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Quantitation of human plasma levels of the anticancer agent carboxyamidotriazole by high-performance liquid chromatography

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ABSTRACT

The predominant cause of death of cancer patients is growth and metastasis of their tumors. By targeting signal transduction pathways as sites of therapeutic intervention, we have identified a novel anticancer drug carboxyamidotriazole (CAI). A straightforward and reliable method of detection and quantitation of human CAI plasma levels using solid-phase organic extraction followed by isocratic reversed-phase chromatography is now reported. This assay detected CAI over the concentration range 0.04–10.0 μg/ml, which brackets the range shown to be physiologically and biochemically effective. Linearity was demonstrated by linear regression analysis of calibration curves (r^2 = 0.999). Equivalence of recovery of extracted *versus* non-extracted CAI over a broad concentration range was demonstrated (r^2 = 0.998, coefficients of variability < 10%). The method was applied to quantitate CAI plasma levels from patients now entered on the Phase I clinical trial underway at the National Cancer Institute.

INTRODUCTION

The most insidious aspect of cancer is its relentless growth and tendency to metastasize [1]. New approaches are needed to combat this process. A new screening method for detection of novel compounds which may combat cancer was identified by targeting signal transduction pathways involved in the growth and metastasis of malignancy. Calcium homeostasis, release of arachidonic acid, and generation of inositol phosphates are important second messengers in malignancy and metastasis [2]. The first agent to be chosen by this signal transduction screen is carboxyamidotriazole (CAI: NSC 609974) [3–5], a

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synthetic agent originally developed as a coccidiostat for chickens [6].

CAI inhibited proliferation, adhesion, and directed motility of a broad range of human cancer cell lines *in vitro* [3,4]. Treatment of tumor cells *in vitro* significantly decreased the development of experimental metastasis after mouse tail vein inoculation. Orally administered CAI inhibited both experimental metastases and growth and metastasis of human ovarian cancer, melanoma, and colon cancer xenografts [4]. Studies addressing the mechanism of action have demonstrated that CAI inhibits receptor-mediated calcium influx, arachidonic acid release, and in selected systems generation of inositol phosphates [3,5]. The concentration of CAI producing these biological and biochemical effects is 1–10 μM.

A simple, accurate, and precise assay system was required for analysis of human plasma CAI levels in support of the clinical Phase I trial of orally administered CAI. The targeted therapeutic window for patients, based on the xenograft model and signal transduction data, was 1–10 μM. In this report, we describe an extraction and reversed-phase high-performance liquid chromatographic (HPLC) assay system for measurement of CAI in human plasma.

EXPERIMENTAL

Reagents and standards

CAI (Fig. 1A) was obtained from the Developmental Therapeutics Program, NCI (Bethesda, MD, USA). Harmine (Fig. 1B), the internal standard, was purchased from Sigma (St. Louis, MO, USA). Virus-free human plasma was obtained weekly from the NIH Blood Bank and kept refrigerated until use. The remainder of the reagents were HPLC grade and all buffers were filtered through 0.2-μm filters before use. Solid-phase extraction cartridges were 300 mg Maxi-Clean C_{18} cartridges from Alltech Assoc. (Deerfield, IL, USA). Gelman acrodisc 0.2-μm filters were used for all sample preparation (Gelman Sciences, Ann Arbor, MI, USA).

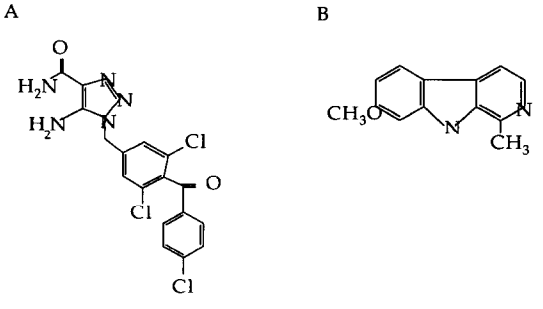

Fig. 1. Structures of CAI (A) and harmine (B).

CAI (1 mg/ml) and harmine (1 mg/ml) stock solutions were made in methanol from powder stock measured to three significant digits on an analytical balance. Aliquots were stored at −70°C, used once, and discarded. Buffer A, 0.1 *M* ammonium acetate pH 6.5, was used for sample extraction and for the chromatography buffer (buffer B). Buffer B consisted of 73% methanol and 27% buffer A.

Apparatus and chromatographic conditions

Chromatographic separation of CAI was done using a computerized Gilson dual-pump HPLC system with autosampler (Middleton, WI, USA). CAI was separated by reversed-phase chromatography over an irregular 10-μm analytical C_{18} column (250 mm × 4.6 mm I.D., Thomson Scientifics, Springfield, VA, USA) preceded by a spherical C_{18} guard cartridge and 100-μl injection loop. The guard column was changed approximately monthly (150 injections) to maintain solvent pressure ($\leq$100 bar) and to prolong the life of the analytical column. The analytical column was reversed and washed with methanol, water, and methanol–water–acetic acid (50:49:1, v/v) approximately every 150 runs and was changed after 1200 runs.

Harmine was used as the internal standard because of its hydrophobic nature, stability under these reaction conditions, and ease of separation from CAI. Optimal chromatographic separation of CAI and harmine was accomplished during an 18-min run using an isocratic gradient of buffer B at a flow-rate of 1.0 ml/min, followed by a 3-min equilibration phase. The mobile phase was prepared, filtered, and degassed daily. An absorbance scan of CAI in this mobile phase demon-

strated a maximum absorbance for CAI at 263 nm. The standard absorbance range used for chromatography was 0.05–0.5 a.u.f.s. CAI and harmine were eluted with this procedure at 6.1 and 9.5 min, respectively.

Extraction procedure

Patients were treated daily with orally administered CAI (100 mg/m^2 per day) on a clinical Phase I study approved by the Institutional Review Board of the National Cancer Institute. The CAI was dissolved in PEG-400 at 100 mg/ml and was taken after an 8-h fast. Patient plasma samples were obtained from either heparinized blood or blood with EDTA as anticoagulant and were centrifuged at 2500 *g* for 10 min. Gravimetric CAI plasma reference samples were made fresh each day. Samples were kept at 4°C until use; up to two freeze–thaw cycles of CAI-containing plasma did not alter CAI detection.

Extraction of CAI was accomplished with a solid-phase matrix attached to a vacuum manifold for simultaneous extraction of up to 24 samples. All samples were analyzed in duplicate unless otherwise stated. The internal standard, harmine, was added to each sample set prior to extraction, at a final concentration of 1 μg/ml. Duplicate 1-ml aliquots of samples were then taken, centrifuged at 2500 *g* for 12 min at 4°C, and filtered. After filtration, samples were applied to a 300-mg C_{18} cartridge that had been pretreated with 10 ml of methanol followed by 10 ml of buffer A. The sample-loaden cartridges were rinsed with 12 ml of buffer A, then CAI and harmine were eluted with 4 ml of methanol. The eluates were dried under a continuous nitrogen stream, resuspended in 500 μl of buffer B, and filtered a final time. Aliquots (50 μl) were drawn up by the autosampler and chromatographed according to the protocol described above.

The CAI concentration range of 0.04–10.0 μg/ml was chosen for the calibration curves. This concentration range bracketed the therapeutic window defined by the signal transduction and animal model experiments [3–5]. The standards were prepared in duplicate in fresh plasma and extracted according to protocol. Standard curves were repeated weekly and fresh standard samples were included with each chromatography series. These consisted of duplicate aliquots of 0.5 and 2.0 μg/ml CAI in plasma which were extracted in parallel with the unknown samples. Calibration curves were constructed by plotting the CAI concentration *versus* area of the CAI peak. Quantitation of CAI concentrations in patient samples and assay performance samples were calculated using the equation derived from the single variable linear regression of the plot of the calibra-

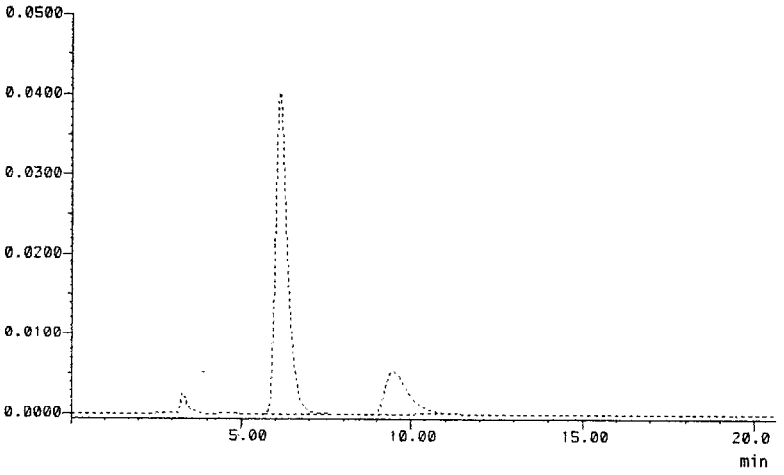

Fig. 2. Sample chromatogram. Gravimetric samples of CAI (3 μg) and harmine (1 μg) were chromatographed as described in Experimental. CAI eluted at 6.09 min and harmine at 9.45 min.

TABLE I

EVALUATION OF COMPOSITION OF THE MOBILE PHASE YIELDING OPTIMAL SEPARATION OF CAI AND HARMINE

Methanol (%)	0.1 *M* NH_4OAc (%)	Retention time (min)		Separation factor (α)
		CAI	Harmine	
70	30	12.3	16.4	1.33
73	27	6.1	9.5	1.56
75	25	5.5	8.5	1.54

tion curves, $y = mx + b$, where y is the peak area of the 6.1-min peak, and m and b were calculated by linear regression analysis.

RESULTS

Assay development

A reversed-phase chromatographic separation approach was chosen because of the hydrophobic nature of CAI. Fig. 2 shows a sample chromatogram of CAI and harmine. The methanol–0.1 *M* ammonium acetate (73:27) mobile phase (buffer B) was chosen after it was shown that this mobile phase composition yielded optimum separation from the void volume, sharpest peaks of CAI and harmine, and could be accomplished within a 20-min chromatographic run period (Table I). These results demonstrate a clear separation of CAI from the internal standard harmine during an 18-min isocratic reversed-phase procedure.

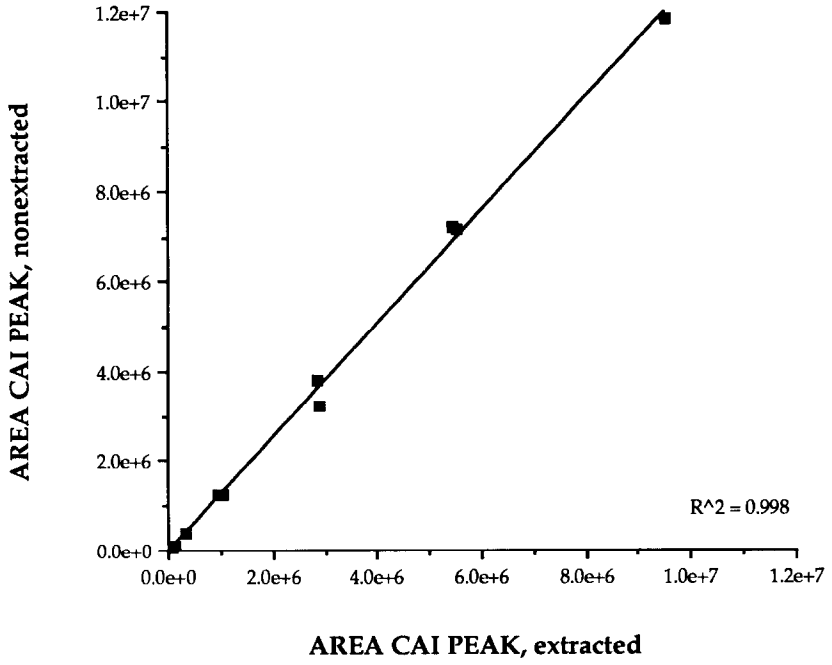

Fig. 3. Efficiency of recovery. Gravimetric samples of CAI with harmine (internal standard) were chromatographed. The equivalence of unextracted (y-axis) and extracted (x-axis) CAI samples was evaluated by linear regression ($r^2 = 0.998$) demonstrating equivalent loss across the standard curve concentration range.

Efficiency of recovery

The efficiency of the extraction procedure was investigated by analyzing recovery of CAI from extracted plasma samples containing gravimetric amounts of CAI. Recovery of CAI was determined by comparison of the CAI peak area measured from extracted plasma samples with the CAI peak area measured from equivalent gravimetric standards diluted directly in mobile phase. Consistency of harmine results were observed within and between runs (coefficient of variability $\leq 6\%$, recovery 80%). The recovery of CAI from plasma samples was consistent over the full concentration range, as shown in the equivalence graph ($r^2 = 0.998$, Fig. 3).

Precision and accuracy assessments

The reproducibility and validity of this assay system were determined by repetitive sampling of plasma containing known CAI concentrations. A set of fifteen replicate gravimetric samples of 2.0 μg/ml CAI in fresh plasma were extracted and then chromatographed daily for five days. The reproducibility of the extraction and detection protocols was determined over the set of samples each day (within-batch accuracy) and between batches over the five-day assay period (precision). The mean (± S.D.) peak area and coefficients of variability presented in Table II and Fig. 4 demonstrate the reproducibility of the chromatographic analysis of each of the fifteen samples over the five-day sampling period. These data demonstrate the reliability of this assay and were confirmed by an additional experiment. Chromatographic analysis of a series of five samples each of 0.05 μg/ml, 0.5 μg/ml, and 5.0 μg/ml CAI (gravimetric) in plasma was performed. The coefficients of variability for this series of samples were 9.7% for 0.05 μg/ml, 4.7% for 0.5 μg/ml, and 5.7% for 5.0 μg/ml.

TABLE II

ACCURACY AND PRECISION OF CHROMATOGRAPHY OF REPETITIVE SAMPLES OF KNOWN CAI CONCENTRATIONS IN HUMAN PLASMA

Day/sample	Area (mean ± S.D.) (a.u.f.s.)	Coefficient of variability (%)
A. Within run (n = 15 samples)		
1	2232090 ± 128650	5.3
2	2154886 ± 128650	6.0
3	2177826 ± 137749	6.3
4	2230454 ± 81978	3.7
5	2226915 ± 152479	6.8
B. Between run (n = 5 days)		
1	2338591 ± 43700	1.8
2	2114022 ± 159000	7.5
3	2224707 ± 105000	4.7
4	2188818 ± 101000	4.6
5	2173757 ± 85400	3.9
6	2309301 ± 39000	1.7
7	2065886 ± 83300	4.0
8	2227422 ± 94900	4.3
9	2190967 ± 95100	4.3
10	2176976 ± 123000	5.6
11	2022007 ± 70100	3.5
12	2218563 ± 108000	4.9
13	2233748 ± 94800	4.2
14	2209108 ± 96700	4.4
15	2411476 ± 8290	0.3

Linearity of CAI detection

The detection of CAI using this reversed-phase chromatographic system yielded linear results over a greater than one thousand-fold concentration range. The choice of the concentration range was based upon the *in vitro* and animal data and ranged from 0.04 μg/ml up to 10 μg/ml [3–5]. A series of standard curves for CAI extracted from plasma was linear over the concentration range 0.04–10.0 μg/ml CAI ($r^2 = 0.999$, $n = 8$). The slope and y-intercept of this series of standard curves are shown in Table III. The lower end of reliable detection was 0.04 μg/ml and the upper limit has not been determined. With each run of unknowns, a set of CAI standards has been included. Known samples of CAI at 0.5 and 2.0 μg/ml were extracted in parallel with unknown samples. The analyses yielded concentrations of 0.47 + 0.018 and 2.07 + 0.157 μg/ml (mean + S.E.M., $n = 7$), respectively, and verified the accuracy of the standard curve.

Patient samples

This chromatographic assay was used to determine if CAI could be detected in the plasma of

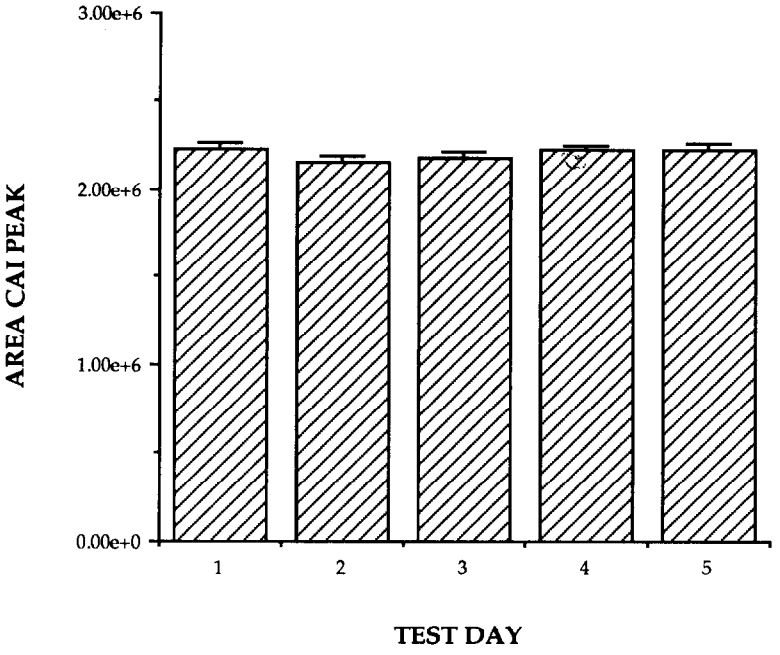

Fig. 4. Precision and accuracy of CAI quantitation. The results shown in Table IIA are presented graphically. Fifteen replicates of CAI (2.0 μg/ml) were chromatographed daily for five days. Mean ± standard error of the mean are shown.

patients receiving orally administered CAI as part of the ongoing Phase I clinical protocol and to quantify measurable amounts for later pharmacokinetic analysis. CAI was solubilized in PEG-400 for oral dosing. Heparinized patient blood samples were processed in duplicate as discussed in Experimental and the extracted, dried samples were chromatographed along with a set of standards for a calibration curve or with the defined known samples. The CAI concentration was calculated from the peak area using the linear regression equation for the given standard curve. Blood samples taken prior to drug administration constituted negative controls and were always devoid of CAI on chromatographic analysis. The chromatographic assay was further vali-

TABLE III

LINEARITY OF CAI STANDARD CURVES OVER THE CONCENTRATION RANGE 0.04–10 μg/ml

Curve	y-Intercept[a] ($\times 10^5$)	m ($\times 10^5$)	r^{2}[b]
1	3.8163	9.222	0.994
2	3.7477	8.404	0.993
3	2.1997	10.987	0.997
4	2.3732	10.418	0.998
5	1.2274	10.986	0.998
6	1.6565	9.893	0.999
Composite (n = 8)	1.6565	9.893	0.999

[a] Equation from linear regression: $y = mx + b$.

[b] Linear regression statistic, CricketGraph (Malvern, PA, USA).

TABLE IV

CHROMATOGRAPHIC QUANTITATION OF UNKNOWN CAI LEVELS IN INDIVIDUAL PATIENT SAMPLES (n = 2)

Patient	CAI level (μg/ml)			
	0 h	4 h	24 h	28 days
1	0	0.434	0.274	1.075
2	0	0.923	1.152	3.610
3	0	0.433	0.233	3.528
4	0	0.421	0.073	3.078

TABLE V

ACCURACY AND PRECISION OF REPLICATE ANALYSES OF 28-DAY SAMPLES

Patient	CAI level (mean ± S.D.) (μg/ml)	Coefficient of variability (%)
1	0.981 ± 0.056	5.6
2	3.974 ± 0.204	5.1
3	3.629 ± 0.057	1.56
4	3.125 ± 0.028	0.89

dated by repetitive analysis of the patient samples. Table IV shows the results of 0-, 4-, 24-h, and 28-day CAI blood samples for the first four patients on the clinical trial of daily oral administration of CAI. The 28-day plasma sample duplicates were assayed five times each and the results are shown in Table V. Fig. 5 demonstrates the negative control (time = 0) and highest level (time = day 28) for patient 2. Comparison of Fig. 5A and B suggests additional small peaks between CAI and the void volume; these are under further investigation. The reliability in assessment of these patient unknowns supports the accuracy and precision results determined with the gravimetric CAI plasma samples.

DISCUSSION

Identification and development of new approaches to inhibit malignant growth and metastasis remain an important goal. We have used selected signal transduction pathways as target sites against which to test potential anticancer compounds. This screening tool has identified CAI as the first in this new class of signal transduction inhibitors [2–5]. A reliable, accurate assay was required for measurement of CAI in patient and laboratory samples. The assay described herein represents a procedure for the resolution and quantification of CAI in plasma; this protocol has been used successfully to measure CAI in tissue culture specimens also (data not shown). This method is straightforward and is currently in use for clinical monitoring of patients on the CAI phase I clinical trial.

The reversed-phase protocol designed for CAI detection incorporates a solid-phase organic extraction [7,8] followed by an isocratic chromatographic separation. Reversed-phase chromatography was chosen as the separation tool because of its superior separation of hydrophobic compounds such as CAI which is non-polar and protic [9]. The goal of chromatography was to cleanly separate CAI and harmine from each other and elements removed in the void volume, using the shortest separation time. This protocol was chosen for its simplicity over a gradient reversed-phase chromatographic method previously employed in the laboratory.

This system has been used successfully to measure CAI in clinical samples. It is being used to support the first human clinical trial of CAI administration. It was not known whether CAI would be present in detectable levels or, if detectable, to what magnitude these levels would rise. The targeted plasma concentration range, suggested by the *in vitro* and animal experiments, was between 1 and 10 μM (0.4–4.0 μg/ml). The standard curve was designed to accommodate concentrations at both ends of this scale. Patient samples ranged from true zero troughs to measurable levels of 0.015–4.6 μg/ml. These measurements were reproducibly accurate. Additional small peaks were seen between the CAI peak and the void volume (Fig. 5) suggesting metabolites of CAI may be detectable by this HPLC separation method. Further studies are underway to characterize these peaks.

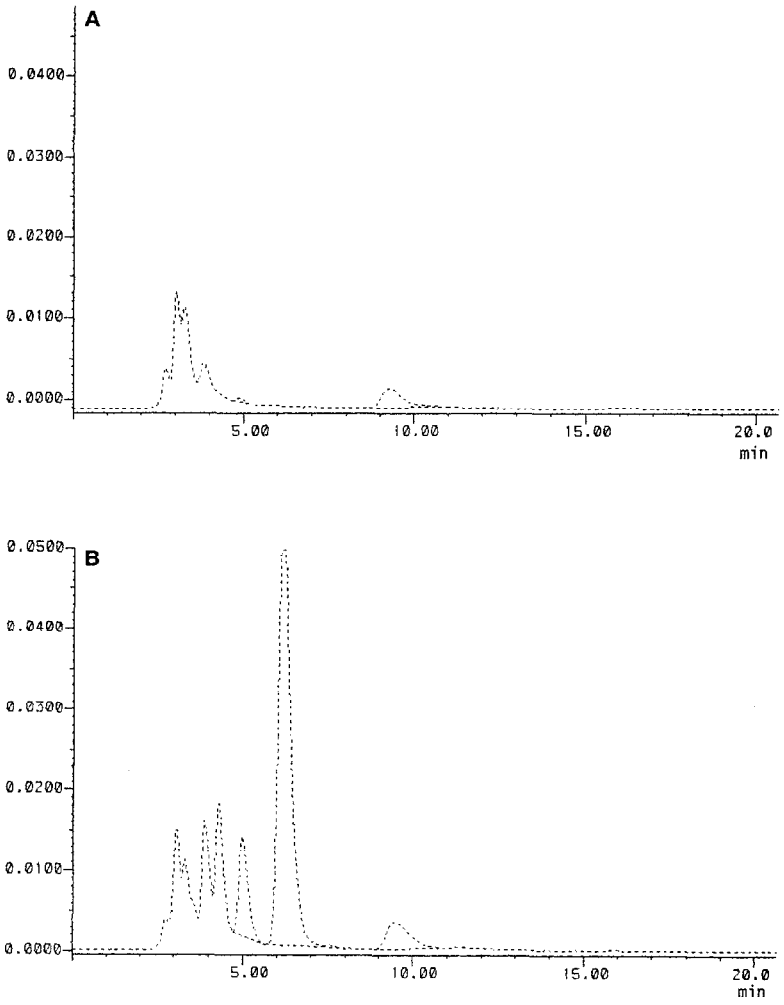

Fig. 5. Sample patient CAI unknown chromatograms. A. Chromatogram of pre-CAI blood sample. Plasma sample was obtained from patient 2 prior to initiation of CAI therapy. Harmine eluted at 9.25 min. B. Chromatogram of 28-day blood sample. This sample was taken at the completion of 28 days of daily oral CAI administration. CAI eluted at 6.12 min and harmine at 9.46 min.

Statistical analysis of repetitive assays of known CAI samples has demonstrated that this extraction and chromatographic method yields accurate, precise, and reproducible results.

While this system is time-consuming, requiring extraction and chromatographic analysis, it can be automated by use of the vacuum manifold with multiple columns and by the use of autosampler injection and computer monitoring. Other methods of CAI detection, such as enzyme-linked immunoabsorbent assay, which may prove to be equally precise but less labor-intensive, are under development.

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