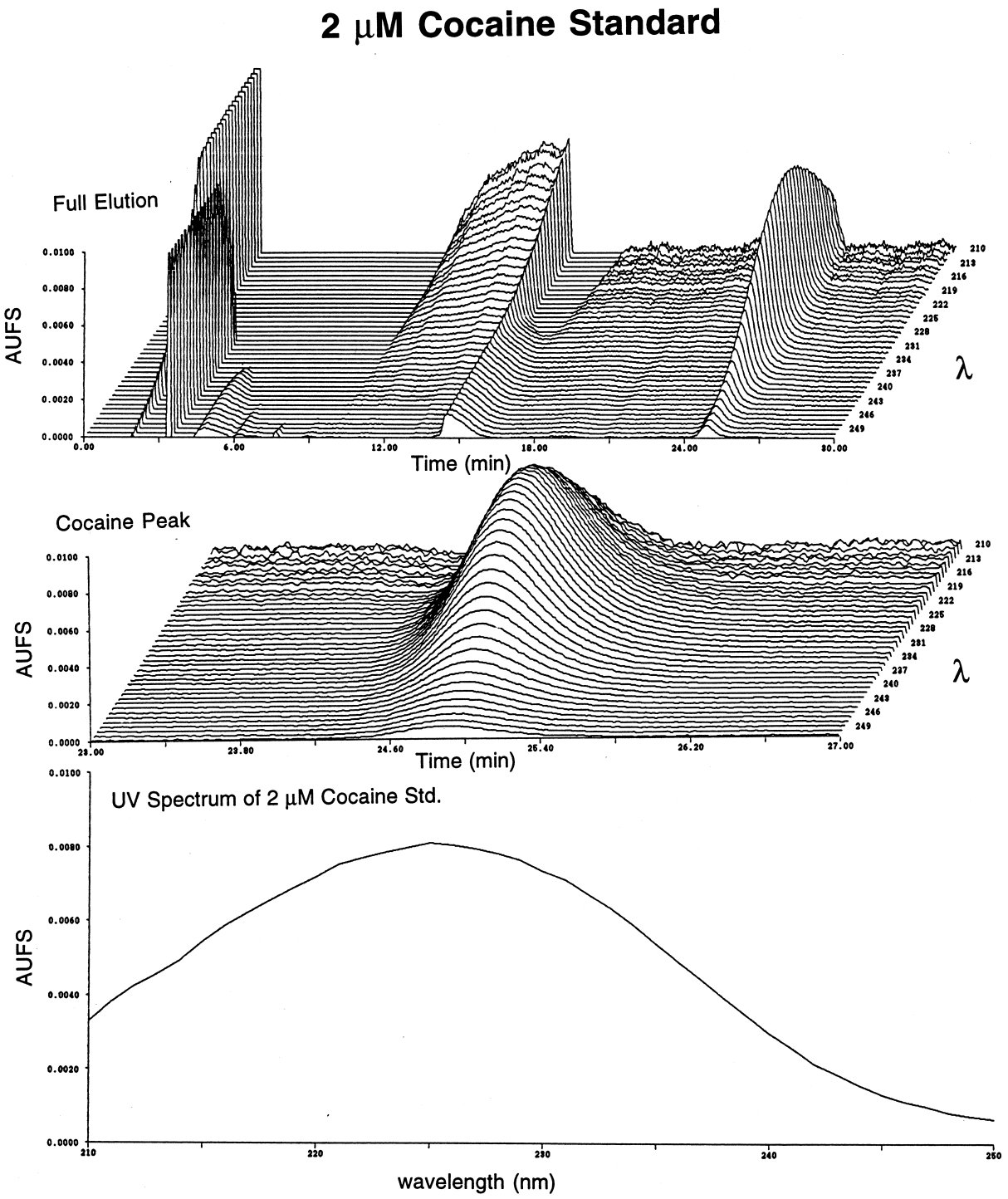

Fig. 3. Representative UV chromatogram of a 2 μ*M* cocaine standard (0.01 AUFS). The full elution pattern is shown in the top panel, with the cocaine peak enlarged for clarity in the middle panel. The UV spectrum (210–250 nm) is shown in the bottom panel.

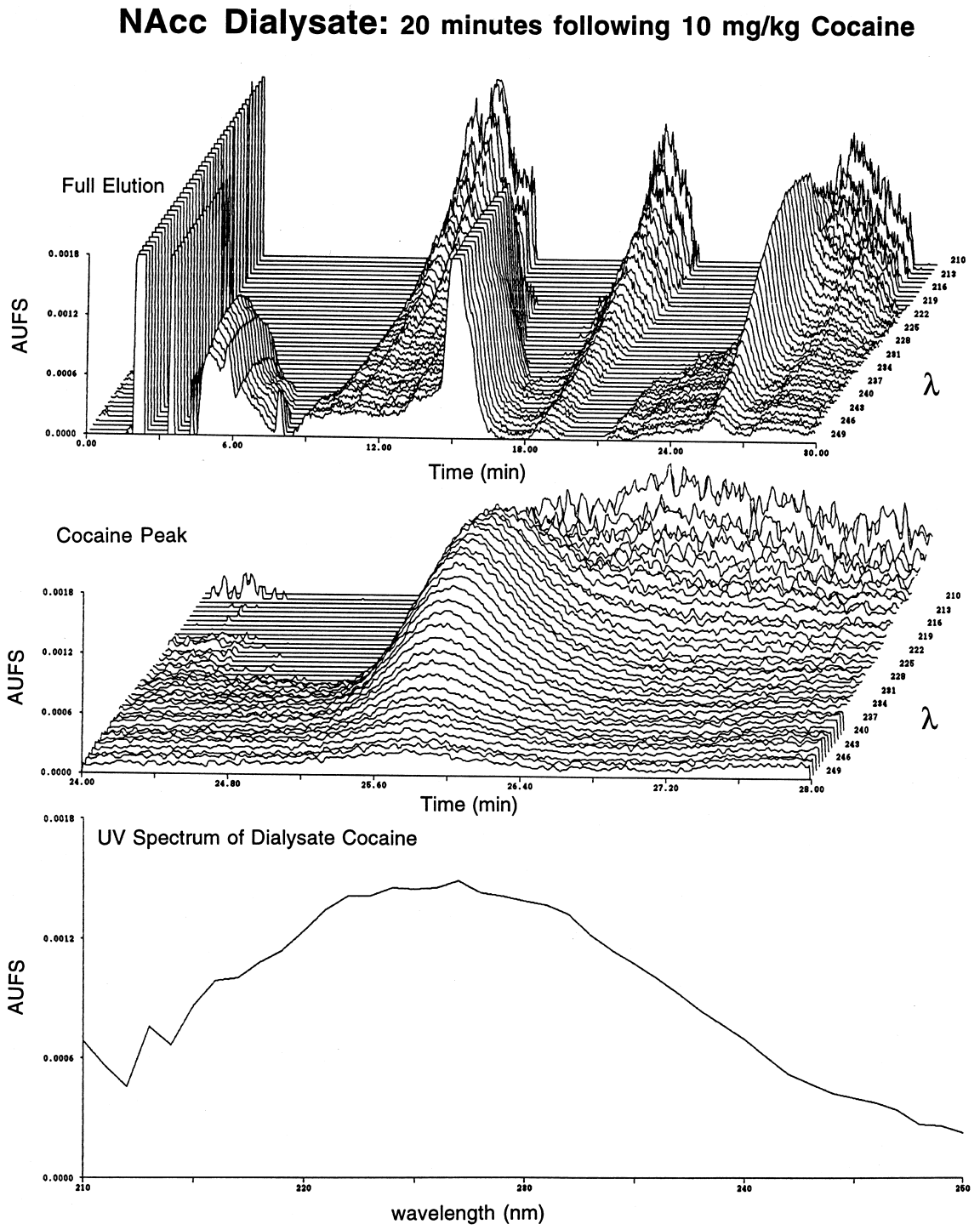

Fig. 4. Representative UV chromatogram (0.001 AUFS) of cocaine in an in vivo microdialysate sample obtained from the nucleus accumbens of a rat during the 10–20 min sampling period following a 10 mg/kg (i.p.) cocaine injection. The full elution pattern is shown in the top panel, with the cocaine peak enlarged for clarity in the middle panel. The UV spectrum (210–250 nm) is shown in the bottom panel.

ence of an active electrochemical cell prior to the UV flowcell produced no effect on either the absorbance spectrum or the absorbance intensity of either cocaine standard concentration. Moreover, when the signal from the first electrode in the flowpath (+700 mV) was monitored, there were no discernible peaks within 5 min of the elution time of cocaine. Together, these observations indicate that cocaine was not significantly affected by the electrochemical cell under the present conditions.

Baseline dialysate samples (i.e., collected before cocaine administration) produced no UV peaks with retention times beyond 18 min (λ=210–310 nm; data not shown). The UV spectrum (λ=210–250 nm) of a 2 μM cocaine standard is shown in Fig. 3, and the UV spectrum of an in vivo microdialysate sample collected from the nucleus accumbens of a rat during the period of 10–20 min following a 10 mg/kg cocaine injection is shown in Fig. 4. As shown in these Figures there were no substantial peaks eluting within 2 min of cocaine. The identical spectra for the dialysate sample and the cocaine standard indicates that there were no UV-absorbing substances in dialysates which co-elute with cocaine (see Figs. 3 and 4, bottom panels). The maximal UV absorbance by cocaine occurred at a wavelength of 225 nm. Therefore this single wavelength was used for all further quantitative analyses of cocaine.

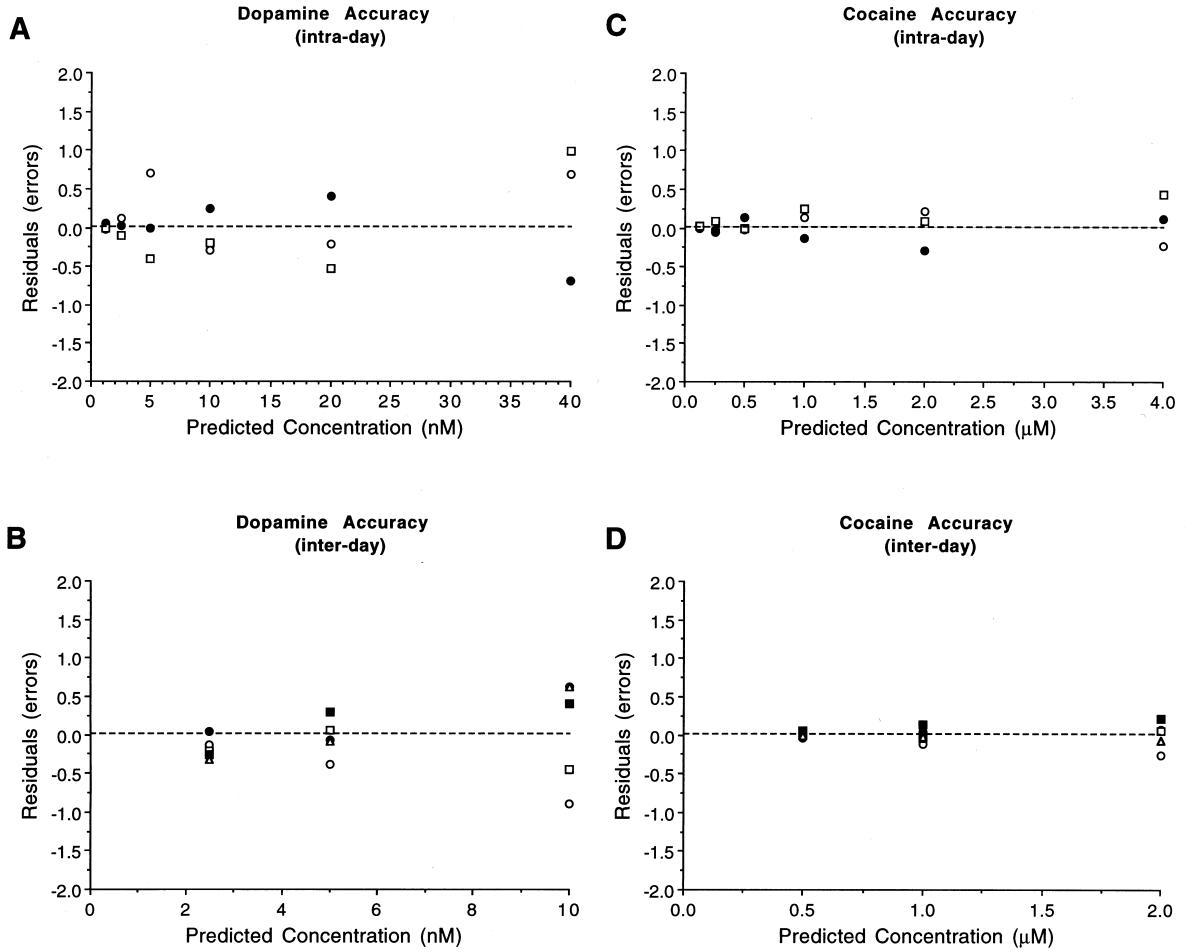

Fig. 5. Residual plots for the intra- and inter-day dopamine and cocaine calibration curves. Panel A: intra-day dopamine; panel B: inter-day dopamine; panel C: intra-day cocaine; panel D: inter-day cocaine.

In preliminary experiments it was determined that there were no differences between calibration curves produced by standards prepared in the dialysis perfusion medium (aCSF) and standards prepared in an aqueous ascorbic acid solution (0.25 m*M*). Subsequently, all standard solutions in the present experiments were prepared in an aqueous ascorbic acid solution (0.25 m*M*). External calibration curves for dopamine and cocaine were constructed by injecting six different standard concentrations in ascending order, with a blank injected between each full iteration. The standard concentrations were 1.25, 2.5, 5, 10, 20 and 40 n*M* for dopamine and 0.125, 0.25, 0.5, 1, 2 and 4 m*M* for cocaine. Three determinations of each concentration were made in this manner, and all data were collected in an 8-h period, thus providing an intra-day determination of linearity, accuracy and precision. These standard concentrations were chosen to encompass a greater analyte concentration range than would be expected from in vivo microdialysate samples. However, these concentrations were not sufficient to establish the outer limits of system linearity. For the dopamine standard curve (peak height vs. concentration) the slope was 12.44 ± 0.24 (average±S.D.), the intercept was -3.16 ± 2.78 with a coefficient of determination of $r^2 = 1.0$. For the cocaine curve (AUe^3 vs. concentration) the slope was 559.2 ± 36.23, the intercept was 74.52 ± 24.15 with a coefficient of determination of $r^2 = 0.998$. Residual plots (Fig. 5, panels A and C) indicated no deviation from linearity (i.e., a normal distribution of the residuals around the zero point), although there was a slight heteroscedasticity. However, the larger residual values at higher analyte concentrations (heteroscedasticity) did not indicate a loss of accuracy (measured concentration as compared with predicted concentration) or precision (coefficient of variation, C.V., at each level; see Table 1). The accuracy of the dopamine estimations ranged from 98.89% to 99.45%, while the accuracy of the cocaine estimations ranged from 92.49 to 100%. The C.V. ranged from 1.56% to 7.79% for dopamine and 5.72% to 19.98% for cocaine.

As would be expected, both accuracy and precision improved with increasing analyte concentrations. Based on these curves, the limits of quantitation for DA and cocaine were approximately 1 n*M* and 0.1 μ*M*, respectively ($S/N = 3$).

The inter-day accuracy and precision of this method was evaluated by analyzing three different standard concentrations of dopamine and cocaine on five separate days (one sample of each concentration on each day) over a 3-week period. The slope of the resulting dopamine standard curve (peak height vs. concentration) was 10.98 ± 0.81, the intercept was -1.69 ± 1.4 and the coefficient of determination was $r^2 = 1.0$. For the cocaine curve (AUe^3 vs. concentration) the slope was 532 ± 38.4, the intercept was

Table 1
Intra- and inter-day accuracy and precision of dopamine and cocaine analysis

Dopamine				Cocaine			
Standard concentration (n*M*)	Measured concentration (n*M*)	S.D.	C.V. (%)	Standard concentration (μ*M*)	Measured concentration (μ*M*)	S.D.	C.V. (%)
Intra-day[a]							
1.25	1.26	0.01	2.1	0.125	0.128	0.01	11.37
2.5	2.52	0.02	3.14	0.25	0.25	0.05	19.98
5	5.10	0.10	7.79	0.5	0.53	0.06	11.52
10	9.92	0.08	2.03	1	1.07	0.13	12.66
20	19.89	0.11	1.71	2	2.00	0.19	9.50
40	40.33	0.33	1.56	4	4.11	0.24	5.72
Inter-day[b]							
2.5	2.32	0.14	6.14	0.5	0.50	0.04	7.72
5	4.96	0.25	5.01	1	1.00	0.09	9.31
10	10.06	0.70	6.91	2	2.00	0.18	9.01

[a] Intra-day estimates are based on three samples of each concentration. See Section 3.1 for details.
[b] Inter-day estimates were determined from five different runs over a 3-week period. See Section 3.1 for details.

40.21±12.3 and the coefficient of determination was $r^2=1.0$. Residual plots of these data indicated no deviation from linearity (Fig. 5, panels B and D). The inter-day accuracy of the dopamine estimations ranged from 92.8 to 99.36% while the accuracy of the cocaine estimations ranged from 99.08 to 99.87%. The inter-day C.V. ranged from 5.01 to 6.91% for dopamine and 7.72 to 9.31% for cocaine (see Table 1).

3.2. Dose–effect analysis of nucleus accumbens dopamine and cocaine levels using in vivo microdialysis

This chromatographic method permitted the reliable detection of baseline dialysate dopamine concentrations in each of the 12 animals analyzed (baseline dopamine concentration=3.2±0.42 n*M*; mean±S.E.M.). Intraperitoneal cocaine injections

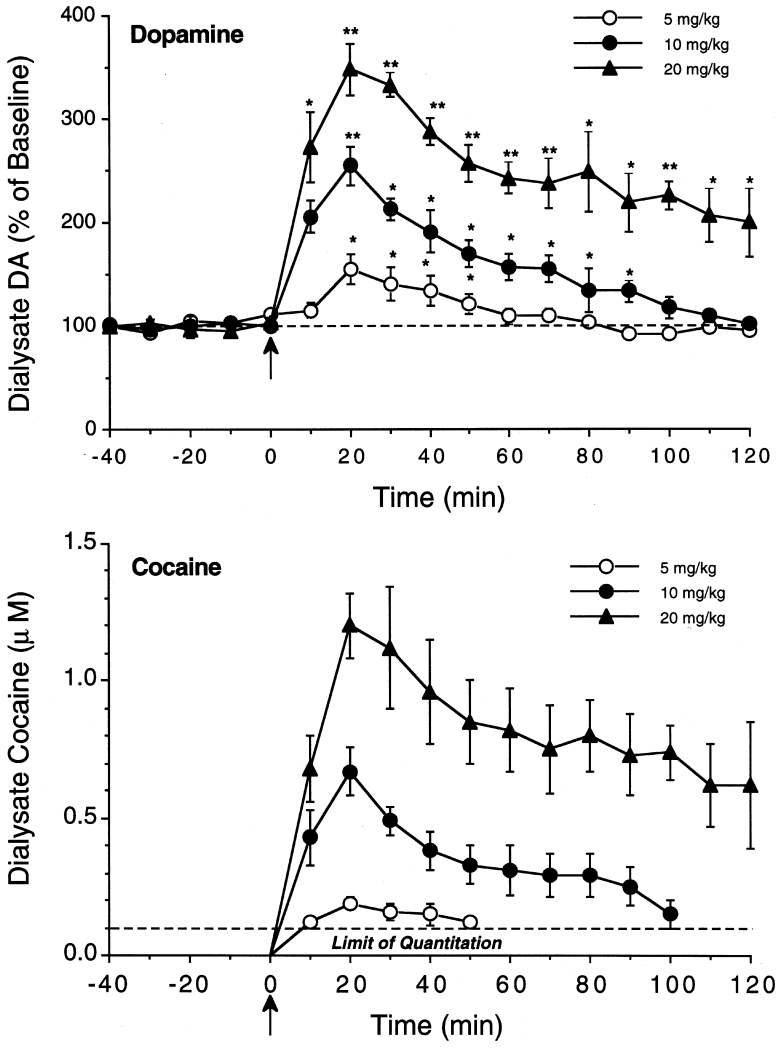

Fig. 6. In vivo dialysate dopamine and cocaine levels from the nucleus accumbens of rats following the i.p. injection of 5, 10 and 20 mg/kg doses of cocaine. The baseline dialysate dopamine concentration was 3.2±0.42 n*M* (average±S.E.M. of all animals), and there was no significant difference in baseline dialysate dopamine levels between dose groups. Arrows denote the cocaine injections. * denotes $p<0.05$ and ** denotes $p<0.01$ compared to pre-drug baseline levels as determined by Fisher's PLS post-hoc comparisons.

induced a dose-dependent (ANOVA, $p<0.0001$) increase in dialysate dopamine levels, which peaked during the second collection interval after the cocaine injection ($t=10–20$ min post-cocaine) and remained elevated for an additional 30–100 min, as a function of the administered dose (see Fig. 6). The maximal dopamine increase was 155±14%, 254±18% and 348±25% of baseline for the 5, 10 and 20 mg/kg cocaine doses, respectively. There was a dose-dependent (ANOVA, $p<0.001$) increase in dialysate cocaine levels following each cocaine injection, which also peaked during the second collection interval ($t=10–20$ min post-cocaine) and remained elevated for an additional 30–100 min, depending on the dose administered (see Fig. 6). The maximal dialysate cocaine concentrations were 0.19±0.02, 0.67±0.09 and 1.2±0.12 μ*M* for the 5, 10 and 20 mg/kg doses, respectively. For each cocaine dose examined, the time course of the elevations in extracellular dopamine closely followed the time course of the interstitial cocaine levels, and the maximal increase in dialysate dopamine was highly correlated with the peak dialysate cocaine concentration (see Fig. 7). While this finding is not surprising pharmacologically, it indicates a responsiveness of the analytical system to changes in both dialysate dopamine and cocaine levels sufficient to reliably quantitate physiologically relevant events.

The presently observed dialysate dopamine and cocaine concentrations are comparable with previous reports using similar cocaine doses and different HPLC methods [4–6,11–18], though it should be noted that the direct comparison of dialysate concentrations between reports is somewhat problematic due to the use in the past of ''correction factors'' based on in vitro microdialysis probe recoveries. Regarding the use of correction factors, several studies have demonstrated that the in vivo recovery of substances from the extracellular space is dependent on the presence or absence of active processes such as neuronal uptake mechanisms (for reviews see Refs. [19,20]). Accordingly, the in vivo recovery of a neurotransmitter such as dopamine is likely to be different than the in vivo recovery of an exogenous substance such as cocaine, and thus the relationship of the dialysate concentrations of these analytes to their respective extracellular concentrations is likely different. Moreover, the in vivo recoveries of both dopamine and cocaine have been shown to be significantly higher than their respective in vitro recoveries [9,18] and as such the ''correction'' of dialysate concentrations of these analytes using in

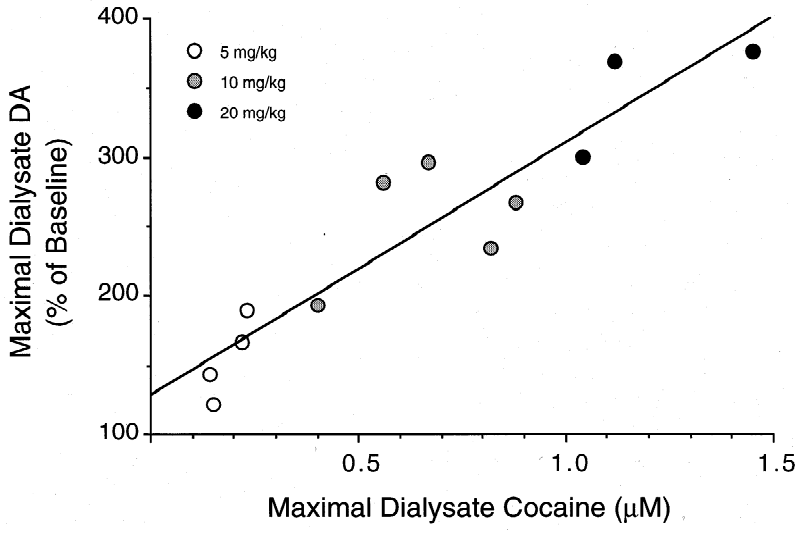

Fig. 7. Correlation between maximal dialysate cocaine concentrations and maximal increases in dopamine levels. Each data point represents an individual animal's cocaine and dopamine levels obtained during the 10–20 min interval following a peripheral cocaine injection. The coefficient of determination of a first order regression of these data was $r^2=0.87$, and a chi-squared test indicated that the data fit a first order regression model [$(\chi^2(0.41, 11)=1.0$].

vitro recovery factors is not appropriate. ''Quantitative'' microdialysis procedures [19,20] should be employed if the experimental intent is to estimate the extracellular concentration of an analyte, rather than simply monitoring relative changes in the dialysate concentration over time.

In conclusion, an analytical method for the simultaneous analysis of both dopamine and cocaine levels in microdialysate samples was developed. This method was found to be viable for the analysis of small sample volumes (5 μl) and to be sensitive, linear, accurate and precise for both intra- and inter-day analyses.

Acknowledgements

This is publication number 10835-NP from The Scripps Research Institute.

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