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Quantification of *S*-Carboxymethyl-(*R*)-Cysteine in Human Plasma by High-performance Ion-exchange Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry

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The determination of *S*-carboxymethyl-(*R*)-cysteine (SCMC) in human plasma during extended bioequivalence studies demands a rapid, accurate and selective assay technique. A liquid chromatographic/mass spectrometric method was developed which involves rough protein precipitation followed by high-performance liquid chromatographic separation with an ion-exchange column and atmospheric pressure ionization (API) mass spectrometric detection, with the instrument operating with electrospray ionization (ESI) and in the selected-ion monitoring mode. The drug and the internal standard *S*-[(*R*)-1-carboxyethyl]-(*R*)-cysteine (SCEC) are detected by focusing the first quadrupole of the triple stage system on MH^+ ions, thus permitting elimination of endogenous interfering substances and allowing a detection limit of 0.05 μg ml^{-1}. The chromatographic run time is 16 min and the method has sufficient sensitivity, precision, accuracy and selectivity for routine analyses of clinical plasma samples containing SCMC at concentrations in the range 0.2–20 μg ml^{-1}. In summary, this LC/MS-based assay of SCMC demonstrates advantages of easy sample preparation, low limit of quantification (200 ng per ml of human plasma) without any derivatization step, high specificity and rapid sample analysis with an overall throughput of more than 60 analyses per day. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

S-Carboxymethyl-(*R*)-cysteine (SCMC, carbocysteine; Fig. 1) lysine salt monohydrate (Fluifort) is a mucolytic agent widely used for the treatment of hypersecretory respiratory diseases.[1–3] A crucial aspect of the correct dosage of this, as for any pharmaceutical preparation, is the quantification of the compound in human plasma, to determine the pharmacokinetic parameters. In the case of SCMC, this task is affected by the lack of absorption in the visible and UV ranges and all reported assays for SCMC rely on the detection of UV-active

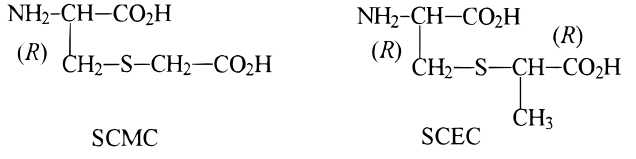

Figure 1. Structures of *S*-carboxymethyl-(*R*)-cysteine (SCMC) and *S*-[(*R*)-1-carboxyethyl]-(*R*)-cysteine (SCEC) (internal standard).

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or volatile derivatives. The number of methods reported include ion-exchange chromatography followed by derivatization prior to UV detection[4–6] and reversed-phase high-performance liquid chromatography (HPLC) with phenyl isothiocianate (PITC)[7,8] or *o*-phthalaldehyde (OPA)[9–11] pre-column derivatization. Two gas chromatographic methods have also been developed for the quantification of SCMC either in human urine, by derivatization with heptafluorobutyric anhydride,[12] or in human plasma, after *N*-acylation with acetic anhydride and esterification with diazomethane.[13] Recently, capillary zone electrophoresis (CZE) has been used to determine SCMC and some of its metabolites in human urine, without prior derivatization.[14] This method uses on-column UV (206 nm) detection, but it has insufficient sensitivity (30 μg ml^{-1}) to be usable for pharmacokinetic monitoring.

Since the accurate determination of SCMC in human plasma was fundamental for our studies on the bioavailability of the drug administered as different pharmaceutical forms, we focused our attention on the development of a rapid and easy method, also reliable in terms of sensitivity and reproducibility. The prerequisites of the method were minimum sample preparation and high detection response, without derivatization steps. HPLC analysis, after precipitation of proteins

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from the plasma matrix, coupled with on-line atmospheric pressure ionization (API) mass spectrometry (MS) in the electrospray (ES) version,[15,16] operating in the positive selected ion monitoring (SIM) mode, fulfilled the required needs. Thus SCMC and *S*-[(*R*)-1-carboxyethyl]-(*R*)-cysteine (SCEC; Fig. 1) as the internal standard are detected, with fairly good sensitivity, by focusing the instrument on the MH^+ ions.

EXPERIMENTAL

Chemicals

S-Carboxymethyl-(*R*)-cysteine (SCMC) lysine salt and drug-free human plasma were provided by Dompé (L'Aquila, Italy). HPLC-grade acetonitrile and methanol and reagent-grade glacial acetic acid were obtained from Carlo Erba (Milan, Italy); water was of MilliQ grade. Other reagents (from Fluka, Buchs, Switzerland or Aldrich, Steinheim, Germany) were of either HPLC or analytical grade and were used as received. *S*-[(*R*)-1-Carboxyethyl]-(*R*)-cysteine (SCEC) was synthesized as described below.

Synthesis of the internal standard *S*-[(*R*)-1-carboxyethyl]-(*R*)-cysteine (SCEC)

A 0.4 g (3.8 mmol) amount of (*R*)-cysteine was dissolved in 7.2 ml of water and the pH was raised to 7.6 with a few drops of 1 M NaOH. A solution containing 0.62 g (4.0 mmol) of (*S*)-(−)-2-bromopropionic acid in 0.4 ml of water (the pH was adjusted to 7.6 with 1 M NaOH) was added dropwise. The solution was then stirred at room temperature for 12 h while maintaining the pH constant at 7.6 by adding, when necessary, a sufficient amount of 1 M ammonia solution. After evaporation of water under vacuum, the dry residue, still containing unreacted starting material, was purified by ion-exchange liquid chromatography using 50 g of Amberlite IRA-910 strongly basic ion-exchange resin (Fluka) as the stationary phase and methanol–water (1:1) containing a small amount of acetic acid (2%, v/v) as the mobile phase. A 0.13 g amount of SCEC was thus isolated as a highly hygroscope white powder in a 10:1 diastereoisomeric excess: *S*-[(*S*)-1-carboxyethyl]-(*R*)-cysteine in fact also formed as a minor (HPLC analysis) isomer. SCEC was not purified further. ^{1}H NMR of the mixture (300 MHz, Bruker ARX 300, D_2O): major isomer, $\delta = 1.53$ (d, $J = 7.2$ Hz, 3H), 3.4–3.3 (m, 2H), 3.74 (q, $J = 7.2$ Hz, 1H), 4.14 (m, 1H); minor isomer, $\delta = 1.51$ (d, $J = 7.1$ Hz, 3H), 3.2–3.0 (m, 2H), 3.79 (q, $J = 7.1$ Hz, 1H), 4.14 (m, 1H).

HPLC/MS equipment, instrumental and analytical conditions

A Waters 600 MS pump and Model 717 automatic sample injector were used for all HPLC analyses. The chromatographic system consisted of a Supelcosil LC-SAX column (250 × 4.6 mm i.d., d_p 5 μm) (Supelco, Bellefonte, PA, USA). For optimum performance, the column needed first to be conditioned once by passing 0.025 M KH_2PO_4 (pH adjusted to 4.0 by adding a few drops of concentrated H_3PO_4)–acetonitrile (90:10, v/v) through it for 12 h at a flow rate of 1.8 ml min^{-1}. Following this treatment the column was used for 1000–1500 of separations without further conditioning.

The eluent was methanol–water (50:50, v/v, containing 2% acetic acid; filtered through a 0.45 μm HV filter; Millipore) delivered at a flow rate of 0.4 ml min^{-1} (back-pressure 950 psi). The eluate from the HPLC column was directly passed into the ionization region of the mass spectrometer without flow splitting. The HPLC autoinjector was set at a 16 min run time at a sample volume of 5 μl. Mass spectrometric detection was carried out with a TSQ 700 triple-stage quadrupole instrument (Finnigan MAT, San Jose, CA, USA) operating in the API mode (electrospray version), with the stainless-steel capillary needle held at +4.5 kV (ionspray, spray current 3.5 μA). The temperature and voltage of the heated capillary were set at 240 °C and 21 V, respectively. The nebulizing sheath gas pressure and auxiliary gas flow (nitrogen) were set at 40 psi and 20 units of the ball manometer, respectively. The tube lens, octapole offset, electron multiplier and conversion dynode settings were 70, −2.7, −1000 and −15 kV. Quantitative data were obtained using SIM. The ions selected to run were *m/z* 180 (protonated molecular ion MH^+ for SCMC) and *m/z* 194 (protonated molecular ion MH^+ for SCEC) with a total scan time of 0.8 s and peak widths of 0.6 u. The output signal from the mass spectrometer was elaborated with a Digital DEC station 5000/125 operating ULTRIX 4.0 and ICIS 7.0 for data collection, peak integration and analysis. Peak areas were utilized for the construction of the calibration graphs, using non-weighted least-squares linear regression of the values, and for quantification of the plasma drug concentrations.

Standard solutions

A stock standard solution of SCMC (500 μg ml^{-1}) was prepared by dissolving 96.23 mg of SCMC lysine salt in 100 ml of water. This solution was further diluted with water to give a series of working standard solutions with concentrations of 250, 125, 50, 12.5 and 5 μg ml^{-1}. A stock standard solution of the internal standard (SCEC) was prepared in water (1.23 mg ml^{-1}), and diluted to 245 μg ml^{-1} to give a working standard solution. Stock and working standard solutions were prepared monthly and stored at 4 °C.

Sample preparation and calibration

The clinical studies were performed at the Guildford Clinical Pharmacology Unit (GCPU), Investigation Unit of the Royal Surrey County Hospital (UK). Blood samples were routinely worked up to obtain plasma. Plasma samples were then frozen at −80 °C and sent to Dompé Research Center in L'Aquila for quantitative determinations.

In an Eppendorff conical tube, 1 ml of acetonitrile was added to 250 μl of unknown plasma sample, pre-

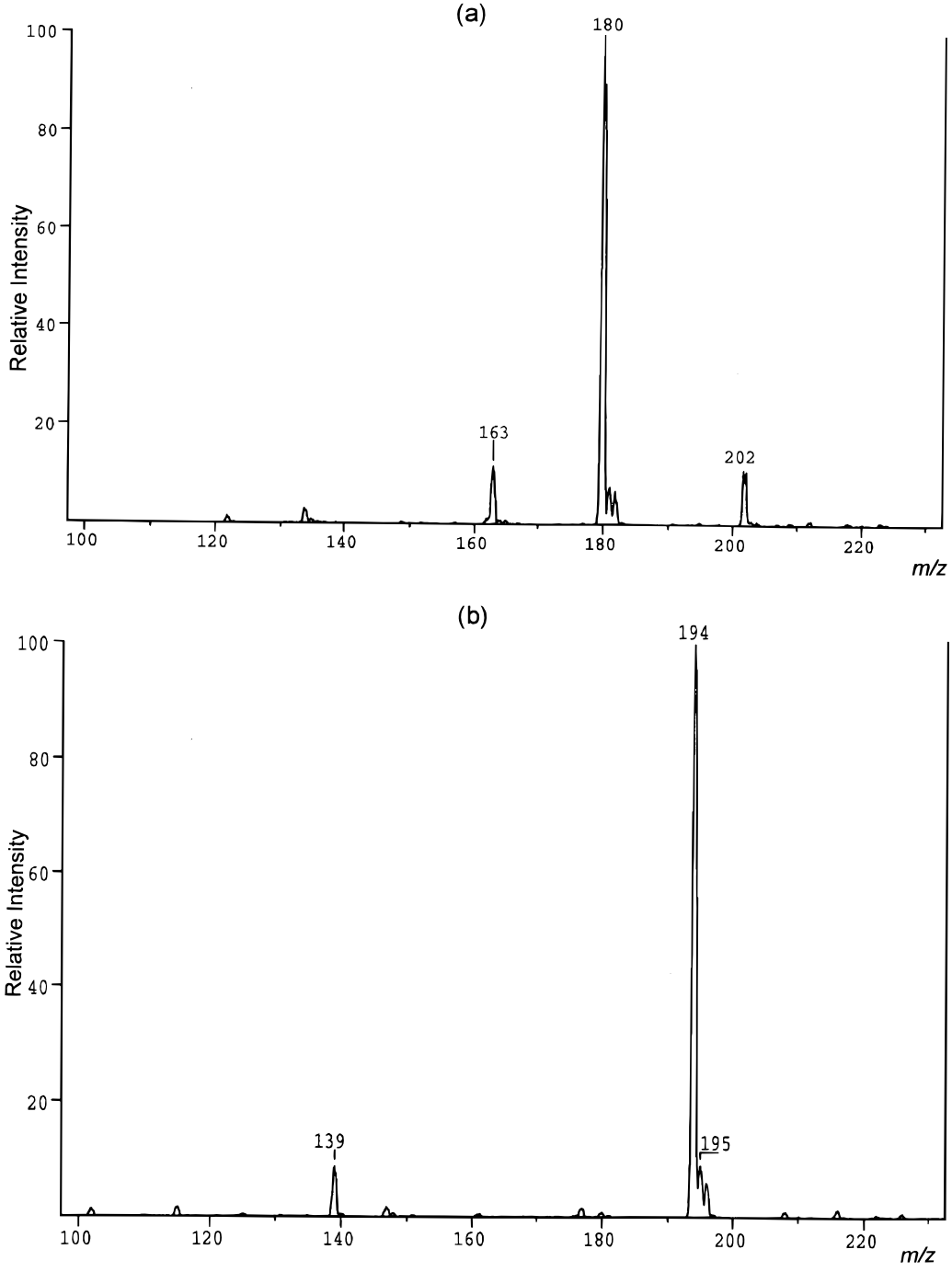

Figure 2. Positive-ion API-ES mass spectra of (a) SCMC and (b) SCEC.

viously spiked with 2.5 μg of SCEC (10 μl of the working solution) as the internal standard. After vigorously mixing on a vortex mixer (5 s), the tube was centrifuged at 10 000 rpm for 10 min using an Eppendorff centrifuge. A 600 μl volume of the surnatant solution was dried under vacuum in a SpeedVac vacuum evaporator and the dried residue was dissolved in the chromatographic mobile phase (200 μl), 5 μl of which were automatically injected into the LC-MS system.

The calibration graph for SCMC in plasma was constructed by spiking drug-free human plasma samples (250 μl) with the appropriate amounts of the drug (final concentrations ranging from 0.2 to 20 μg ml^{-1}: seven different samples, including blank plasma, were used) and with the internal standard (final SCEC concentration: 10 μg ml^{-1}).

Precision, accuracy, linearity, recovery and specificity

Peak area ratios of SCMC to the internal standard, measured at each nominal concentration, and the corresponding nominal concentration were used to obtain the equation of the plasma calibration graph:

$$y = a + bx$$

where x = nominal plasma concentration of SCMC (μg ml^{-1}), y = peak area ratio of SCMC to the internal

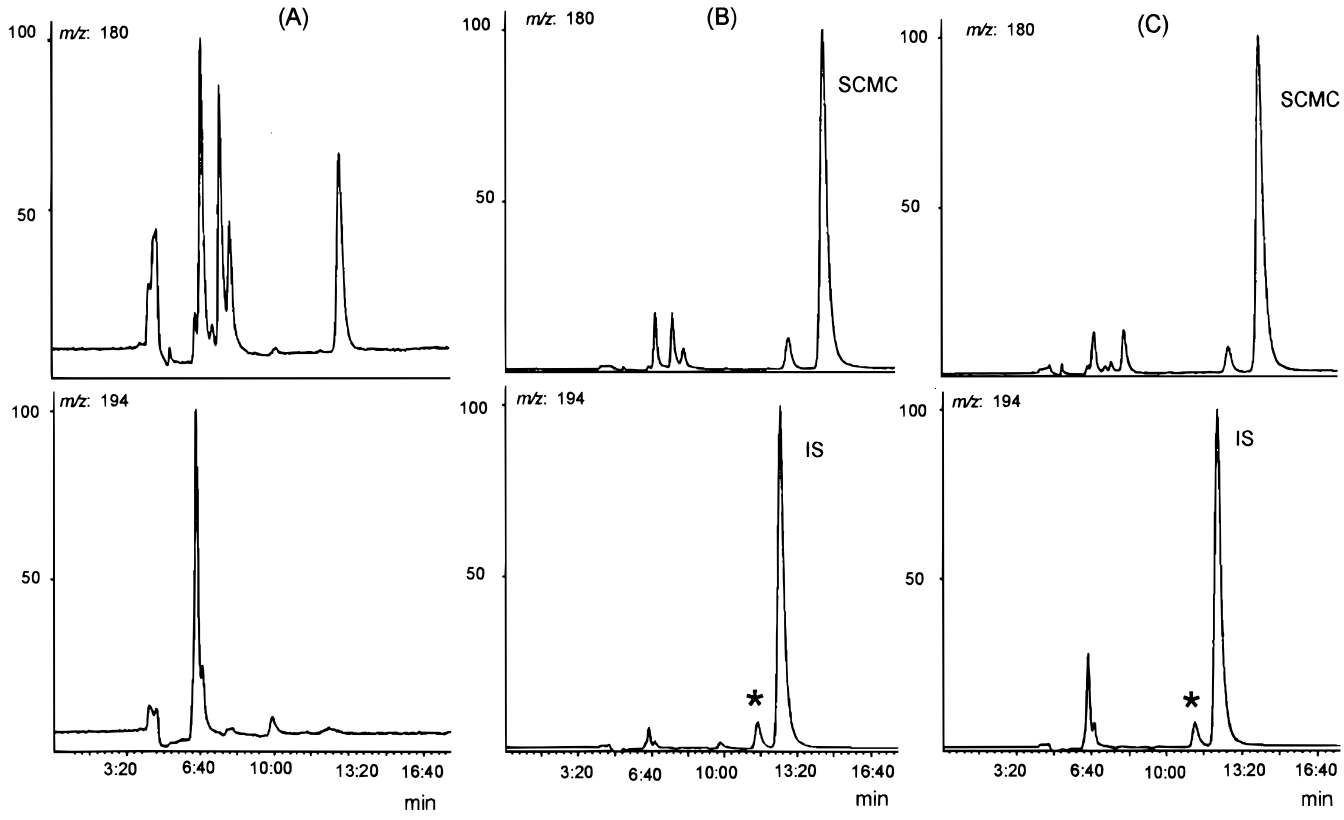

Figure 3. SIM chromatograms [upper trace, *m/z* 180 (MH^+ of SCMC); lower trace, *m/z* 194 (MH^+ of the internal standard SCEC)] of samples of plasma (A) before administration of SCMC, (B) drug spiked (10 μg ml^{-1}) and (C) from a treated volunteer, 1 h after single-dose oral administration of SCMC lysine salt. SCEC as internal standard was added to plasma in (B) and (C) (10 μg ml^{-1}). The starred peak refers to the minor diastereoisomer of SCEC.

standard, a = intercept and b = slope of the calibration graph.

A non-weighted least-squares linear regression procedure was used to obtain the equation of the calibration graph, along with the value of the regression coefficient (r^2). The linearity of the method was assayed by plotting peak area ratios of SCMC to the internal standard, measured at each nominal concentration (three replicate analyses per concentration), *vs.* the corresponding nominal concentration (μg ml^{-1}). A calibration graph was also prepared and checked daily with quality control (QC) samples during the analysis of unknown samples.

The intra-run precision and accuracy of the method were determined by five replicate analyses of human plasma containing SCMC at concentrations of 0.5 and 10 μg per ml of biological matrix. The inter-run precision and accuracy were evaluated over a period of 2 months by analyzing daily quality control plasma samples at SCMC concentrations of 0.5 and 10 μg ml^{-1} (three replicate analyses per concentration).

The extraction recovery was measured by comparing peak areas corresponding to SCMC and SCEC, both extracted from biological samples, to the corresponding working solutions directly diluted with the HPLC eluent at the same nominal concentrations (0.5 and 10 μg ml^{-1} for SCMC and 10 μg ml^{-1} for the internal standard). The specificity of the assay was evaluated by analyzing drug-free plasma samples obtained from untreated subjects.

RESULTS AND DISCUSSION

Optimization of the analytical procedure

The chromatographic and mass spectrometric conditions needed to be accurately selected. The best chromatographic conditions for the separation and quantification of SCMC in human plasma were realized by using a strong anion-exchange column; unfortunately, under these conditions, a broad negative peak due to endogenous chloride ion was present, centered at 110 min. This caused a drastic reduction in the ionization efficiency of the cysteine derivatives, in the event of co-elution of Cl^- and the analyte during multiple chromatographic runs. This problem forced us to choose an internal standard with a retention time as close as possible to that of SCMC, in order to minimize the differences in the ionization conditions between the two compounds. Accordingly, either *S*-[(*R*)-2-carboxyethyl]-(*R*)-cysteine and α-aminoadipic acid, previously described as suitable internal standards for SCMC quantification,[9–11] were discarded since, with the chosen chromatographic conditions, their elution times were too much different from that of SCMC (about 7 min *vs.* 14.5 min needed for SCMC to elute). Also, a bis-deuterated analog of SCMC, as a synthetically easily accessible isotope-labeled internal standard, was not taken into consideration: the mass increment in fact

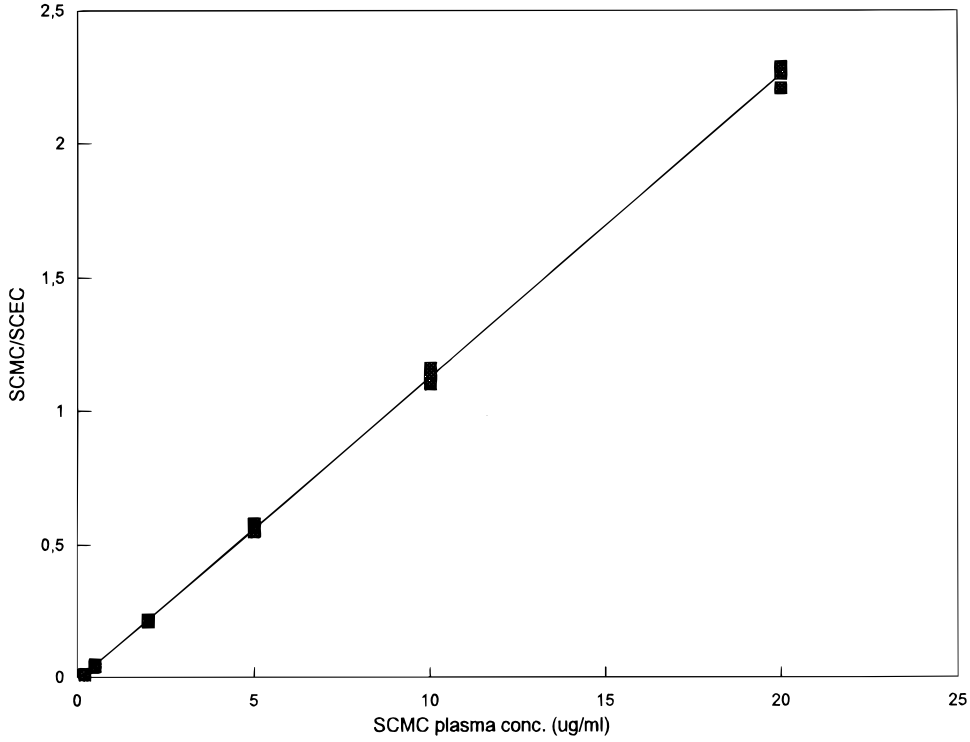

Figure 4. Calibration graph for SCMC in human plasma in the range 0.2–20 μg ml^{-1} ($y = 0.113x - 0.009$). Regression coefficient (r^2) = 0.999; confidence limit for the slope ($P = 95\%$) = 0.001; confidence limit for the intercept ($P = 95\%$) = 0.015; degrees of freedom = 16.

is only 2 mass units with respect to the compound of interest, not being compatible with the quantification of a sulfur-containing analyte. SCEC was finally synthesized (retention time 12.7 min) and used throughout. The proximity of the retention times of SCMC and SCEC allowed an easy injection time setting of the autosampler device, thus permitting interference from the chloride ion peaks coming from previous injections to be avoided. It should also be pointed out that SCEC did not interfere with the endogenous substances present in all blank and treated human plasma samples (see below).

Concerning the mass spectrometric detection, it is worth noting that a second, negative effect of the long-lasting presence of the chloride ion in the eluate is also apparent when trying to work in the multiple reaction monitoring (MRM) mode, with a faster chromatographic run time. Unfortunately, under these conditions, the signal was not only greatly reduced but also too unstable to permit reproducibility, possibly because of the interference of the chloride ion itself with the spray current. Having lost the advantage of an improved rate of analysis, we turned to SIM methodology, which allowed sufficient sensitivity for the post-dose quantification of SCMC and was also easier to handle with respect to the MS/MS technique.

The chromatographic efficiency and selectivity of the method were good enough (number of theoretical plates = 9550 for the SCMC peak with a resolution of 2.8 between SCMC and the closest endogenous substance), although a relatively low flow rate (0.4 ml min^{-1}, giving maximum ionization efficiency with the API-MS detection) was applied, compared with the internal diameter of the analytical column. Operating in the positive-ion mode, both SCMC and the internal standard generate prominent protonated molecular ions, at *m/z* 180 and 194, respectively (Fig. 2). It is worth considering that, although SCMC and SCEC are acid species, putative candidates for negative-ion detection, the chromatographic need for acetic acid as a separation additive permitted a particularly efficient positive-ion detection.

Selected-ion detection on the protonated molecular ions of SCMC and SCEC allowed an LOQ of 200 ng ml^{-1}, which was sufficient for our pharmacokinetic study; a higher response, although easily achievable, was beyond our purposes since the SCMC concentrations in plasma samples ranged from 0.4 to 18.0 μg ml^{-1}. The LOQ was defined here as the lowest point of the calibration graph at which precision [expressed as relative standard deviation, RSD (%)] and accuracy [expressed as percentage recovery (see below)] were $\leqslant 10\%$ and $100 \pm 15\%$, respectively. The extraction recovery of the method, calculated for concentrations of SCMC and SCEC of 5 and 20 μg ml^{-1} respectively, was $>20\%$ in both cases (most of the compounds were lost during protein precipitation). The overall throughput was about 60 clinical samples per day, without detriment to the chromatographic performance, and it was only limited by the sample work-up procedure.

The SIM chromatograms of a typical blank plasma sample, of a spiked plasma sample and of a sample from a volunteer treated with a single dose of 2.7 g of SCMC lysine salt (1 h after administration p.o.) are shown in Fig. 3; the chromatograms relate to both the protonated molecular ions of the drug (*m/z* 180) and of the internal standard (*m/z* 194). As is apparent from the SIM profiles, both SCMC and the internal standard are

Table 1. Intra-run precision and accuracy of the HPLC/API-ESMS method for the determination of *S*-carboxymethyl-(*R*)-cysteine (SCMC) in human plasma

	Nominal concentration (μg ml^{-1})	
	0.5	10.0
Measured concentration (μg ml^{-1})	0.495	9.84
	0.515	9.79
	0.520	9.84
	0.485	9.75
	0.506	9.73
Mean	0.504	9.79
SD	0.0143	0.0505
Accuracy[a]	100.8	97.9
Precision[b]	3.20	0.588
Number of experiments	5	5

[a] Expressed as percentage recovery (mean calculated concentration/nominal concentration × 100).
[b] Expressed as confidence limit ($P = 95\%$).

well resolved with respect to endogenous substances with the exception of a substance, roughly co-eluting with the internal standard (chromatogram A, upper trace) but totally absent at its MH^+ value (chromatogram A, lower trace). The internal standard SIM signal is also accompanied by a faster peak, which is attributed to the *S*,*R*-diastereoisomer of SCEC (formed during its synthesis in a known percentage; see Experimental), which, because of its position on the chromatogram, does not interfere with the quantitative measurements.

Method validation

A typical calibration graph for SCMC in plasma is shown in Fig. 4 along with calculated linearity parameters: it was derived by plotting the drug *vs.* internal standard peak area ratio as a function of drug concentration. The intra-run linearity data were obtained by replicate analyses (three measurements per nominal concentration) of spiked plasma containing SCMC at concentrations of 0.2, 0.5, 2.0, 5.0, 10 and 20 μg ml^{-1}, to which the internal standard had been added to give a concentration of 10 μg ml^{-1}. The mean slope and regression coefficient (r^2) of the calibration graphs, constructed over a period of 2 months, were 0.122 and 0.999, respectively, with RSD of 7.55% and 0.151%, respectively.

The analytical data for intra-run precision and accuracy of the method, both determined by five replicate analyses of spiked human plasma samples containing SCMC at concentrations of 0.5 and 10 μg ml^{-1}, are reported in Table 1. Summarizing, the intra-run precision based on peak area ratios (SCMC *vs.* internal standard) was 3.1% (as a confidence limit $P = 95\%$) for 0.5 μg ml^{-1} samples and 0.59% (as a confidence limit $P = 95\%$) for 10 μg ml^{-1} samples; the intra-run accuracy, expressed as percentage recovery (mean measured concentration/nominal concentration × 100), was 100.8% at 0.5 μg ml^{-1} and 97.9% at 10 μg ml^{-1}.

The inter-run precision, expressed as the RSD (standard deviation/mean × 100), calculated on QC plasma standards (0.5 and 10 μg per ml of biological matrix) analyzed over a period of 2 months, was below 10% (see Table 2). The inter-run accuracy, determined as percentage recovery (mean measured concentration/nominal concentration × 100) was 102% at 0.5 μg ml^{-1} and 98.8% at 10 μg ml^{-1}.

Table 2. Inter-run precision and accuracy of the HPLC/API-ESMS method for the determination of *S*-carboxymethyl-(*R*)-cysteine (SCMC) in human plasma (QC samples)

Nominal concentration (μg ml^{-1})	Mean concentration (μg ml^{-1})	Recovery (%)[a]	RSD (%)[b]
0.5	0.510	102	9.8
10	9.88	98.8	5.0

[a] Mean value calculated for 54 analyses performed over a period of 2 months.
[b] $n = 54$.

CONCLUSION

An HPLC/API-ESMS assay for *S*-carboxymethyl-(*R*)-cysteine in human plasma, with a range from 0.2 μg ml^{-1} to 20 μg ml^{-1}, was developed. Owing to the excellent specificity of the SIM mass spectrometric detection, coupled with on-line separation by ion-exchange HPLC with an API interface, the method has the characteristics of sensitivity, selectivity, reproducibility and accuracy. Moreover, the easy and fast sample processing makes the method suitable for pharmacokinetic and bioavailability studies.

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REFERENCES

1. G. Edwards, *Chest* **70**, 506 (1976).
2. E. Puchelle, F. Aug and J. M. Polu, *Eur. J. Clin. Pharmacol.* **14**, 177 (1978).
3. L. Allegra, C. I. Cordaro and C. Grassi, *Respiration* **63**, 174 (1996).
4. W. H. Stein and S. Moore, *J. Biol. Chem.* **211**, 915 (1954).
5. H. Engelhart, J. Asshauer, U. Neue and N. Weigand, *Anal. Chem.* **46**, 336 (1974).
6. E. Bayer, E. Grom, B. Kaltengeger and R. Uhman, *Anal. Chem.* **48**, 1106 (1976).
7. E. Reid, T. McDonald and J. S. Burton, *Anal. Lett.* **14(BB)**, 615 (1981).

8. E. Gaetani, C. F. Laureri, M. Vitto and F. Bordi, *Farmaco. Ed. Prat.* **37**, 235 (1982).
9. C. Dubruc, Ph. Hermann, A. Haddouche, M. M. Badarani and J. P. Thénot, *J. Chromatogr.* **417**, 208 (1987).
10. P. Guinebault, C. Dubruc, A. Haddouche, C. Colafranceschi and G. Bianchetti, *Chromatographia* **26**, 377 (1988).
11. J. A. De Schutter, G. Van der Weken, W. Van den Bossche and P. De Moerloose, *J. Chromatogr.* **428**, 301 (1988).
12. E. Dolfini and R. Testa, *Farmaco. Ed. Prat.* **34**, 1338 (1979).
13. W. R. Maynard, R. B. Bruce and G. C. Fox, *J. Pharm. Sci.* **67**, 1753 (1978).
14. Y. Tanaka and W. Thormann, *Electrophoresis* **11**, 760 (1990).
15. T. R. Covey, E. D. Lee and J. D. Henion, *Anal. Chem.* **58**, 2453 (1986).
16. E. D. Lee, W. Muck and J. D. Henion, *Biomed. Environ. Mass. Spectrom.* **18**, 253 (1989).