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Note

Determination of S-carboxymethyl-L-cysteine in plasma by high-performance liquid chromatography with column switching following precolumn derivatization

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S-Carboxymethyl-L-cysteine (SCMC), Rhinathiol®, is a mucolytic agent widely prescribed in bronchitis and other respiratory diseases [1, 2]. Like aspartic and glutamic acids this amino acid with two carboxylic functions (Fig. 1) cannot be extracted from biological fluids by conventional liquid–liquid procedures, and its lack of absorption in the visible and UV range prevents its detection without prior derivatization.

Since the original work of Stein and Moore [3], many modifications of methods based on ion-exchange chromatography followed by derivatization have been described [4, 5]. Although powerful enough to separate all the amino acids, these ion-exchange methods tend to be rather lengthy for the determination of a single amino acid in a complex mixture.

A gas chromatographic (GC) method has been developed for the quantification of SCMC in human urine, using a two-step derivatization procedure involving esterification with isobutanol–3 *M* hydrochloric acid and acylation by heptafluorobutyric anhydride [6]. Another GC method for the determination of SCMC in human plasma involves acylation with acetic anhydride and subsequent methylation with diazomethane, followed by sulphur photometric detection [7]. These two techniques lack the sensitivity needed for pharmacokinetic studies and involve tedious sample preparation.

A reversed-phase high-performance liquid chromatographic (HPLC) method for the quantification of SCMC in plasma as its phenylthiohydantoin derivative has been described [8]. However, the proposed UV absorption is not a specific mode of detection and late-eluting peaks derived from plasma increase the anal-

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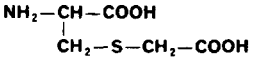
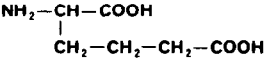
FORMULAE

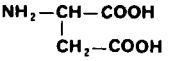
S-Carboxymethyl-L-cysteine



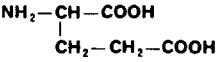
α-Aminoadipic acid (internal standard)



Aspartic acid



Glutamic acid



DERIVATIZATION REACTION WITH O-PHTHALALDEHYDE

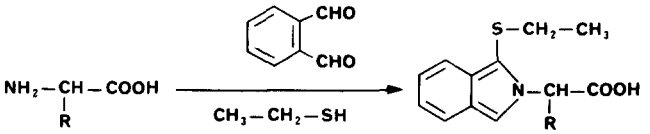


Fig. 1. Formulae and derivatization reaction with *o*-phthalaldehyde.

ysis time. In order to shorten the latter, Gaetani et al. [9] have used gradient conditions, but the analysis still requires 30 min. Following derivatization with *o*-phthalaldehyde (OPA) a limit of quantitation of 500 ng/ml for SCMC in plasma was obtained. Reversed-phase chromatography of OPA derivatives, described for twenty common amino acids by Hill et al. [10], seems to be convenient for the determination of SCMC in biological fluids.

The aim of this study was to improve upon the latter procedure by reducing the routine analysis run-time by column switching.

EXPERIMENTAL

Standards and reagents

SCMC was provided by Synthélabo (Mourenx, France), α-aminoadipic acid and ethanethiol (97%) were purchased from Aldrich (Beerse, Belgium). Glutamic and aspartic acids and OPA were obtained from Serva (Heidelberg, F.R.G.). Analytical-grade potassium dihydrogen phosphate, orthophosphoric acid, sodium hydroxide, potassium chloride, boric acid, perchloric acid (62%) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Merck (Darmstadt, F.R.G.) as well as methanol, LiChrosolv-grade acetonitrile and tetrahydrofuran.

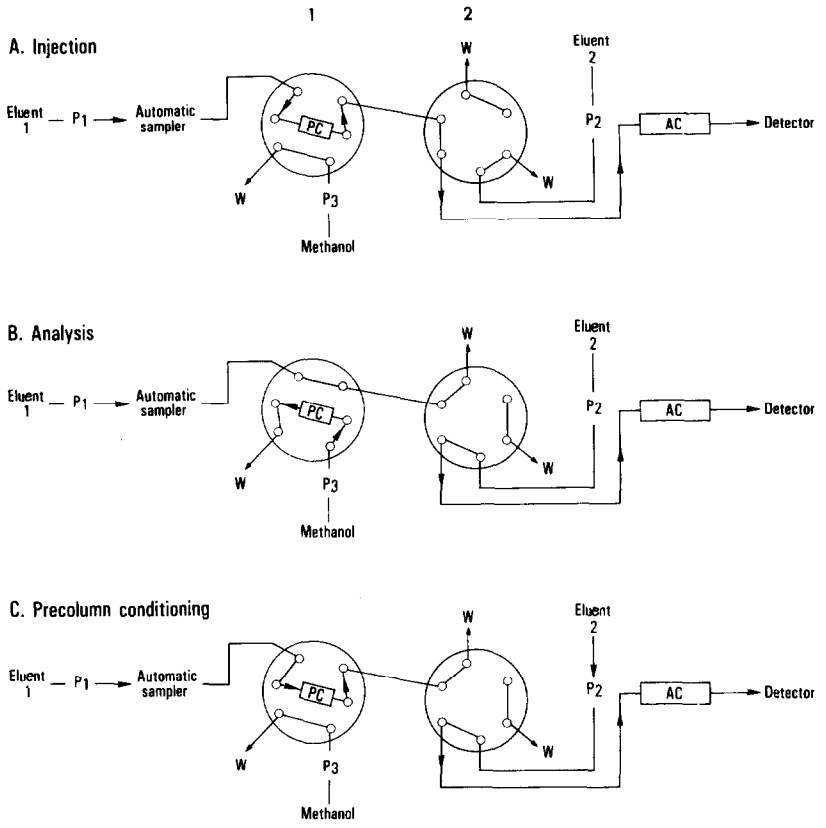


Fig. 2. Valves positions during (A) injection, (B) analysis and (C) precolumn equilibration. P = pump; PC = precolumn; AC = analytical column; W = waste.

Instrumentation

The chromatographic system (Fig. 2) consisted of: two Constametric III high-pressure pumps (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) for eluents 1 and 2; a Dosapro-Milton Roy minipump for rinsing the precolumn with methanol; two Rheodyne 7010 high-pressure switching valves (Cotati, CA, U.S.A.) motorized by electrical jacks (Touzart et Matignon, France) and controlled, through time relays, by a Sigma 10 data station (Perkin-Elmer, Norwalk, CT, U.S.A.); a Schoeffel FS 970 (Cunow, Clichy, France) or Kontron SFM 23B (Montigny le Bretonneux, France) fluorescence detector; a Sedex 100 automatic sampler (Sedere, France) with a 10-μl loop; a Perkin-Elmer 56 recorder.

Chromatographic conditions

Both eluents were mixtures of 0.4% aqueous TEMED solution, adjusted to pH 6.5 with phosphoric acid–methanol–acetonitrile–tetrahydrofuran. The relative composition of eluent 1 (pump P_1) was 85:7:7:1 (v/v) and of eluent 2 (pump P_2) 83:9:8:2.5 (v/v).

The analytical column, a stainless-steel tube (10 cm × 4.6 mm I.D.), was packed with Hypersil ODS (3 μm) (Shandon Southern, Runcorn, U.K.) at SFCC

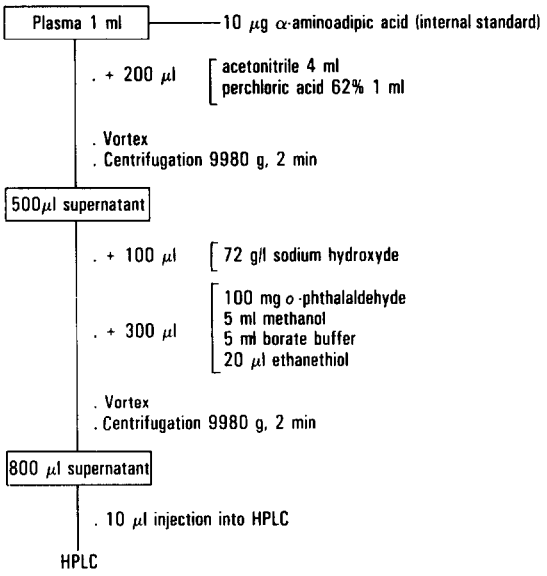


Fig. 3. Sample preparation scheme.

(Gagny, France) and the precolumn (3 cm × 4.6 mm I.D.) was filled with Spheri-5 C_8 (5 μm) from Brownlee (Santa Clara, CA, U.S.A.).

Detection was performed on a Schoeffel FS 970 fluorimeter with an excitation wavelength at 339 nm and a KV 470 emission filter. When the Kontron SFM 23B spectrofluorimeter was used the excitation and emission wavelengths were set at 339 and 455 nm, respectively.

A flow-rate of 1 ml/min was used throughout the separation.

Sample preparation

In an Eppendorf conical tube, 200 μl of a mixture containing acetonitrile and 62% perchloric acid (4:1, v/v) were added to a 1-ml plasma sample spiked with 10 μg of α-aminoadipic acid as the internal standard. After vigorous mixing, the tube was centrifuged at 9980 *g* for 2 min in an Eppendorf top bench centrifuge. To 500 μl of the supernatant were added 100 μl of a 2 *M* sodium hydroxide aqueous solution and 300 μl of the reagent. After vigorous mixing and centrifugation at 9980 *g* for 2 min, 800 μl of the supernatant were transferred into a brown bottle and 10 μl were injected with the automatic sampler onto the column. This preparation is outlined in Fig. 3.

Reagent preparation

The reagent was prepared by dissolving 100 mg of OPA in 5 ml of methanol and 5 ml of a borate buffer (pH 10) containing 20 μl of ethanethiol. The composition of the borate buffer (pH 10) was as follows: 50 ml of 0.1 *M* boric acid in 0.1 *M* potassium chloride are added to 43.9 ml of 0.1 *M* sodium hydroxide and diluted to 100 ml with distilled water.

Column-switching procedure

At the beginning of the cycle, when the sample was automatically injected, valve 1 and valve 2 were set such that eluent 1, with a weaker elution power than eluent 2, flowed through the precolumn and the analytical column (Fig. 2). Since the relays could be operated only sequentially with the Sigma 10, there was a slight delay ($\simeq 0.03$ min) between the two valve switchings. As soon as the compounds to be analysed reached the analytical column ($t = 4.70$ min), valve 1 was switched so the precolumn could be backflushed with methanol. The valve 2 switching allowed eluent 2 to flow through the analytical column.

Before the end of the chromatographic run, the precolumn was reequilibrated with eluent 1 by switching valve 1 to the "inject" position. This last step required only 1–2 min.

RESULTS

Without precolumn switching many peaks eluting over a 50-min period were observed following separation of a blank plasma extract with eluent 2 (Fig. 4A). The same plasma sample injected through the precolumn with eluent 1 and analysed on the same column with eluent 2 gave the chromatogram in Fig. 4B. Late-eluting peaks from the precolumn are cut off by the switching procedure and do not enter the analytical column. Fig. 4C shows a chromatogram obtained from a plasma sample from a healthy volunteer (1 ml) obtained 3 h after administration of a 3-g oral dose of SCMC. SCMC and α-aminoadipic acid are well resolved and no interference from endogenous compounds can be observed.

The stability of OPA derivatives was assessed by injecting the same sample containing SCMC and α-aminoadipic acid, kept for 21 h in a brown bottle. No difference with time was observed in the peak-height ratio of SCMC to α-aminoadipic acid.

The linearity for SCMC, aspartic and glutamic acids in water was investigated with nine data points from 100 ng/ml to 50 μg/ml, using 10 μg/ml α-aminoadipic acid as internal standard. The peak height ratio (h), used for quantitative measurement was linearly correlated to the concentration (C), according to the following equations: $h = 0.1278C - 0.0044$ ($r = 0.99975$) for SCMC; $h = 0.1634C - 0.0112$ ($r = 0.99997$) for aspartic acid; $h = 0.181C - 0.0023$ ($r = 0.99993$) for glutamic acid.

The reproducibility of the quantitative determination of SCMC in plasma samples was followed over a two-month period. At each concentration from 2 to 50 μg/ml the coefficients of variation were smaller than 10%. Extreme values ranged from 3.6% at 50 μg/ml to 8.5% at 5 μg/ml (Table I).

The stability of SCMC in plasma at 37°C was assessed by preparing a 10-ml pool spiked with 100 μg of SCMC. Every hour for 24 h 1 ml was sampled and analysed as described above. SCMC was found to be stable in plasma at 37°C for that length of time.

This method allows quantitative determination of SCMC down to 250 ng/ml with a signal-to-noise ratio of 10, which is sensitive enough for pharmacokinetic studies. However, the full sensitivity limit of the method was not reached as only 10 μl (out of 800) were injected.

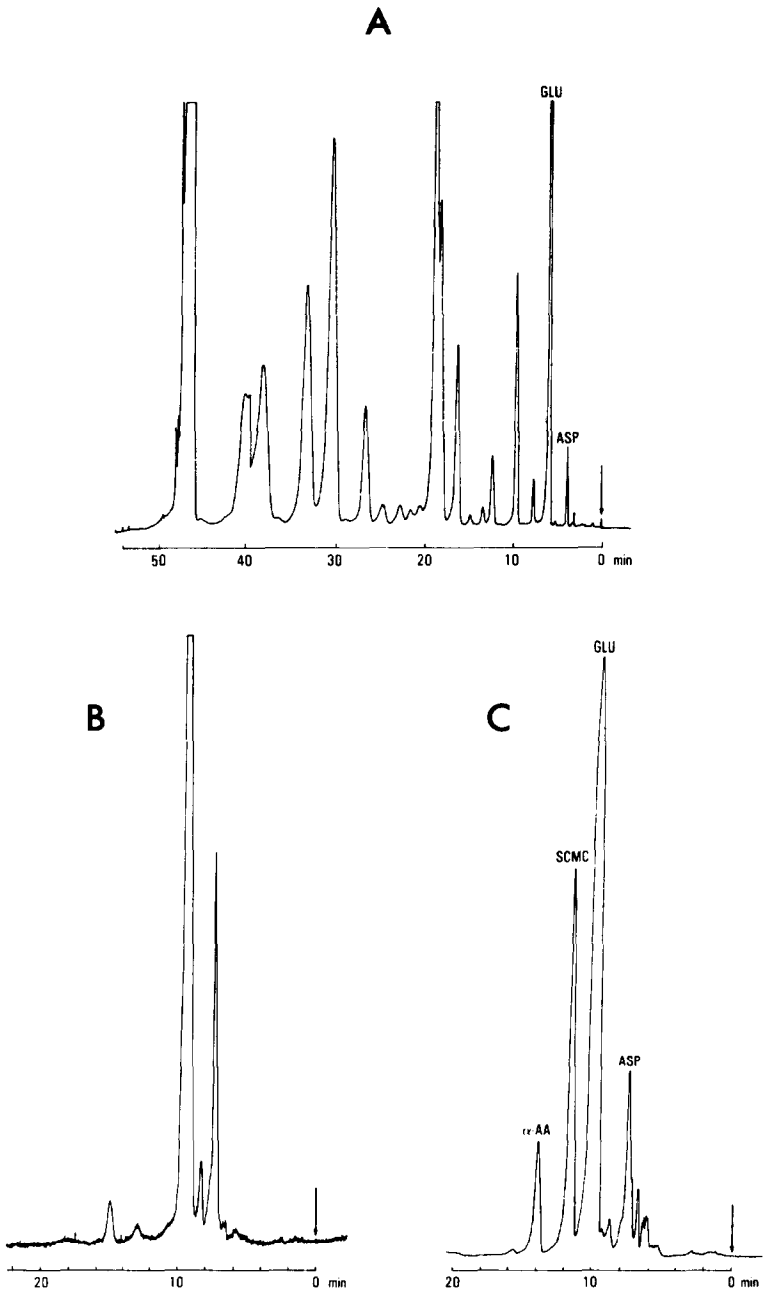


Fig. 4. Chromatograms of (A) a blank plasma sample without column switching (eluent 2), (B) a blank plasma sample using column switching (eluents 1 and 2), (C) a plasma sample (1 ml) obtained from a healthy volunteer 3 h after administration of a 3-g oral dose of SCMC (chromatographic conditions B). Peaks: α-AA = α-aminoadipic acid; SCMC = S-carboxymethyl-L-cysteine; GLU = glutamic acid; ASP = aspartic acid.

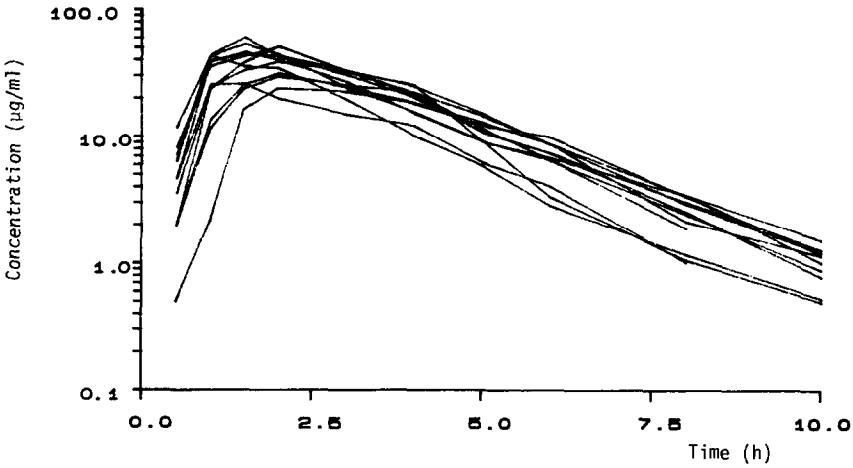


Fig. 5. Individual pharmacokinetic profiles obtained from twelve healthy volunteers, following administration of a 3-g oral dose of SCMC (as a 5% syrup).

Plasma concentrations obtained in twelve healthy volunteers following a 3-g oral administration of SCMC as a 5% syrup are shown in Fig. 5. Maximum plasma concentrations ranged from 23.7 to 59.5 μg/ml. Following a mono-exponential decrease, plasma concentrations of ca. 1 μg/ml were observed 10 h after intake.

DISCUSSION

The present method can be applied to the quantification of other dicarboxylic acids, such as aspartic and glutamic acids, as shown by the linearity test. However, some modifications to eluent 2 may be required in order to separate the endogenous peak interfering with glutamic acid in plasma, seen as a shoulder in Fig. 4B.

The use of eluent 1, which has a weaker eluting power than eluent 2 since it contains more water and a smaller percentage of organic modifiers, prevents peak broadening and allows all the endogenous monocarboxylic amino acids from plasma to be retained on the precolumn. As the latter are backflushed with meth-

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF S-CARBOXYMETHYL-L-CYSTEINE IN PLASMA AT DIFFERENT CONCENTRATIONS

Added concentration (μg/ml)	*n*	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
2	6	1.98 ± 0.14	7.35
5	7	4.73 ± 0.40	8.45
10	11	9.59 ± 0.54	5.59
25	7	25.57 ± 1.13	4.43
50	11	51.7 ± 1.88	3.64

anol without being injected onto the analytical column, the run-time for complete sample chromatography is significantly reduced.

This time saving can be partially achieved with a gradient system, as shown by Hill et al. [10]. But precolumn switching gives a more stable baseline and is more convenient to set up than gradient conditions for routine analyses of a large number of samples, since it avoids reequilibration of the analytical column between each injection.

Eluent 2 was chosen after several trials to get the best separation for SCMC and α-aminoadipic acid on the analytical column without using buffer solutions, which may damage the pumps when used continuously in an automatic system.

Organic modifiers, such as acetonitrile, methanol and tetrahydrofuran, are useful in achieving selectivity, short retention times and sharp peaks. The role of 0.4% TEMED at pH 6.5 has not been fully elucidated. It is supposed either to form ion pairs with the ionized dicarboxylic functions of the amino acids, or to mask the residual silanols on the bonded silica gel stationary phase, as described by Gill et al. [11].

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