Journal of Chromatography, 428 (1988) 301–310
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4190

DETERMINATION OF S-CARBOXYMETHYLCYSTEINE IN SERUM BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY WITH COLUMN SWITCHING FOLLOWING PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE

J.A. DE SCHUTTER*, G. VAN DER WEKEN, W. VAN DEN BOSSCHE and P. DE MOERLOOSE

Department of Pharmaceutical Chemistry and Drug Quality Control, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

(First received December 23rd, 1987; revised manuscript received February 26th, 1988)

SUMMARY

A method is described for the determination of S-(carboxymethyl)-L-cysteine in serum. After addition of S-(carboxyethyl)-L-cysteine as internal standard, both compounds are extracted into methanol, converted into fluorescent derivatives with *o*-phthalaldehyde and quantitatively determined by reversed-phase high-performance liquid chromatography. Chromatography of unwanted amino acid derivatives is avoided by column switching, thereby shortening analysis time and increasing column lifetime. The technique was applied in a study of the bioavailability of S-(carboxymethyl)-L-cysteine after oral administration to humans. The concentration–response curve was linear from 2 to 16 μg/ml; mean serum concentrations are reported.

INTRODUCTION

S-(Carboxymethyl)-L-cysteine (SCMC) is a mucolytic drug widely used in the treatment of chronic bronchitis, either as such (Siroxyl®, Rhinathiol®, Pulmoclase®) or in combination preparations with tetracycline or promethazine. SCMC has properties similar to those of amino acids, and its determination in human serum always has to deal with possible interference from amino acids.

A gas chromatographic determination for SCMC in human plasma using a sulphur photometric detector has been described [1]. Apart from papers [2] that mention the (seldom baseline) separation of SCMC from other amino acids in a rather qualitative way, one paper described a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the determination of SCMC in human plasma [3]. An HPLC method for the simultaneous determination of

0378-4347/88/$03.50

theophylline and SCMC in tablets and suppositories [4] and another for the determination of SCMC in syrup formulations [5] have been reported.

Our goal was the development of a fast, accurate and reliable HPLC method that would permit a study of SCMC pharmacokinetics with two different pharmaceutical dosage forms in adult healthy subjects. The ion-pair method for SCMC in serum that we developed was based on that described by Gaetani et al. [3], with several modifications. A suitable internal standard, S-(carboxyethyl)-L-cysteine (SCEC) was added, isocratic rather than gradient elution was performed, and a column-switching technique was introduced to prevent the elution of *o*-phthalaldehyde (OPA) derivatives of endogenous amino acids on the analytical column, thereby shortening the analysis time by a factor of 3. Ethanethiol (ET), which was used as a reducing agent to form a highly fluorescent product of the adduct formed in the reaction between OPA and SCMC, was replaced by 2-mercaptoethanol (ME) since this thiol yielded derivatives of SCMC and SCEC that were more readily separated from the bulk of endogenous amino acid derivatives and thus facilitated the column switching.

The resulting analytical methodology permits a fast and simple determination of SCMC in serum, sufficiently sensitive to be applicable to pharmacokinetic studies.

Very recently, an ion-pair RP-HPLC method with column switching, similar to the one described here, has been reported [6]. The methodology and results obtained will be discussed and compared with those reported in this paper (see Results and discussion).

EXPERIMENTAL

Reagents and materials

SCMC (purum) reference compound and SCEC (purum), internal standard, were obtained from Fluka (Buchs, Switzerland). OPA (p.a.) was obtained from Serva (Heidelberg, F.R.G.), and tetramethylammonium bromide (98% p.a.) from Janssen Chimica (Beerse, Belgium). Water was purified by ion-exchange chromatography and subsequent distillation. ME, ET and methanol, all of analytical reagent grade, were obtained from U.C.B. (Leuven, Belgium).

All other chemicals and solvents used were of analytical-reagent grade except for acetonitrile, which was of HPLC grade (Carlo Erba, Milan, Italy).

Equipment

Chromatography was performed with two Waters M 6000A pumps and a Waters Model 660 solvent programmer, fitted with a Waters Model 420 E fluorescence spectrophotometer, a Spectra Physics Minigrator integrator (Darmstadt, F.R.G.) and a Model A40 single-channel recorder (Kipp & Zonen, Delft, The Netherlands).

For column-switching, the above equipment was used in conjunction with a column-switching valve system consisting of two Valco six-port injection valves connected to the analytical column and a 25-μl sample loop (Fig. 1). First, 1 min after the injection of the sample [VAL 1 in inject position (step A), dark lines in

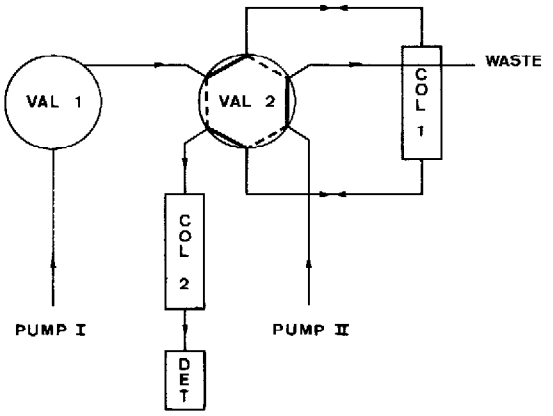

Fig. 1 Schematic diagram of the column-switching valve system: COL 1 = pre-column; COL 2 = analytical column; DET = detector; VAL 1 = injection valve; VAL 2 = switching valve.

Fig. 1], VAL 2 was switched from inject to load position (step B, dotted lines in Fig. 1). Then, 8 min after switching VAL 2, this valve was switched back to the inject position and, after VAL 1 had been switched to the load position, the next injection was made without delay (step C). In step A the columns were connected in series and eluted with pump I at a flow-rate of 2.0 ml/min , while pump II was pumping directly to the waste at a flow-rate of 2.5 ml/min. In step B, the pre-column was disconnected from pump I and connected to pump II in such a way that the pre-column was back-flushed by pump II. At the same time, the analysis was being performed on the analytical column still connected to pump I. In step C, both columns were again connected in series to pump I and the initial conditions were restored.

All analyses were performed manually. However, using appropriate devices, i.e. an autosampler and pneumatic valves controlled by time relays, automation of the method should be possible.

The pre-column (50 mm × 4.6 mm I.D.) was laboratory-made and packed with 10-μm irregular C_{18} material (LiChrosorb) using a conventional slurry-packing technique. The analytical column was a prepacked LiChrosorb 10-μm RP 18 (250 mm × 4.6 mm I.D.) column (Alltech-Europe, Eke, Belgium). The analytical column was maintained at 25 °C using a water-bath.

Chromatography

The mobile phase consisted of acetonitrile–0.01 *M* sodium dihydrogenphosphate buffer adjusted to pH 7.0, with 0.1 *M* sodium hydroxide (105:895, v/v) containing 5 m*M* tetramethylammonium bromide. The flow-rate was 2.0 ml/min for pump I and 2.5 ml/min for pump II (back-flushing the pre-column). The column effluent was monitored with a fluorescence detector fitted with a 360-nm excitation filter and a 460-nm emission filter (0.64 a.u.f.s.).

Preparation of standards

Stock solutions containing ca. 40 mg of SCMC reference compound per 100 ml and ca. 10 mg of SCEC (internal standard) per 100 ml were prepared in water,

previously made alkaline by the addition of 0.15 ml of dilute ammonia (10%, v/v) per 100 ml of water. The SCMC stock solution was further diluted with water to give solutions ranging from 2 to 16 μg per 100 ml (working standards). Serum standards were freshly prepared each day by spiking blank serum with working standards.

Extraction and derivatization procedure

To 1-ml aliquots of blank serum, 100 μl of the working standards and 100 μl of the internal standard stock solution were added. This mixture was shaken for 30 s on a Vortex mixer, followed by the addition of 4.0 ml of methanol and by further shaking for 1 min on the Vortex mixer. To 50-μl aliquots of the supernatant, 500 μl of a mixture consisting of 50 mg of OPA, 1.0 ml of methanol, 9 ml of 0.40 *M* borate buffer (pH 9.5), 50 μl of ME and 10 μl of Bry 35 (10% aqueous solution) were added [7,8]. After a reaction time of 1 min at room temperature, aliquots of the mixture were analysed by HPLC.

Serum samples were extracted and derivatized as described for the blank serum, except that the 100 μl of the working standards were replaced by 100 μl of water.

Recovery

The percentage recovery of SCMC was determined by comparison of peak areas of SCMC obtained after injection of extracted and derivatized spiked samples with those obtained after injection of comparable unextracted standards.

Quantitation

Standard curves were generated over the concentration range 2–16 μg/ml SCMC in serum on a daily basis. Quantitation was achieved using peak-area ratios of SCMC to the internal standard (SCEC). Linear regression analysis was used to compute the standard curves and the SCMC serum concentrations in the unknown samples.

RESULTS AND DISCUSSION

Optimization of the analytical procedure

When the OPA–ET derivatization mixture and an appropriate gradient elution similar to that described in ref. 3 were used, SCMC and SCEC could be eluted as pure compounds (Fig. 2). However, only a limited number of such injections could be made before the column performance deteriorated, owing obviously to an accumulation of co-extracted serum components. Therefore a column-switching procedure, involving selective introduction of effluents from the pre-column containing the drugs of interest onto the analytical column, was developed. Since the separation of SCMC and SCEC and the bulk of unwanted endogenous amino acid derivatives was critical to allow convenient column switching, better separation conditions were selected by varying the eluent parameters. The retention of both drugs is increased by decreasing the buffer concentration and by lowering the eluent pH. Increasing the acetonitrile content and decreasing the tetramethylammonium bromide concentration reduces the retention of SCMC and SCEC.

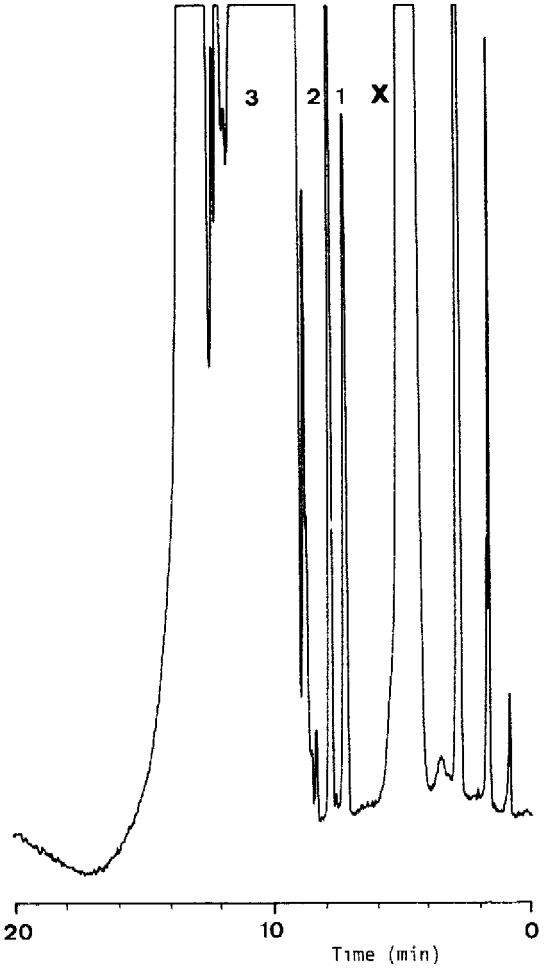

Fig. 2. Representative chromatogram following extraction and derivatization of a serum sample spiked with SCMC and SCEC. Column, LiChrosorb RP 18 (10 μm particle size, 250 mm × 4.6 mm I.D.) at 25 °C; mobile phase A, a mixture of 0.01 *M* sodium dihydrogenphosphate buffer (pH 7.0) with 5% (v/v) acetonitrile, containing 5 m*M* tetramethylammonium bromide; mobile phase B, acetonitrile; gradient program 8 (concave profile) on the Waters Model 660 solvent programmer, 0 to 80% B in 10 min. Peaks: 1 = SCMC; 2 = SCEC (internal standard); 3 = bulk of endogenous amino acid derivatives; X = unidentified product formed in the derivatization process using ET as reducing agent.

The effect on chromatographic retention resulting from the addition of tetramethylammonium bromide to the eluent is most probably two-fold. A reduction of the interaction of the amino acid derivatives with residual silanol groups will result in a decrease of retention as the concentration of the amine additive increases. However, ion-pair formation with the dissociated carboxylic acid groups will have the opposite effect, i.e. a retention increase. Since in our chromatographic system, SCMC is only slightly retained (and other dicarboxylic acids not at all), the contribution to retention of the polar interactions is small and the retention increase observed with increasing concentration of the amine must be caused by ion-pair interaction.

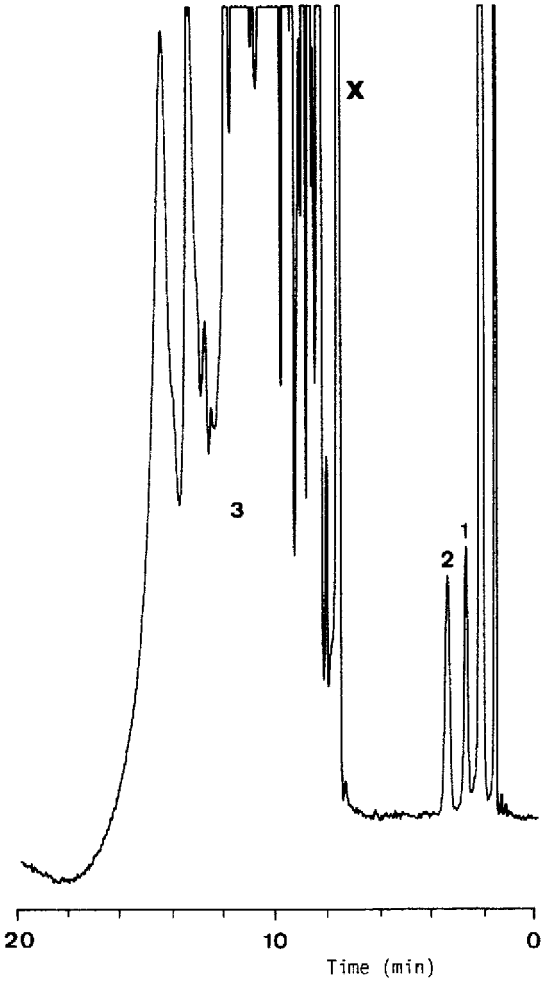

Fig. 3. Representative chromatogram following extraction and derivatization of a serum sample spiked with SCMC and SCEC. Column and peaks as in Fig. 2. Mobile phase as in Fig. 2 except that solvent A contained 5% B. Gradient elution as in Fig. 2: 5 to 80% B in 10 min. The derivatization mixture is described in Experimental.

A gradient elution with the conditions of Fig. 2 except for solvent mixture A, which now contained 5% B (in addition to the initial 5% acetonitrile) did indeed allow more convenient column switching by decreasing the retention of the drugs, whereas the elution of unwanted material was virtually unaffected. However, interference with the elution of both drugs by an unidentified peak (X in Fig. 2) now occurred. Replacing ET by ME in the derivatization mixture resulted in derivatives with markedly different chromatographic properties, and the unknown peak X no longer caused interference. The use of ME instead of ET only affects the chromatographic retention of the OPA derivatives, since the stability and the relative fluorescence of the derivatives remain virtually constant [8]. A separation of SCMC and SCEC in blank serum spiked with both drugs and analysed with the (chromatographic) conditions of Fig. 2, except for solvent mixture

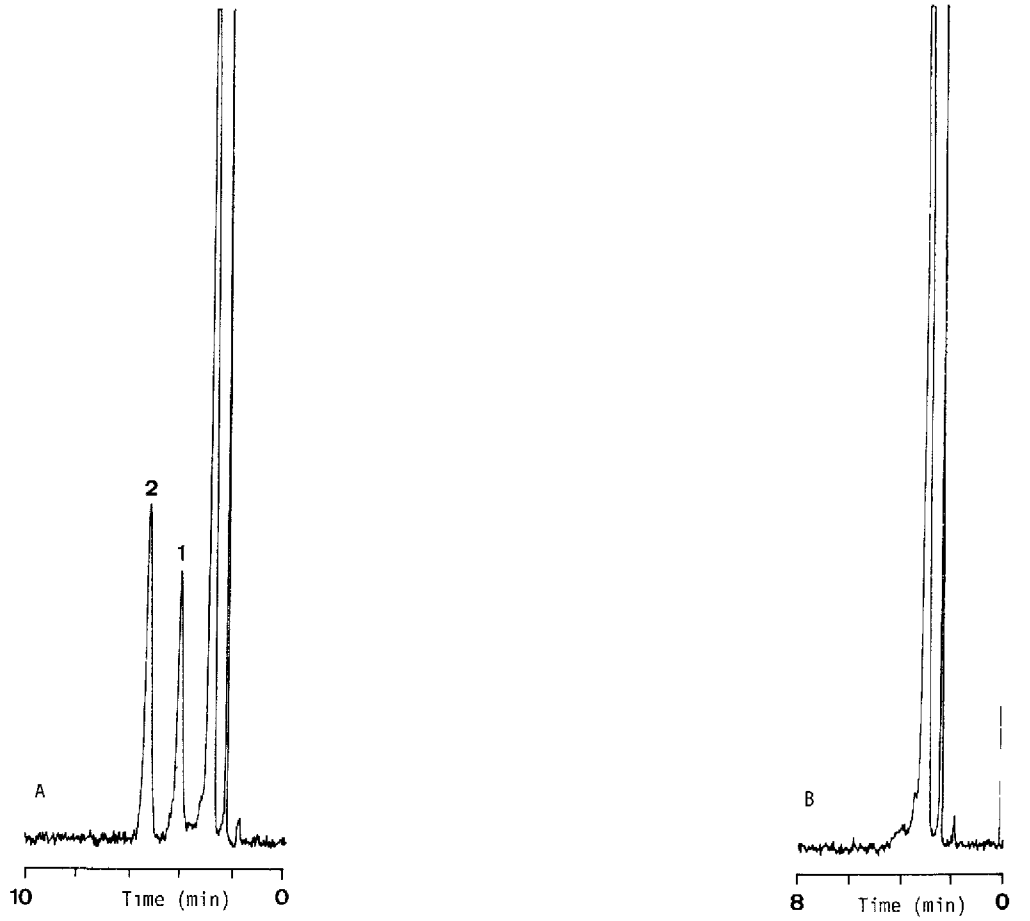

Fig. 4. Representative chromatograms of (A) a derivatizated serum extract from a patient, 3 h after receiving 1.5 g of SCMC test preparation, obtained with column switching and isocratic elution, and (B) a blank serum sample taken from the same patient. Column and peaks as in Fig. 2. Mobile phase, a mixture of 0.01 *M* sodium dihydrogenphosphate buffer (pH 7.0) and acetonitrile (895:105, v/v) containing 5 m*M* tetramethylammonium bromide. Chromatographic conditions: column temperature, 25 °C; flow-rate for pump I, 2.0 ml/min; flow-rate for pump II, 2.5 ml/min (see Experimental). Fluorescence detection: excitation, 360 nm; emission, 450 nm (0.64 a.u.f.s.).

A and for ET which was replaced by ME, is shown in Fig. 3. An excellent separation of SCMC and SCEC (internal standard) from unwanted amino acid derivatives was then obtained.

From these gradient elution conditions (Fig. 3), a mobile phase was selected that permits the isocratic separation of both drugs from the less retained peaks and, by column switching, from the unwanted strongly retained material, in less than ca. 10 min (pump I). At the same time, this unwanted material that is retained on the pre-column is eluted (back-flushed) to the waste by a mobile phase with the same solvent composition (pump II). Thus no re-equilibration time is needed, and by the time the sample has been analysed on the analytical column,

the pre-column (and thus the whole system) is flushed and ready for the next injection. The chromatographic conditions presented in Fig. 4A were considered to meet the above criteria. The chromatogram depicted in this figure was obtained after the injection of 25 μl of a derivatized and extracted serum sample taken from a patient 3 h after oral administration of 1.5 g of SCMC.

Using this column-switching procedure, several hundred samples were analysed over a period of two months without loss in resolution or without a significant decrease in efficiency of the analytical column. Only a flushing of the column with ca. 100 ml of pure acetonitrile was required after analysis of ca. 50 samples to avoid any baseline drift.

Resolution, specificity and sensitivity

With the chromatographic conditions indicated in Fig. 4A the retention times are ca. 4 min for SCMC and ca. 5.5 min for SCEC, with a resolution of at least 1.5. A blank serum sample, taken from the patient before the start of the bioavailability study, was run with each series of patient serum samples; the blank serum was free of interfering peaks in all cases (Fig. 4B).

The limit of detection was ca. 0.5 μg/ml of serum (signal-to-noise ratio 3). This sensitivity can be increased 100-fold using the Perkin Elmer 650-10 S fluorescence spectrophotometer fitted with a xenon power supply (excitation wavelength 340 nm, emission wavelength 440 nm). This detector, however, was not available during the study.

Stability of SCMC

The influence of storage of room temperature on the stability of SCMC in serum was not investigated. All (patient) samples were extracted and derivatized within ca. 2 h after storage at room temperature. The stability of the derivatives of SCMC and SCEC was investigated for 3 h after the derivatization with OPA. No change in the peak-area ratio of SCMC and SCEC was observed during that period.

Method validation

Calibration curves for SCMC in serum were linear over the concentration range 2–16 μg/ml. The regression equation of the curve calculated on a daily basis from data for standards in serum was $y=0.0742x+0.0312$ ($r=0.9996$). The slope of the calibration curves constructed over two months showed a relative standard deviation (R.S.D.) of 6.06%. The precision of the method was determined from repeated analyses of aliquots of pooled control (drug-free) serum spiked with known concentration of SCMC (10 μg/ml). The within-day precision based on peak-area ratios was 10.1 μg/ml $\pm$ 2.7% (mean $\pm$ R.S.D., $n=20$). The day-to-day precision calculated on three different days and expressed as the R.S.D. of the results obtained, varied from 2.9 to 4.3%. Extraction recoveries from serum analysis averaged 103.8% $\pm$ 5.5% R.S.D. ($n=20$) for SCMC at a serum level of 10 μg/ml and 101.8% $\pm$ 4.7% R.S.D. ($n=20$) for SCEC at a serum level of 15 μg/ml.

Application of the method

The RP-HPLC column-switching method was used in a study of SCMC pharmacokinetics with two different pharmaceutical dosage forms. Serum concentrations of patients receiving a single oral dose of 1.5 g of SCMC test preparation (powder) were determined. After one week, the same group of patients received a single oral dose of 1.5 g of the SCMC reference preparation, i.e. four capsules labelled at 375 mg per unit, and serum concentrations were determined again. Blood samples were collected 20 min, 40 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h and 10 h after medication, allowed to coagulate, then decanted and centrifuged. The serum was then separated and frozen at −20°C. Fig. 5 illustrates the results of the analysis, plotted as concentration–time curves. Fig. 5A shows mean serum concentrations from eight healthy adults following oral administration of the test preparation. A maximum concentration of SCMC of 13.9 μg/ml at 2 h post dosing and a mean concentration of 0.87 μg/ml persisting at 10 h after administration were obtained. Fig. 5B shows comparable mean serum concentrations of SCMC from the same group of patients following oral administration of the reference preparation, with a maximum concentration of 10.8 μg/ml at 2 h post dosing and a mean serum concentration of 1.82 μg/ml, persisting at 10 h after administration. From Fig. 5 it can be seen that the inter-individual variations of the serum concentrations were wide: the R.S.D. on the mean peak concentration varied from 23.2 to 52.2% for the test preparation and the reference preparation, respectively. The question now arose whether the bioavailability was the same for both compounds. Application of the *t*-test to the mean peak concentration obtained after 2 h and of Wilcoxon's non-parametric signed rank test on the concentration obtained after 10 h with both pharmaceuticals showed that there was no statistical difference at the 95% confidence limit between the two series. Hence it was concluded that the dose–response curves depicted in Fig. 5, and thus the bioavailabilities of both dosage forms, were the same.

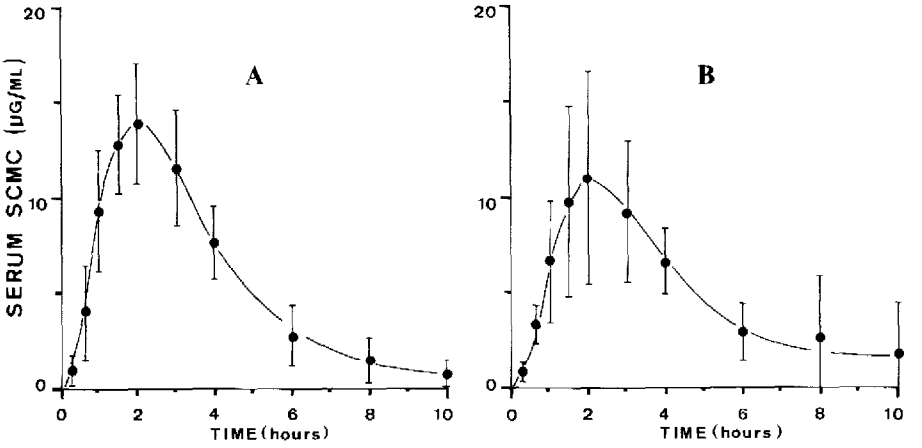

Fig. 5. Serum concentration–time curves for SCMC after oral administration of (A) 1.5 g of SCMC of the test and (B) 1.5 g of SCMC of the reference preparation. Each point represents the mean ± S.D. of eight subjects.

The HPLC method described by Dubruc et al. [6] allowed the determination of SCMC in plasma with about the same methodological error and a comparable detection limit as the present method. The use of three pumps and two eluents with different composition makes the method more complicated, but on the other hand allowed its full automation. It also appeared that the separation of SCMC from endogenous dicarboxylic amino acids (especially from glutamic acid) is more critical. With our procedure, these compounds are only slightly retained and hence did not interfere at all. Consequently the analysis time is much shorter, but since the method described in ref. 6 is automated, its longer analysis time is not really a disadvantage. We, therefore, conclude that the method developed by Dubruc et al. [6] is a valuable alternative to the one described here and by its automation more suitable for the handling of a large number of samples. Moreover, minor modifications to the eluent will result in chromatographically useful systems for the determination of other dicarboxylic amino acids. However, the method seems to be somewhat less robust than the one described here, mainly due to the more critical separation of SCMC from glutamic acid mentioned above.

The mean peak concentration of SCMC in serum and the time of obtain this concentration reported here are in good agreement with results reported earlier for the determination of SCMC in plasma, i.e. a mean peak concentration of ca. 13 μg/ml at 2 h post dosing [1] following oral administration of 1.5 g of SCMC.

CONCLUSION

Owing to the excellent separation of SCMC and SCEC from endogenous amino acids, and to the column-switching technique, the method presented is selective, reproducible, fast and robust, and is suitable for pharmacokinetic and bioavailability studies following a single therapeutic dose.

REFERENCES

1 W.R. Maynard, R.B. Bruce and G.G. Fox, J. Pharm. Sci., 67 (1978) 1753.
2 H.V.J. Kolbe, R.C. Lu and H. Wohlrab, J. Chromatogr., 327 (1985) 1.
3 E. Gaetani, C.F. Lameri, M. Vitto and F. Bondi, Il Farmaco, Ed. Prac., 37 (1982) 235.
4 G. Caccialanza and C. Gandini, Il Framaco, Ed. Prac., 36 (1981) 396.
5 C.K. Melucci, G.W. Lyman, A.D. Bond and R.N. Johnson, J. Chromatogr., 391 (1987) 321.
6 C. Dubruc, Ph. Hermann, A. Haddouche, M.M. Badarani and J.P. Thenot, J. Chromatogr., 417 (1987) 208.
7 L. Knesel, University of Veterinary Medicine, Brno, personal communication.
8 D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.