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Journal of Chromatography B, 654 (1994) 257–262

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of L-2-oxothiazolidine-4-carboxylic acid (Procysteine®) in human plasma by high-performance liquid chromatography

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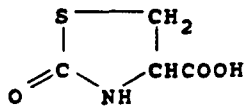
(First received October 21st, 1993; revised manuscript received January 12th, 1994)

Abstract

A new high-performance liquid chromatographic (HPLC) method has been developed for the determination of the cysteine prodrug, L-2-oxothiazolidine-4-carboxylic acid (Procysteine®), in human plasma. Samples were mixed with 5% metaphosphoric acid and the supernatants chromatographed using a reversed-phase analytical column. Procysteine was detected spectrophotometrically at 230 nm and quantitated by comparison with a standard curve of known amounts of Procysteine (20–1000 μM) in plasma. The coefficients of variation for 48.6 μM and 676 μM control pools were 6.5 and 4.0% respectively ($n = 46$). Deviations from the expected concentrations were less than 2%. The method has been utilized to evaluate the pharmacokinetics of a wide range of oral and intravenous doses.

1. Introduction

Procysteine® (L-2-oxothiazolidine-4-carboxylic-acid) is a 5-oxoproline analog with the molecular formula shown in Scheme 1. Procysteine is useful as an intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis [1,2]. In a reaction catalyzed by 5-oxo-L-prolinase, Procysteine is converted to S-carboxy-L-cysteine, which spontaneously decarboxylates to L-cysteine, facilitating glutathione synthesis [1]. Glutathione, like other antioxidants such as N-acetyl cysteine and glutathione monoethyl ester, has been reported to have ameliorative effects on *in vitro* HIV (human immunodeficiency virus) expression [3–5]. However, glutathione is depleted in plasma and peripheral blood mononuclear cells of patients infected with HIV [6,7]. The potential benefits of Procysteine treatment, such as the maintenance of intracellular glutathione levels, have led to the evaluation of this cysteine prodrug in healthy human volunteers [2] and in HIV seropositive patients [8].



Scheme 1.

Methods for the assay of Procysteine in biological fluids have been based on scintillation counting of the ^{35}S-labelled molecule and have

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0378-4347/94/$07.00
SSDI 0378-4347(94)00021-V

been limited to animal studies [9]. A high-performance liquid chromatography (HPLC) method for the determination of Procysteine in human plasma has recently been briefly described [2]. Procysteine was isolated from plasma by anion-exchange solid-phase extraction (SPE) prior to quantitation by ion-pairing reversed-phase high-performance liquid chromatography (HPLC). To address the need for a precise and accurate method which eliminates SPE and is more suitable for the rapid measurement of Procysteine in large numbers of plasma samples, we have developed a reversed-phase HPLC method that requires only acid deproteinization for sample preparation. This method has been used to support the Procysteine pharmacokinetics portion of multi-center clinical trials [10,11].

2. Experimental

2.1. Reagents and samples

Procysteine, greater than 99% pure, was provided by Free Radical Sciences (Cambridge, MA, USA). The sodium salt of 1-heptane sulfonate (HSA) was obtained from Alltech (Deerfield, IL, USA). Reagent grade metaphosphoric acid, phosphoric acid, and sodium phosphate monobasic were from Mallinckrodt (St. Louis, MO, USA). HPLC-grade methanol was from Burdick and Jackson (Muskegon, MI, USA). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Blood samples were collected by venipuncture into an EDTA containing Vacutainer tube (Becton Dickinson, Rutherford, NJ, USA) and kept on ice. Plasma was separated within one hour and stored at −70°C. For method evaluation, human plasma samples were obtained from volunteers on site.

To test the stability of Procysteine in whole blood, approximately 10 ml of blood from a healthy volunteer was drawn, immediately mixed with a 50-μl aliquot of an aqueous 100 m*M* Procysteine solution, and placed on ice for approximately 15 min. An aliquot of the blood was then centrifuged (10 min, 1000 *g*) to obtain plasma. The remainder of the blood was left on ice for an additional 75 min before another aliquot was centrifuged to separate plasma. The remainder of the blood was then stored at room temperature for 2.5 h before separation of plasma. All plasma samples were deproteinized without delay for HPLC analysis.

2.2. Preparation of standards and controls

Aqueous 100 m*M* and 20 m*M* Procysteine standards were prepared and aliquots stored at −70°C until use. For each day's run, additional standards (0.4–10 m*M*) were prepared by dilution of the 100 m*M* standard with water. Plasma standards (20–1000 μM) were also prepared daily by mixing 475-μl aliquots of a plasma pool with 25 μl of the appropriate aqueous standard. Quality control (QC) samples were prepared by adding a known amount of Procysteine to a different plasma pool. QC aliquots were assayed immediately or stored at −70°C if assayed on more than one day.

2.3. Sample preparation for HPLC

Plasma (200–500 μl) in a microfuge tube was deproteinized by the addition of an equal volume of ice-cold 5% (w/v) metaphosphoric acid, followed by vigorous mixing. The plasma was incubated at room temperature for 10–15 min and then centrifuged at approximately 16 000 *g* for 5 min in a microfuge. The supernatants were transferred to autosampler vials.

2.4. HPLC procedure

The HPLC equipment consisted of a Model 9095 Autosampler, Model 9010 solvent delivery system, and Model 9050 variable wavelength UV-Vis detector, all from Varian (Walnut Creek, CA, USA). Procysteine in plasma was separated at ambient temperature on an Adsorbosphere HS C_{18} column, 250 × 4.6 mm I.D., 5 μm (Alltech, Deerfield, IL, USA). A Brownlee 15 × 2.2 mm I.D. NewGuard® cartridge packed with 7-μm RP-18 Aquapore (Brownlee Labs., Santa Clara, CA, USA) served as a guard column. The

mobile phase was 0.1 *M* sodium phosphate monobasic, adjusted to pH 3.0 with phosphoric acid. Samples (25 μl) were chromatographed at a flow-rate of 1.0 ml/min.

Eluted compounds were detected at 230 nm. Peak areas were determined and the assay calibrated using the Hewlett-Packard Laboratory Automation System (Avondale, PA, USA). For each run, a linear regression line was obtained by least squares analysis of the peak areas and concentrations of the plasma standards. The equation of this line was used to calculate Procysteine concentrations from peak areas of the plasma samples.

3. Results and discussion

The chromatograms in Fig. 1 are shown at high sensitivity to illustrate the specificity of the method. There was a window in the 7–10 min region in which Procysteine was essentially baseline resolved. Some new Adsorbosphere HS C_{18} columns were initially conditioned once, when new, with a solution of 0.5 m*M* HSA in 0.1 *M* phosphate buffer, pH 3.0 to reduce the Procysteine retention time to less than 10 min. After conditioning with at least 60 ml of this solution, the column was washed with the same volume of water and then purged with 90% (v/v) aqueous methanol. After the initial conditioning, HSA was never added to the mobile phase. Other columns were used as received from the manufacturer, with no conditioning.

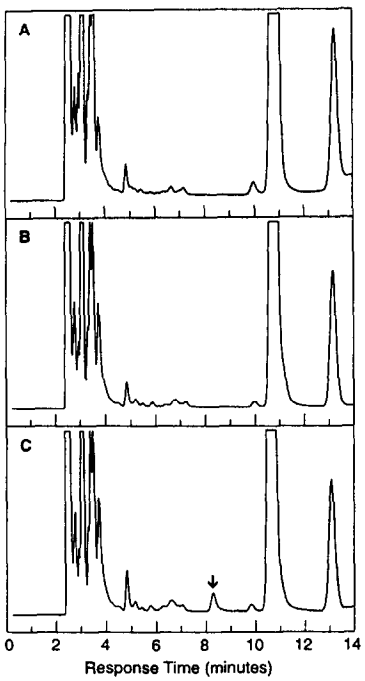

Fig. 1. Chromatograms of (A) pooled normal plasma; (B) plasma obtained from an HIV-positive asymptomatic patient prior to receiving 500 mg oral Procysteine; (C) plasma obtained from the same patient 0.5 h after therapy, containing 30 μ*M* Procysteine. The Procysteine peak, at approximately 8.3 min in chromatogram C, is indicated. All chromatograms are 0.01 AUFS.

3.1. Interferences

Chromatograms derived from plasma samples which were obtained from normal volunteers who had ingested common medications indicated that acetaminophen, aspirin, caffeine, ibuprofen and various antihistamines including dextromethorphan-HBr, pseudoephedrine-HCl and doxylamine succinate do not interfere. As expected, relatively nonpolar compounds, including antiretroviral nucleosides, such as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI) were eluted much later than Procysteine (>30 min). There were no false-positive measurements of Procysteine in 135 samples from 16 patients in the placebo group of clinical trials, including patients who were receiving AZT or ddI. Each HPLC run (50–100 injections of plasma standards and samples) included two plasma blanks. Procysteine was not detected in any of these blanks. We found that interferences from previous injections could be avoided by adjusting the sample analysis time to *ca.* 23 min. Columns could be used for at least 1500 injections of plasma supernatants if the column was purged for 1–2 h with water and 90% aqueous methanol after each HPLC run.

3.2. Linearity, accuracy, precision, sensitivity

Duplicate aliquots of plasma standards in the 20–1000 μ*M* range were prepared and processed in each day's run with each set of unknown samples analyzed. A single standard curve for

the run was calculated using the peak areas obtained by chromatographing the first replicate of the standards at the beginning of the run and the second replicate at the end of the run. Peak areas were a linear function of concentration in the 20–1000 μM range. The equation of the line was $y = -958 + 292x$ where 292 and 958 are averages of slopes (area counts/μM) and intercepts determined for 21 standard curves on 21 different days. The metaphosphoric acid supernatants of samples containing Procysteine concentrations above the linear calibration range were diluted to within the range and reassayed.

The calculated slopes of standard curves had low variability and ranged from 282 to 305 area counts/μM for curves determined on the 21 different days. On one day, the slope for the plasma standard curve was 279, and the slope for aqueous standards prepared and analyzed in the same way as the plasma standards was 303. The ratio of the two slope values indicated that the recovery of Procysteine from metaphosphoric acid precipitated plasma samples was 92%. Correlation coefficients for all standard curves were greater than 0.995, and the average value of all correlation coefficients was 0.9995 ($n = 21$). The observed mean concentrations of the standards, calculated from the standard curves and the Procysteine peak areas, were within 1% of the prepared values, except for the lowest standard (Table 1). Coefficients of variation (C.V.s) were less than 6% except that determined for the lowest standard.

We initially estimated the lower limit of quantitation (LOQ) to be the concentration of the lowest standard (20 μM), using the criteria that the mean value should not deviate more than ± 20% and the C.V. should not exceed 20% [12]. When the full 20–1000 μM range of standards was included in the standard curves, the negative intercept (equivalent to about 3 μM) precluded a reduction of the LOQ to a concentration closer to the lower limit of detection, which was 2 μM (signal-to-noise ratio 2). However, we found that the occasional sample in the 5–20 μM range could be quantitated when the high standards were eliminated and the calibration curve was based on 20, 50, and 100 μM standards. The equation of the line for these standards was $y = -225 + 284x$ where 284 and 225 are the averages of slopes and intercepts determined for 13 standard curves on 13 different days.

There was excellent agreement between the prepared and observed concentrations of QC samples (Table 2). At least two aliquots of two of the QC samples (48.6 and 676 μM) were included with each set of unknown samples analyzed over a one year period. The overall C.V.s for these two controls were 6.5% and 4.0% respectively. Between-day and within-day C.V.s for all QC samples are summarized in Table 2.

3.3. Analyte stability

Procysteine concentrations in plasma separated within one hour after blood drawing did not differ from Procysteine concentrations in

Table 1
Calculated concentrations of calibration standards for determination of Procysteine in plasma

Prepared standard concentration (μM)	Number of observations	Mean concentration found (μM)	Deviation (%)	C.V. (%)
1000	41	1000.9	+0.1	1.9
500	41	499.1	−0.2	3.0
250	41	248.0	−0.8	3.7
100	40	99.4	−0.6	5.1
20	42	21.9	+9.3	16.5

A separate standard curve was determined each day from standards freshly prepared and processed in duplicate

Table 2
Reproducibility and accuracy of Procysteine assay determined by analysis of quality control samples

Concentration (μM)		Deviation (%)	Number of observations		C.V.[a] (%)	
Prepared	Measured		Total	Days[b]	Intra	Inter
48.8	48.0	−1.7	46	21	4.0	5.2
676	663	−1.9	46	21	0	4.6
600	601	+0.2	5	1	0.7	–
150	147	−1.8	5	1	0.9	–

[a]Intra-day and inter-day C.V.
[b]One to six assays per day on different days.

plasma separated 15 min after blood drawing (Table 3). The Procysteine concentration dropped significantly in the plasma that was not separated until the blood stood at room temperature for 2.5 h. The data support the conclusion that plasma Procysteine concentrations do not decrease if the blood specimen is stored on ice and plasma is separated within 1.5 h after blood drawing. The data used for Table 2 included plasma pools (prepared concentration = 48.8 and 676 μM) which were stored at −70°C and assayed over a period of 10 months. There was no observable degradation of Procysteine in these quality control samples. No significant changes in Procysteine concentrations were observed in plasma samples which were allowed to remain at room temperature for several hours and were then frozen and reassayed the following day. Repeated analyses of the metaphosphoric acid supernatants prepared from standards and controls revealed no decreasing trend in Procysteine peak areas in 2–3 day time periods, indicating that the analyte was stable in the supernatants.

3.4. Clinical trial applications

The method described has been used for the analysis of *ca.* 1200 plasma samples collected from normal, HIV infected, and ARDS (acute respiratory distress syndrome) patients during Phase I/II clinical trials. Pharmacokinetic analyses for a wide range of oral and intravenous Procysteine doses administered in these studies are presented elsewhere [10,11]. In a study of the oral pharmacokinetics of single dose Procysteine following acute and chronic administration in 27 asymptomatic HIV-infected subjects, an initial single dose of 50, 1500, or 4500 mg Procysteine was administered to nine subjects at each dose level [11]. After the chronic phase of the study, on day 42, the same nine subjects received a single dose of Procysteine equivalent to the initial dose of drug that they

Table 3
Effect of blood sample storage on plasma Procysteine concentrations

Length of time blood stored before plasma separation begun	Storage conditions	Plasma Procysteine concentration[a] (mean ± S.D.) (μM)	n
15 min	On ice	807.3 ± 8.4	3
75 min	On ice	815.1 ± 8.9	5
3.75 h	75 min on ice then 2.5 h at room temperature	763.2 ± 16.8	5

[a]The mean concentration for the 15 and 75 min samples was 812 ($n = 8$) and was significantly higher than the mean for the 3.75 h samples ($p < 0.0005$, unpaired t-test).

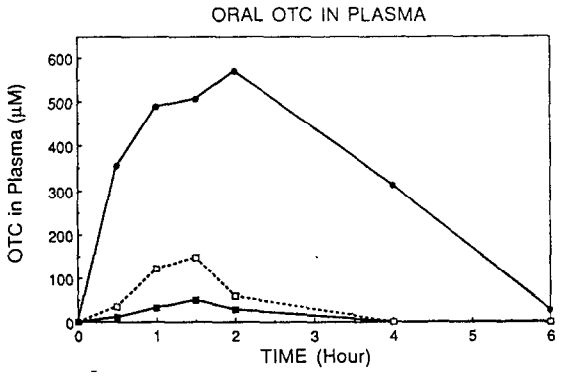

Fig. 2. Plasma drug concentration after a single oral dose of Procysteine to three subjects at three dose levels: 500 mg (■), 1500 mg (□), 4500 mg (●).

had received. Pharmacokinetic indices were similar at both time points. For example, maximum plasma concentration (C_{max}) values for each dose group were 73, 201, and 612 μM on day 1, and 63, 180, and 655 μM on day 42; these values increased proportionally with dose. Representative plasma concentration *vs.* time profiles for the three dose levels are shown in Fig. 2. The appearance of these profiles is similar to those reported by Porta *et al.* [2], who used their HPLC assay to measure plasma Procysteine following oral administration of this drug to six healthy volunteers.

4. Conclusions

The HPLC method reported here has proven to be reliable, rapid and suitable for the support of pharmacokinetic studies. The preparation of plasma samples by acid precipitation greatly reduces the analysis time relative to that required for methods which utilize SPE, and the cost of extraction columns is eliminated. We selected metaphosphoric acid as the precipitating agent, because we had found, as reported previously [13], that the Procysteine metabolites, glutathione and cysteine, are stable in supernatants of 5% metaphosporic acid-treated plasma, blood, and tissues. Glutathione, cysteine and other thiols and disulfides in these supernatants may be determined by ion-pairing reversed-phase HPLC with electrochemical detection [13]. We have found that the Alltech HS C_{18} column may be used to separate these thiols when HSA is added to the mobile phase that we use for Procysteine analyses. This finding would be expected from the separation of thiols previously achieved with this column [14]. Thus, the combination of our chromatographic procedure with UV and electrochemical detection may prove useful for the simultaneous determination of Procysteine and its metabolites.

5. Acknowledgements

We thank Free Radical Sciences for providing the clinical samples.

6. References

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