Table I—Addition of Ferricyanide and Ferrocyanide to I

Amount Added, %			Number of
Ferricyanide	Ferrocyanide	Recovery, %	Runs
0.05	—	0.047 ± 0.004	7
—	0.05	0.047 ± 0.005	7
0.04	—	0.037 ± 0.008	5
—	0.04	0.041 ± 0.014	5
0.03	—	0.031 ± 0.005	6
—	0.03	0.024 ± 0.004	6

viously described with the exception of the final wash. Here the nitric acid concentration was varied on individual columns from 1.0 to 2.5 *N*, and 25.0 ml of eluate was collected. A similar experiment using ferrocyanide was carried out using nitric acid strengths up to 6 *N*.

At nitric acid concentrations of 1.0 *N* and below, no ferricyanide was eluted; full recovery was obtained at an acid strength of 1.8 *N* and above. Ferrocyanide started eluting when the nitric acid concentration reached 2.5 *N*. At nitric acid strengths of 4.0 *N*, there was evidence that elution was still incomplete. An acid strength of 6.0 *N* eluted ferrocyanide completely. As a result of these data, nitric acid concentrations of 2.0 and 6.0 *N* were chosen for the ferricyanide and ferrocyanide elutions, respectively.

To verify the column results, a TLC separation of I, ferricyanide, and ferrocyanide was performed on aluminum oxide plates with 2.0 *N* HNO_3 as the developer. As with the column separation, I and ferricyanide traveled to the solvent front while ferrocyanide remained at the origin.

The linearity of the spectrophotometric response to changes in ferricyanide and ferrocyanide concentrations was evaluated. Known amounts of potassium ferricyanide and potassium ferrocyanide trihydrate were subjected to chromatography, and the absorbances of the solutions were determined spectrophotometrically. A plot of absorbance *versus* concentration was linear from 2 to 30 μg of ferricyanide and ferrocyanide/ml. Since a sample size of 400 mg was chosen to assure nonoverloading of the column, 8 μg each of ferricyanide and ferrocyanide/ml represented 0.05% of the I content. This concentration is well within the region of linear response.

To determine whether ferricyanide and ferrocyanide could be determined accurately at levels below 0.05%, known concentrations of each were added to I and the solutions were analyzed according to the procedure (Table I). The data indicate that the precision and accuracy of the method are lower than those normally acceptable for a quantitative determination. However, its use as a limit test is justified since the recoveries of both ferricyanide and ferrocyanide are at least semiquantitative when added to I.

Compared to the limit tests currently available for the determination of ferricyanide and ferrocyanide in I, this procedure offers the following advantages:

1. Semiquantitation of the contaminants, if more than a limit test is required, is possible.
2. The results are reproducible.
3. The preparation of reagents, samples, and standards is simple.
4. The procedure can be carried out rapidly.

Since I is now accepted and used as a pharmaceutical preparation, this improved procedure for controlling two possible contaminants will help in maintaining and ensuring the purity and integrity of the product.

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Determination of Carbocysteine from Human Plasma

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Abstract □ A fast and accurate GLC procedure for determining carbocysteine, using a sulfur photometric detector, is described. Interferences from nonsulfur constituents are eliminated. The method is linear from 2 to 15 μg. Results from the application of this method to a bioavailability study in humans are given.

Keyphrases □ Carbocysteine—GLC analysis in plasma □ GLC—analysis, carbocysteine in plasma □ Mucolytic agents—carbocysteine, GLC analysis in plasma

Carbocysteine, *S*-(carboxymethyl)cysteine, has been undergoing clinical evaluation as a mucolytic agent. Therefore, it was of interest to determine the pharmacokinetic parameters of this drug, and an analytical method was needed.

Carbocysteine has properties typical of amino acids and has been determined in biological materials by ion-exchange chromatography (1). That method is time consuming and did not appear practical. GLC procedures are more rapid and have varying degrees of success using trifluoroacetic anhydride derivatization with flame-ionization detection (2–8). In general, these methods require temperature programming, which is undesirable.

Since carbocysteine contains a sulfur atom, the use of a flame photometric detector in the sulfur mode appeared logical. Concentration of the drug through extraction into an organic solvent would also help in derivatization. Such a method was found to be of value by adopting Maynard's (9) procedure for extracting piperazine from an aqueous solution. That this method may be applicable to the analysis of other amino acids was demonstrated by

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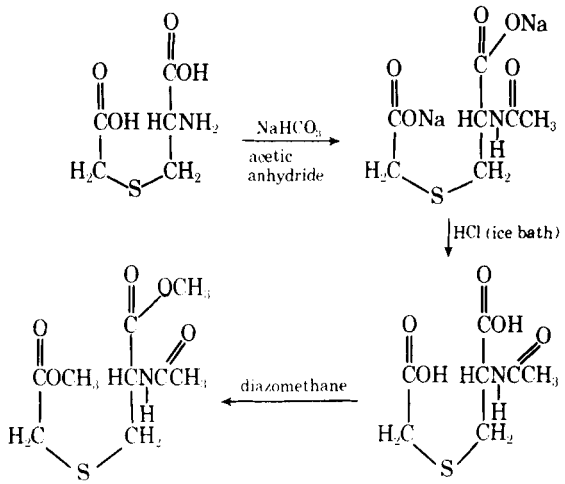

Scheme I

applying it to methionine, cystine, and cysteine.

EXPERIMENTAL

Reagents—Ethyl acetate and chloroform were ultrapure[1]; sodium bicarbonate, hydrochloric acid, and anhydrous sodium sulfate were reagent grade. Diazomethane was generated from nitrosomethylurea with 40% potassium hydroxide in chloroform and redistilled (10).

S-(2-Carboxyethyl)cysteine, the internal standard, was synthesized by refluxing a sodium hydroxide solution of L-cysteine hydrochloride with chloropropionic acid under nitrogen. The resulting compound was precipitated by adjusting to pH 2–3 with 6 *N* HCl. It was further purified by reprecipitation from water with 6 *N* HCl, mp 230° dec.

Anal.—Calc. for $C_6H_{11}NO_4S$: C, 32.29; H, 5.74; N, 7.25. Found: C, 36.88; H, 5.74; N, 7.20.

Equipment—The gas chromatograph[2] was equipped with a sulfur–phosphorus emission detector[2]. The column was glass, 1.2 m × 2 mm i.d., packed with 15% Apiezon-L on 100–120-mesh Gas Chrom P and silanized[3]. The column temperature was 185°; the injector and detector were at 295°. The carrier was nitrogen at a flow rate of 25 ml/min.

Structures of all compounds and their derivatives (Scheme I) were confirmed with a mass spectrometer[4] by a direct probe method.

Drug Administration—Eleven healthy, normal male subjects[5] received 1.0-g doses of carbocysteine orally. On 1 day, they received the drug as a capsule; 3 days later, they received it as a suspension. Blood samples were taken at intervals, and the plasma was separated and analyzed for carbocysteine.

Analytical Method—The plasma sample (1.0 ml) was pipetted into a 50-ml glass-stoppered centrifuge tube, and 10 μg of the internal standard in water was added. Then the solution was diluted to 4.0 ml with water and saturated with sodium bicarbonate (Scheme I). An excess of approximately 25 mg of sodium bicarbonate was added. Then 1.0 ml of acetic anhydride was added cautiously; the mixture was shaken gently and then mixed for 1 min on a vortex mixer or until there was no further evolution of carbon dioxide. The sample was then cooled in an ice bath and made acidic with concentrated hydrochloric acid (~2 ml).

The aqueous mixture was extracted with two 20-ml portions of ethyl acetate, and each extract was filtered through a layer of anhydrous sodium sulfate into a large test tube (~25 × 200 mm). The sodium sulfate was washed with 3 ml of ethyl acetate. The solution was evaporated in a hot water bath (about 80°) under a nitrogen stream to 5 ml, and the ethyl acetate solution was transferred to a 15-ml centrifuge tube. This solution was evaporated to dryness using the same conditions. The tube was cooled in an ice bath, and diazomethane in chloroform was added with mixing until the solution remained yellow for 1 min. Then this solution was evaporated to approximately 200 μl and mixed, and 10 μl was injected into the gas chromatograph.

The retention times of the derivatives of carbocysteine and the internal standard were 4.9 and 6.7 min, respectively.

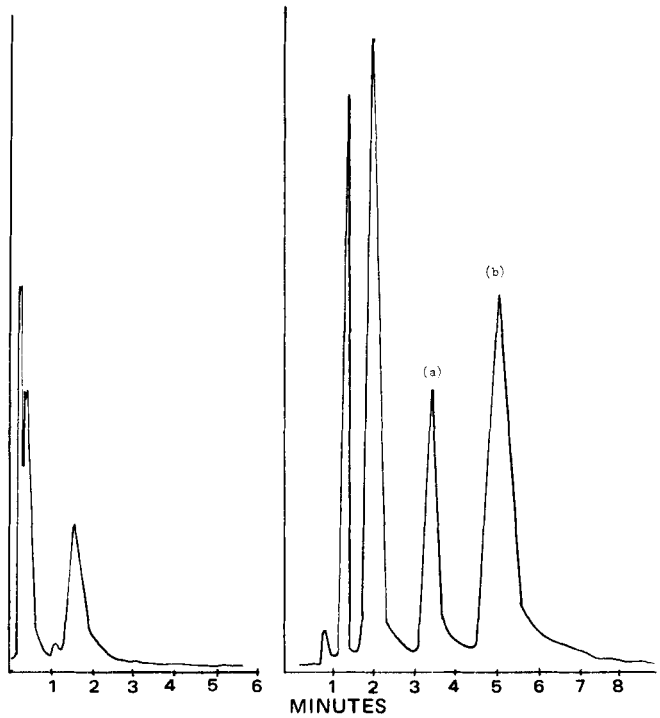

Figure 1—*Chromatograms of control plasma (left) and plasma containing carbocysteine (a) and S-(2-carboxyethyl)cysteine (b).*

Standard Curve and Calculations—A standard curve was prepared using 1.0 ml of plasma for each determination. Known concentrations of carbocysteine from 2.0 to 15.0 μg were added to each tube, followed by 10.0 μg of the internal standard.

The response of the flame photometric detector, as pointed out by Brody and Chaney (11), is exponentially proportional to the number of sulfur atoms in the molecule. To obtain a straight-line relationship between the response and concentration of carbocysteine, the square root of the peak height must be used rather than the simple peak height. The ratio obtained by dividing the square root of the peak height of the known concentration of the sample by the square root of the peak height of the internal standard was plotted against the amount added to obtain the calibration curve. The concentration of the sample in the aliquot taken for analysis was calculated from this calibration curve.

RESULTS AND DISCUSSION

Typical chromatograms obtained following the analysis of control plasma and of plasma containing both the internal standard and carbocysteine are shown in Fig. 1. These chromatograms do not show solvent peaks usually associated with GLC because early eluting gases, including solvents, were vented away from the detector to prevent undue contamination. However, the retention times were specific and easily repeated. No interferences were found from control samples carried through the procedure. It was necessary to silanize the column and to clean the detector following the injection of 150–200 samples.

The possibility existed of interference of other sulfur-containing amino acids whose derivatives might be extracted from blood. Aqueous solutions of methionine, L-cystine, and L-cysteine were carried through the described procedure. The structures of their derivatives were confirmed by mass spectra and then chromatographed. The cysteine derivative had a retention time of 3 min at a column temperature of 160°, the methionine derivative had a retention time of 3 min at 170°, and cystine had a retention time of 4 min at 250°. These compounds would not interfere under the method conditions. Similar GLC procedures for these amino acids probably could be easily developed.

Another source of possible interference is metabolites of the administered drug. Biotransformation of carbocysteine was studied[6] (12, 13). The major metabolites in humans, monkeys, dogs, and rats are inorganic

[1] Nanograde, Mallinckrodt Chemical Co.
[2] Bendix 2500 Series, Bendix Corp., Lewisburg, W. Va.
[3] Rejuv-8, Supelco, Inc.
[4] Hitachi RMU-6.
[5] Informed written consent was obtained.
[6] To be published.

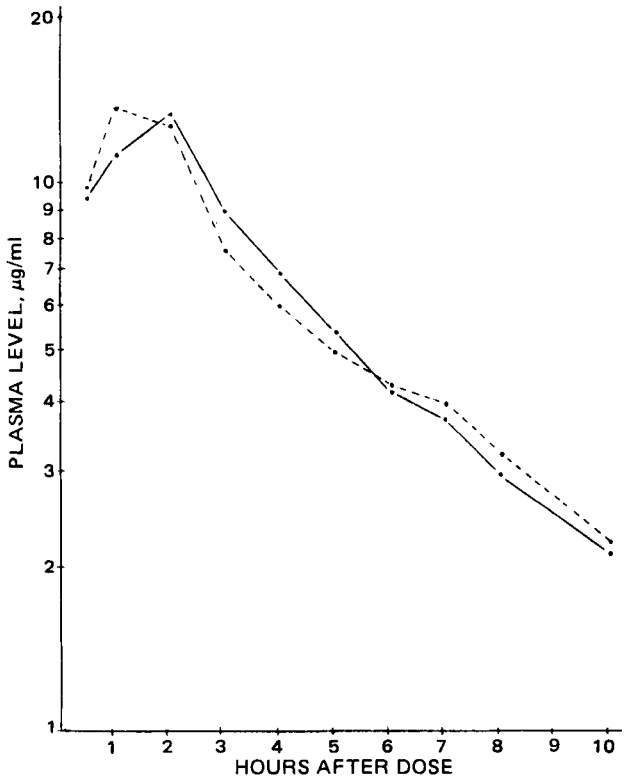

Figure 2—*Mean plasma carbocysteine levels in humans. Key: - - -, suspension; and —, tablet.*

sulfate and thiodiglycolic acid. A sample of this acid was carried through the described procedure and did not interfere. The dimethyl ester formed had a retention time of approximately 4 min at 170°. Like the cysteine and methionine derivatives, this compound would be discarded in the vented gas.

Recoveries of known amounts of carbocysteine added to control plasma are shown in Table I, together with standard deviations at low, medium, and high levels. These results indicate that the described procedure should be satisfactory for the determination of this compound in plasma.

The extraction procedure used for the amino acid was of primary interest and warranted further investigation. A consistent amount is extracted in this procedure, but the absolute amount is not indicated. This value was determined with ^{34}S-carbocysteine. A solution of 15 μg/ml of control plasma was prepared, and the radioactivity was determined before extraction and in the extract. The experiment was done with four samples of plasma; 72.8 ± 5.4% was extracted as the *N*-acetyl derivative.

Table I—Recovery of Known Amounts of Carbocysteine Added to Control Human Plasma

Amount Added, μg/ml	Amount Found (±*SD*[a]), μg/ml
2.0	2.08
2.5	2.58 (±0.050)
3.0	3.27
4.0	3.91
6.0	6.01
7.5	7.58 (±0.136)
9.0	9.23
10.0	10.1
12.0	12.1
15.0	15.3 (±0.39)

[a] From six determinations.

To illustrate the usefulness of this method, the results obtained from a bioavailability study are plotted in Fig. 2. This figure gives the mean values of the plasma levels for the 11 subjects (see *Experimental*). Maximum levels were obtained within 2 hr and were approximately 13 μg/ml; after 10 hr, they had declined to about 2 μg/ml. Standard errors at the two intervals were 1.38 and 0.46, respectively.

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