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Journal of Chromatography B, 768 (2002) 305–313

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of the benztropine analog AHN-1055, a dopamine uptake inhibitor, in rat plasma and brain by high-performance liquid chromatography with ultraviolet absorbance detection

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Received 8 June 2001; received in revised form 10 December 2001; accepted 12 December 2001

Abstract

N-Substituted 3α-[bis(4′-fluorophenyl)methoxy] tropanes represent a series of novel potential cocaine abuse therapeutics. AHN-1055, a member of this series, has been assessed to be the most suitable analog for pharmacokinetic studies. A sensitive and specific high-performance liquid chromatography method was developed to quantitate AHN-1055 in rat plasma and brain tissue. Reversed-phase chromatography with ultraviolet detection (λ=220 nm) was utilized to quantitate the eluate. Plasma or brain tissue samples were prepared by liquid–liquid extraction using hexane, followed by evaporation, reconstitution in mobile phase, and injection onto an ABZ+plus column. AHN-1055 and oxprenolol (internal standard) eluted at ~9.9 and 5.01 min, respectively, without any interfering peaks. The calibration curves were found to be linear in the range of 25–10 000 ng/ml for plasma and 50–5000 ng/g for brain ($r^2 \geq 0.999$). The intra- and inter-day variabilities were ≤10% whereas the intra- and inter-day errors were ≤8.5%. Plasma and brain recoveries of AHN-1055 were 95 and 79%, respectively. Stability studies showed plasma quality control samples to be stable through at least three freeze–thaw cycles (error<3.5%), for at least 24 h when subjected to room temperature (error<3%) and for at least 30 h after loading the processed samples onto the autosampler (error<3%). AHN-1055 stock solution was found to be stable for at least 4 months when stored at 4 °C (error<6%). The validated method accurately quantified AHN-1055 in plasma and brain samples collected from a pharmacokinetic study consisting of an intravenous bolus in the tail vein of adult male Sprague–Dawley rats.

Keywords: Benztropine analogue; AHN-1055; Dopamine uptake inhibitor

1. Introduction

N-Substituted 3α-[bis(4′-fluorophenyl)methoxy] tropanes represent a series of novel potential cocaine abuse therapeutics. Cocaine dependence is the most rapidly spreading form of substance dependence in the United States. At present, there are no suitable medications and the search to find an effective pharmacotherapeutic continues. Research shows that binding to the dopamine transporter (DAT) and subsequent inhibition of dopamine (DA) reuptake is the primary mechanism of action responsible for the

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1570-0232/02/$ – see front matter
PII: S1570-0232(01)00614-6

psychostimulant effects of cocaine [1]. A rapid increase in dopamine levels followed by elimination within a very short time is responsible for the ''euphoria'' followed by craving [2]. This phenomenon is in accordance with the rapid absorption and short half-life of cocaine [3]. One of the strategies for the treatment of cocaine abuse is to develop compounds that would be neurochemically similar to cocaine but not as reinforcing or addictive. This would promote patient compliance as well as allow gradual reduction in addiction. The most important desirable properties of such a medication include high affinity for the dopamine transporter, lack of abuse potential and slow onset and long duration of effect so as to avoid the reinforcing effects as well as craving of cocaine. The *N*-substituted 3α-[bis(4′-fluorophenyl)methoxy] tropanes, obtained by the extensive modification of benztropine, are a series of novel dopamine uptake inhibitors synthesized by Newman and co-workers (National Institute of Drug Abuse) [4,5]. Preliminary studies have shown these compounds to have high affinity and selectivity for the dopamine transporter and a behavioral profile distinct from that of cocaine [6]. The blood–brain barrier (BBB) permeability as well as pharmacokinetic disposition may significantly influence the in vivo potency of these compounds as agents to treat cocaine abuse. AHN-1055 showed the most favorable binding and dopamine uptake inhibition characteristics when tested for affinity to DAT and inhibition of DA uptake in rat caudate putamen. In addition, utilization of a widely accepted in vitro model of the BBB, bovine brain microvessel endothelial cells (BBMECs) assessing permeability of these analogs across cell monolayers showed AHN-1055 to exhibit high permeability in the absorptive direction and an efflux pattern similar to cocaine (unpublished data). Thus, results of binding as well as in vitro transport studies make AHN-1055 a suitable analog for pharmacokinetic studies. To date, no analytical method has been reported for these compounds. A valid, simple, reproducible and sensitive method is needed for the quantitation of these compounds in order to facilitate further research with these compounds. To assess the pharmacokinetic distribution of AHN-1055 in rats, a sensitive and specific analytical method to quantitate AHN-1055 in rat plasma and brain was developed.

2. Experimental

2.1. Materials and reagents

The chemical structures of AHN-1055 {*N*-methyl 3α-[bis(4′-fluorophenyl)methoxy] tropane} and the internal standard oxprenolol (OXP) are shown in Fig. 1. AHN-1055 was synthesized and donated by Amy H. Newman (National Institutes of Drug Abuse, NIH, Baltimore, MD, USA). Oxprenolol, sodium phosphate dibasic, and triethylamine (TEA) were purchased from Sigma (St. Louis, MO, USA). Phosphoric acid was obtained from EM Science (Gibbstown, NJ, USA). Hexane and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals and solvents were ACS analytical grade or HPLC grade. Deionized water was prepared by an ultrapure water system Pyrosystem Plus (Hydro, Research Triangle Park, NC, USA). Heparinized, sterile filtered rat plasma was obtained from Valley Biomedical (Winchester, VA, USA).

2.2. Instrumentation

Plasma samples were analyzed by a high-performance liquid chromatography (HPLC) system consisting of a Waters 2690 liquid chromatograph supplied with a Model 2487 UV detector set at 220 nm (Waters–Millipore, Milford, MA, USA). The chromatographic data was analyzed using Millenium chromatography manager (Waters, version 3.2). Separation was achieved at ambient temperature

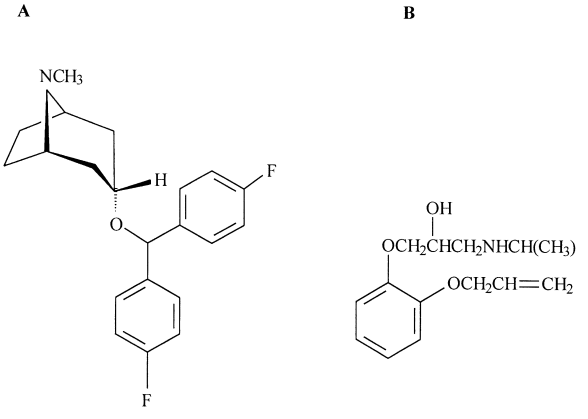

Fig. 1. Chemical structures of (A) AHN-1055 and (B) oxprenolol.

using a reversed-phase Supelcosil LC-ABZ+Plus column (C_{18}, 250×4.6 mm, 5 μm; Supelco, Bellefonte, PA, USA) fitted with a Supelguard 5 μm LC-18, 2 cm guard column (Supelco).

The mobile phase consisted of solvent A [methanol–0.05 *M* Na_2HPO_4, pH 3.0 (40:60, v/v)] and solvent B [methanol–0.05 *M* Na_2HPO_4, pH 3.0 (80:20, v/v)]. The buffer was prepared with deionized water and the pH was adjusted to 3.0 with phosphoric acid. The mobile phase was filtered through a 0.45-μm nylon filter and degassed under ultrasound and vacuum for 15 min. The mobile phase was pumped over a gradient profile (15% B to 80% B) at a flow-rate of 1 ml/min with a pump pressure of approximately 2400 p.s.i. (1 p.s.i.= 6894.76 Pa). The total analysis time for each sample was 15 min.

2.3. Preparation of stock solutions, standards and quality controls (QCs)

Stock solutions of 50 μg/ml for AHN-1055 (in phosphate-buffered saline) and 100 μg/ml for OXP (in water) were prepared and stored at 4 °C. A 50-μl volume of the stock solutions was used to spike blank rat plasma in the preparation of standards (25, 50, 100, 500, 1000, 5000, and 10 000 ng/ml) and QC samples (75, 750, and 7500 ng/ml). Stock solutions for calibration standards and quality controls were prepared separately. Standards were prepared fresh whereas QC samples were stored at −20 °C in 2 ml aliquots. QC samples were brought to room temperature before analysis and carried through the appropriate sample preparation along with the standards. A 100-μl volume of the stock was added to blank rat brain homogenate to obtain concentrations in the range of 50–5000 ng/g.

2.4. Preparation of plasma and brain samples

Aliquots (300 μl) of plasma samples (standards or QC samples) were pipetted into 100×16 mm borosilicate glass disposable culture tubes followed by the addition of 50 μl of the working internal standard solution (OXP—100 μg/ml in water). OXP was chosen as the internal standard as it was found to elute under the same HPLC conditions as those used for AHN-1055 (type of column, mobile phase, UV wavelength, etc.). There was complete resolution between AHN-1055 and OXP with no interference from the biological matrix at the retention time of OXP. Brain tissue was homogenized and based on the weight, diluted with an equal volume of phosphate buffered saline. Aliquots (250 μl) of this brain homogenate mixture was pipetted into 125×16 mm borosilicate glass disposable culture tubes followed by the addition of 50 μl of the working internal standard solution (OXP—100 μg/ml in water). This was followed by the addition of 100 μl of TEA to confer basicity to the biomatrix (pK_a of AHN-1055~ 8–9) after which the samples were vortex-mixed briefly for 20–30 s. For plasma samples, 2 ml of hexane was added, samples were vortex-mixed (30–45 s) and centrifuged at 3000 rpm for 5 min. For brain samples, extraction was carried out twice, with 3 ml of hexane for each extraction. The supernatant was transferred to a clean 100×16 mm glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The resulting residue was reconstituted with 210 μl of mobile phase A (methanol–0.05 *M* Na_2HPO_4, pH 3.0, 40:60, v/v), transferred to a micro-vial (300 μl capacity) and 200 μl was injected into the HPLC system.

2.5. Calibration and linearity

Calibration curves were constructed over 3 consecutive days using triplicates ($n=3$) of seven standards (25, 50, 100, 500, 1000, 5000, and 10 000 ng/ml) of plasma and five standards (50, 100, 500, 1000, and 5000 ng/g) of brain homogenate. The response values for the assay were determined by calculating the ratio of peak area of AHN 1055 to that of the internal standard, OXP and the response was correlated against analyte concentration by least-squares regression. A weight of $1/y$ was utilized to determine slopes, intercepts, and correlation coefficients. The minimum acceptable coefficient to establish linearity was set at 0.95 a priori. The lower limit of quantitation (LOQ) for AHN 1055 was selected as the concentration at which the assay precision was within 15% and the signal-to-noise ratio exceeded 4:1.

2.6. Accuracy and precision

Accuracy and precision of the assay were determined through analysis of plasma QC samples (75, 750, and 7500 ng/ml) and brain standards. Six replicates of ($n=6$) of each concentration of plasma QC samples and three concentrations ($n=3$) of brain standards were analyzed on 3 consecutive days, after which intra- and inter-day means, standard deviations, relative standard deviations (RSDs; measure of precision) and % error (measure of accuracy) were calculated. The RSD and % error were determined from the following equations:

$$\text{RSD}\ (\%) = \frac{\text{SD}}{\text{Mean}} \cdot 100 \quad (1)$$

$$\%\ \text{Error} = \frac{\text{Theoretical} - \text{mean}}{\text{Theoretical}} \cdot 100 \quad (2)$$

RSDs and % errors less than 10% were considered to be suitable for acceptable accuracy and precision of the assay.

2.7. Recovery

Extraction efficiency was determined by comparing replicate ($n=6$) peak height ratios of extracted plasma samples to those of unextracted water standards for three concentrations of AHN-1055: 75, 750 and 7500 ng/ml. Recovery of AHN-1055 from brain was determined for 50, 500 and 5000 ng/g. Recovery of internal standard OXP was determined by comparing replicate ($n=6$) peak height areas of extracted plasma samples to those of unextracted water standards for a concentration of 10 μg/ml. The percent recovery was determined by the following equation:

$$\%\ \text{Recovery} = \frac{\text{Peak area ratio (plasma or brain standard)}}{\text{Peak area ratio (water standard)}} \quad (3)$$

2.8. Stability

2.8.1. Freeze–thaw stability

Stability of AHN-1055 plasma samples after consecutive freezing and thawing was determined by subjecting six aliquots ($n=6$) of all three QC concentrations to three freeze–thaw cycles and comparing the concentrations with those of freshly thawed QC samples. Samples that were initially frozen at −20 °C were thawed at room temperature for 20–30 min and this procedure was repeated for a subsequent freeze–thaw cycle. Free flow of samples with the absence of any frozen mass was used as the criterion for complete thawing. The last thawing cycle was just before analysis. Freshly thawed QC samples were analyzed along with the test QC samples for comparison. An error of >10% from the expected concentrations for the test QC samples was considered to be unacceptable for this stability test.

2.8.2. Short-term stability

Stability of AHN-1055 plasma samples at room temperature was determined by subjecting three aliquots ($n=3$) of all three QC concentrations to room temperature for 12 and 24 h and comparing the concentrations with those of freshly thawed QC samples. QC samples were thawed and left on the bench at room temperature for the appropriate period of time and then analyzed along with freshly thawed QC samples for comparison. An error of >10% from the expected concentrations for the test QC samples was considered to be unacceptable.

2.8.3. Processed sample stability

Stability of AHN-1055 in processed samples was assessed after samples were loaded onto the autosampler. Two aliquots of low and high QC samples were processed using the established sample preparation procedure, placed into HPLC vials, loaded onto the autosampler and analyzed at 0, 6, 12, 15, 18, 24, and 30 h. The concentration of AHN 1055 at each time period was determined and an error of >10% from the expected concentrations for the test QC samples was considered to be unacceptable.

2.8.4. Stock solution stability

The stability of AHN-1055 in phosphate-buffered saline (50 μg/ml) was determined when stored at 4 °C. A standard curve was prepared in phosphate-buffered saline from a fresh stock solution of AHN-1055 (50 μg/ml) at the following concentrations: 2.5, 4, 6, 7.5 and 10 μg/ml and each standard was run in duplicate. A 5 μg/ml dilution was made from the stock solution and six replicates ($n=6$) were analyzed on day 1. A fresh stock solution was

prepared on days 7, 14, 21, 28, 42, 70 and 4 months and was used to construct a standard curve. Six replicates ($n=6$) of the original stock solution (prepared on day 1) were analyzed using the new standard curve prepared on each of the 6 days. Mean AHN-1055 concentration was determined on each day and compared to the average concentration measured on day 1. A difference of $>10\%$ between the fresh and test stock solution was considered unacceptable.

2.9. Pharmacokinetic study

A study was designed to investigate the pharmacokinetics of AHN-1055 in adult male Sprague–Dawley rats (Hilltop Labs., PA, USA). The protocol was approved by the University of Maryland, School of Pharmacy IACUC. Sprague–Dawley rats approximately 6–8 weeks of age (mass = 250–275 g) were administered a 10 mg/kg intravenous (i.v.) bolus dose via the tail vein. Cohorts of three animals were sacrificed by CO_2 asphyxiation at the following time points: 5, 15, 30, 60, 120, 360, 600, 720, 1080, 1440 and 2100 min. Blood samples were centrifuged, plasma and brain tissue samples were collected and stored at $-70\,^{\circ}C$ until analysis.

3. Results and discussion

3.1. Specificity and resolution

Fig. 2A and B represent chromatograms of extracted blank rat plasma and a 1000 ng/ml plasma standard with internal standard (OXP; 14.3 μg/ml), respectively. Fig. 2C is a representative chromatogram of an extracted pre-dose rat plasma sample and Fig. 2D displays a chromatogram from the 60 min sample after intravenous administration of AHN-1055 (481 ng/ml).

Fig. 3A and B are representative chromatograms of extracted blank rat brain tissue and a 1000 ng/g brain standard with internal standard (OXP; 16.7 μg/ml), respectively. Fig. 3C is a representative chromatogram of an extracted pre-dose rat brain tissue sample and Fig. 3D displays a chromatogram from the 60 min sample after intravenous administration of AHN-1055 (4160 ng/g). For both plasma and brain tissue, the assay was found to be specific for AHN-1055 with no interference from the biomatrix at the retention time of AHN-1055 or OXP. Additionally, OXP and AHN-1055 were adequately separated with typical retention times of approximately 5.01 min and 9.9 min for OXP and AHN-1055, respectively.

3.2. Calibration and linearity

The plasma standard curve for AHN-1055 was found to be linear in the range of 25–10 000 ng/ml with a consistent slope (0.00011 ± 0.00001) and a correlation coefficient of 0.9993 ± 0.002 ($n=9$). The brain standard curve was found to be linear in the range of 50–5000 ng/g with a consistent slope (0.000027 ± 0.000003) and a correlation coefficient of 0.9986 ± 0.001 ($n=9$). Based on the signal-to-noise ratio and the assay precision, the limit of quantitation of the assay was estimated to be 25 ng/ml in plasma and 50 ng/g in brain.

3.3. Accuracy and precision

The intra- and inter-day accuracy and precision data for AHN-1055 are listed in Tables 1 and 2. The intra-day variability (RSD) was less than 4.5% ($n=6$) and the error in accuracy was less than 3.5% ($n=6$) for plasma. Also, the inter-day imprecision (RSD) was less than 5% ($n=18$) whereas the inter-day error was less than 1%. For brain tissue samples, the intra-day variability (RSD) was less than 10% ($n=3$) and the error in accuracy was less than 8.5% ($n=3$) for plasma. Also, the inter-day imprecision (RSD) was less than 9.4% ($n=9$) whereas the inter-day error was less than 8.2%.

3.4. Recovery

Comparison of the peak height ratios of extracted plasma and unextracted water standards showed that the extraction recoveries of AHN-1055 as well as OXP were $95.9\pm3.2\%$ ($n=18$) and $96.5\pm4.5\%$, respectively ($n=6$). Recovery of AHN-1055 from brain tissue was found to be $79.0\pm2.4\%$.

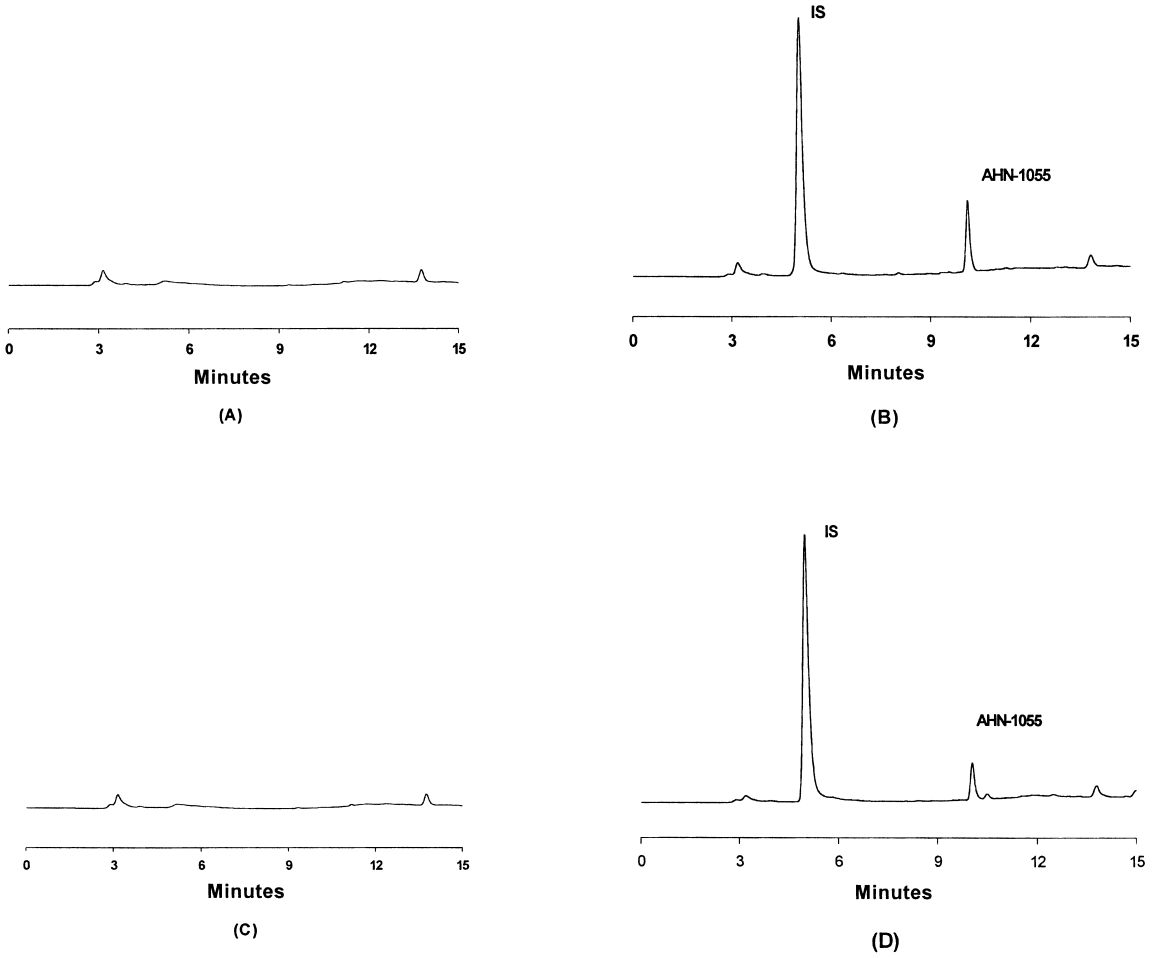

Fig. 2. Representative rat plasma chromatograms: (A) extracted blank rat plasma; (B) extracted rat plasma spiked with AHN-1055 at 1000 ng/ml and oxprenolol at 14.3 μg/ml; (C) extracted pre-dose plasma sample and (D) extracted rat plasma at 60 min.

3.5. Stability

Stability data of AHN-1055 QC samples are summarized in Table 3. Plasma QC samples ($n=6$ for each of the three concentrations) were found to be stable through all three freeze–thaw cycles with % errors ranging from 0.1 to 3.5%. QC samples ($n=3$ for all three concentrations) were also found to be stable for at least 24 h at room temperature with $<3\%$ error in calculated concentration from the expected concentration. Processed plasma QC samples ($n=2$ for low and high concentrations) were stable for at least 30 h after being loaded onto the autosampler (error $<3\%$). AHN-1055 stock solution was found to be stable for at least 4 months in phosphate buffered saline when stored at 4 °C ($<5\%$ difference between fresh and test solution).

3.6. Pharmacokinetic study

A representative plasma and brain concentration vs. time profile for AHN-1055 following a bolus dose (10 mg/kg) to an adult male Sprague–Dawley

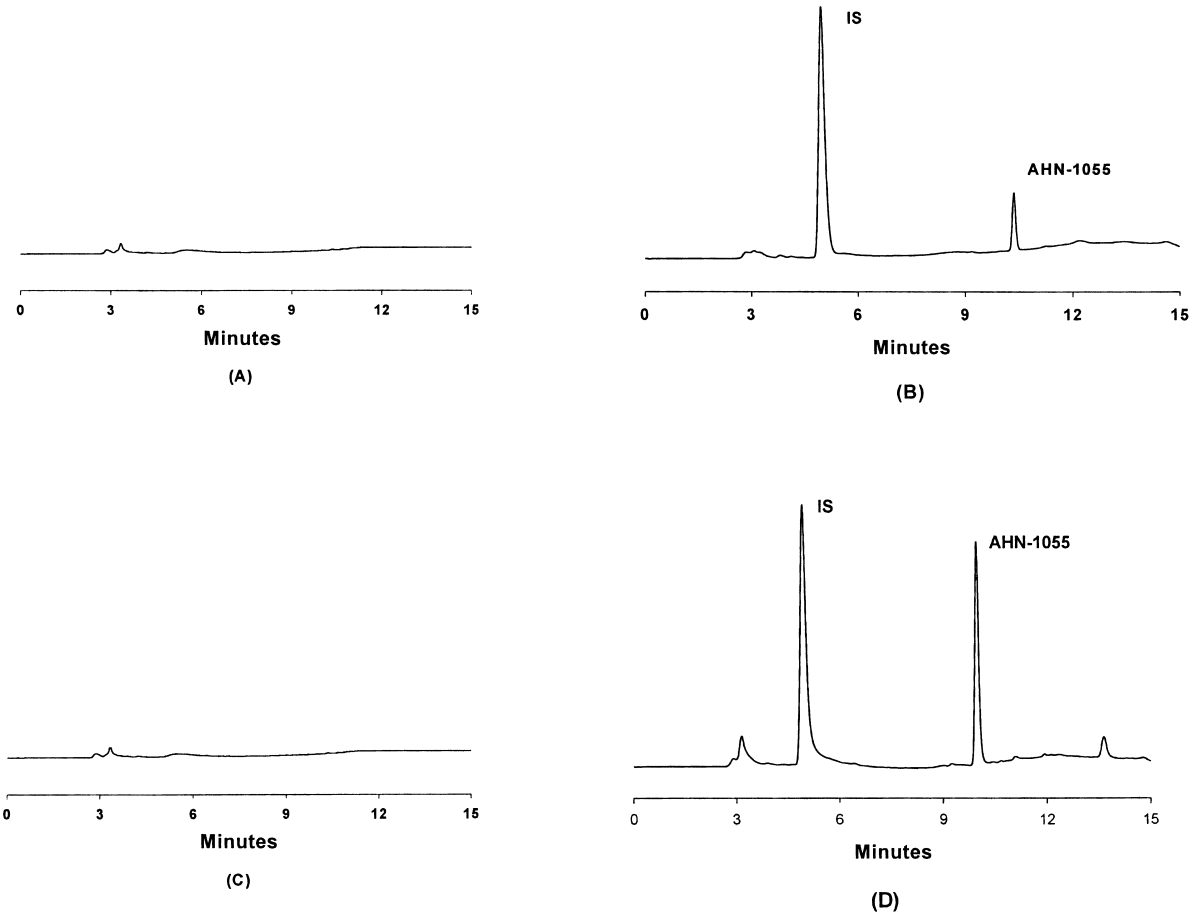

Fig. 3. Representative rat brain tissue chromatograms chromatograms: (A) extracted blank rat brain tissue; (B) extracted rat brain tissue spiked with AHN-1055 at 1000 ng/g and oxprenolol at 16.7 μg/ml; (C) extracted pre-dose brain tissue sample and (D) extracted rat brain tissue at 60 min.

Table 1
Intra-day precision and accuracy for AHN-1055 in plasma QC samples and brain standards

Parameter	Plasma[a]			Brain[b]				
	75 ng/ml	750 ng/ml	7500 ng/ml	50 ng/g	100 ng/g	500 ng/g	1000 ng/g	5000 ng/g
Precision								
Mean	73.4	773.8	7510.8	46.0	101.6	505.2	990.4	4639.3
SD	3.2	24.5	212.0	1.4	10.1	32.6	17.9	93.4
RSD (%)	4.3	3.2	2.8	3.0	9.9	6.5	1.8	2.0
Accuracy								
Mean % error	−2.1	3.2	0.1	−8.1	1.6	1.0	−1.0	−7.2
SD	4.2	3.3	2.8	2.8	10.1	6.5	1.8	1.9

[a] Data for $n=6$.
[b] Data for $n=3$.

Table 2
Inter-day precision and accuracy for AHN-1055 in plasma QC samples and brain standards

Parameter	Plasma[a]			Brain[b]				
	75 ng/ml	750 ng/ml	7500 ng/ml	50 ng/g	100 ng/g	500 ng/g	1000 ng/g	5000 ng/g
Precision								
Mean	74.4	756.6	7432.4	45.9	97.1	502.4	1051.5	5027.7
SD	3.3	34.6	274.9	1.9	9.1	26.1	50.3	342.7
RSD (%)	4.4	4.6	3.7	4.2	9.3	5.2	4.8	6.8
Accuracy								
Mean % error	−0.7	0.9	−0.9	−8.2	−2.9	0.5	5.1	0.6
SD	4.4	4.6	3.7	3.9	9.1	5.2	5.0	6.9

[a] Data for $n=18$.
[b] Data for $n=9$.

Table 3
Stability of AHN-1055 plasma QC samples

Stability test		75 ng/ml	750 ng/ml	7500 ng/ml
Freeze–thaw stability	Mean±SD	75.0±1.9	736.9±44.8	7291.84± 323.9
(3 cycles)	RSD (%)	2.6	6.1	4.4
	% Error	0.1	−1.8	−2.8
Short-term stability	Mean±SD	77.2±3.1	753.5±26.6	7692.4±44.7
(24 h)	RSD (%)	4.0	3.5	0.6
	% Error	2.9	0.5	2.6
Processed sample	Mean±SD	75.6±2.0	Not determined	7697.7±185.5
stability (30 h)	RSD (%)	2.7	–	2.4
	% Error	0.8	–	2.6

$n=6$ for freeze–thaw stability; $n=3$ for short-term stability; $n=2$ for processed sample stability.

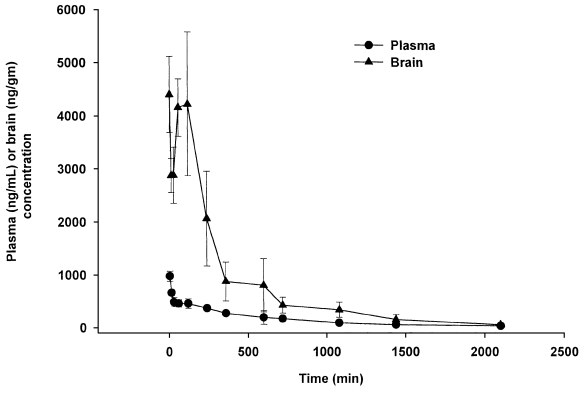

Fig. 4. Plasma and brain concentration vs. time curves for AHN-1055 in rats after a single i.v. bolus dose of 10 mg/kg.

rat is shown in Fig. 4. Brain tissue concentrations were much higher than plasma concentrations at all time points. This concentration vs. time profile highlights the sensitivity of the assay as well as its utility since it is possible to follow the time course of AHN-1055 in plasma and brain after a single bolus dose.

4. Conclusion

In summary, a valid, sensitive, specific and reproducible assay for the quantitation of the novel cocaine-abuse therapeutic AHN-1055 in both plasma

and brain has been developed. The method employs reversed-phase HPLC with liquid–liquid extraction of AHN-1055 followed by UV detection. Rendering the plasma basic with TEA facilitates efficient extraction of AHN-1055 from the biomatrix. There is no chromatography method for the determination of the benztropine analogs currently reported in literature. Several analytical methods for benztropine mesylate utilizing HPLC with UV detection have been reported, but most of these methods are very cumbersome and include several steps for extraction [7–9]. Simplified sample preparation as well as excellent specificity and sensitivity are significant advantages of this assay over methods that have reported for benztropine and will allow for rapid and accurate analysis of samples from pharmacokinetic studies. Sample processing consists of only a single liquid–liquid extraction step (plasma) or a double liquid–liquid extraction step (brain), thus minimizing sample processing time. This analytical method will also be used for quantitation of other potent tropane analogs of the *N*-substituted 3α-[bis(4′-fluorophenyl)methoxy] tropane series.

Acknowledgements

The authors would like to thank Dr. Amy H. Newman, NIDA, for providing the tropane analog.

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