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Ion-chromatographic determination of carbocisteine in pharmaceuticals based on non-suppressed conductimetric detection

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Abstract

A novel method for the determination of carbocisteine (S-CMC), a mucolytic and expectorant drug with an acidic amino acid structure, was developed and validated, using non-suppressed ion-chromatographic system with conductimetric detection, and anion or cation exchange columns. Among the various combinations of column type and eluent composition tested, a cation exchange column with a 0.25 mM trifluoroacetic acid (TFA) as eluent in isocratic mode at 1.2 ml/min gave the best results. S-CMC was very well separated from all common amino acids (resolution > 2.6). The retention time was 3.5 min and the asymmetry factor 1.1. A linear calibration curve from 17 to 400 μg/ml ($r = 0.99994$), with a detection limit of 0.14 μg (5.6 μg/ml–25 μl injection volume) and a precision of 1.5% R.S.D. (100 μg/ml, $n = 3$) was achieved. The proposed method was applied for the determination of S-CMC content in intensely colored commercial formulations (syrups). No interference from excipients was found and the only pretreatment step was the appropriate dilution with the mobile phase. Recovery from standard additions was ranged from 96.0 to 104.9% and precision (R.S.D., $n = 3$) 1.8–3.6%.

Keywords: Non-suppressed conductimetric detection; Detection; LC; Carbocisteine; *S*-Carboxymethyl-L-cysteine

1. Introduction

Carbocisteine (*S*-carboxymethyl-L-cysteine, S-CMC) (Fig. 1) has long been employed as a mucolytic and expectorant drug, administered in the form of syrup and oral granules under several commercial names (it must be differentiated from *N*-acetylcysteine [HOOC-CH(NHCOCH$_3$)CH$_2$SH] with the same therapeutic use) [1]. The official analytical methods for the determination of S-CMC in crude material are based on potentiometric titrimetry in anhydrous formic acid–acetic acid with perchloric acid titrant [2]. Available analytical methods are based on kinetic ion-selective potentiometry (reaction with 1-fluoro-2,4-dinitrobenzene accelerated by micellar catalysis) [3], spectrophotometry after formation of colored products (with Ni(II) [4] and *p*-benzoquinone [5]), HPLC with precolunm derivatization (with phenylthiohydantoin [6], phthalaldehyde [7–9] and 9-fluorenylmethyl chloroformate [10]), direct HPLC using ion-exchange or ion-pairing reverse phase partition mechanisms [11–14], ion-exchange chromatography with post column ninhydrin derivatization [15] or atmospheric pressure ionization MS [16], isotachophoresis [17] and capillary electrophoresis [18]. Since S-CMC exhibits low UV-Vis absorptivity, the available direct chromatographic methods are not suitable due to poor detectability. Tedious precolumn derivatization is usually employed to decrease considerably the detection limits below 0.5 μg/ml. With MS detector traces of carbocisteine can be determined in biological fluids with a detection limit of 0.05 μg/ml [16].

In this study, the conductivity of S-CMC, due to the two carboxylate and one (protonated) primary amino groups, was taken advantage to develop a novel non-suppressed ion-chromatographic method with conductimetric detection, without the necessity of any derivatization step. Since S-CMC is possible to have both positive and negative charged groups, it appears retention by anion-exchange as well as cation exchange columns. An optimization study concerning the composition of the mobile phase was carried out for both columns and the method was applied for content assay of commercial formulations.

Furthermore, the response of common amino acids to the conductimetric detector operating in non-suppressed mode and their resolution from S-CMC was examined. Therefore, this paper appears to be the first study in literature describing

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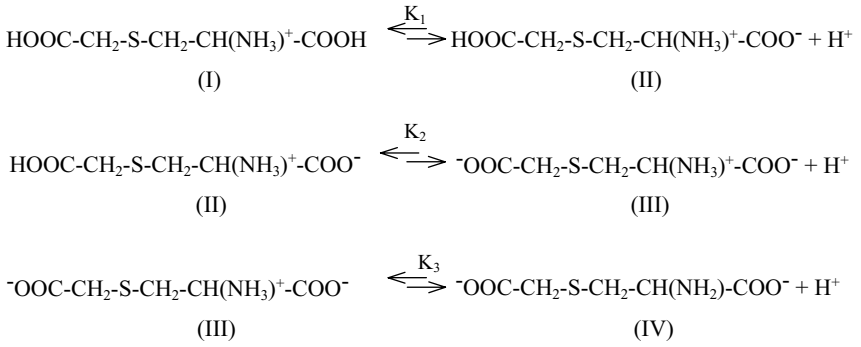

Fig. 1. Structure and ionization equilibria of carbocisteine (S-CMC). pK_a values are not available in the bibliography but they are expected to be analogous to the closely related acidic amino acids, aspartic and glutamic acids, taking into account the negative inductive effect (−1) of S atom, which results in a moderate acid-strengthening. pK_1: Asp 1.88, Glu 2.19, pK_2: Asp 3.65, Glu 4.25, pK_3: Asp 9.6, Glu 9.67.

ion-chromatographic determination of amino acids and related substances based on direct conductimertic detection.

2. Experimental

2.1. Instrumentation

Dionex DX-100 ion-chromatographic system, consisting of: DX-100 high pressure one piston pump, a sample injector equipped with a 25 μl loop, Ion pac AG-14 guard (4 mm × 50 mm) and AS-14 analytical columns (4 mm × 250 mm) for anion exchange separations, Ion pac CG-14 guard (4 mm × 50 mm) and CS-14 analytical columns (4 mm × 250 mm) for cation exchange separations, ASRS-I and CSRS-I micro-membrane suppressors operating in the auto suppression recycle mode (selectable current intensity 50–500 mA), and a conductimetric detector (dead volume 1.25 μl) equipped with a thermistor for compensation of temperature variations. Selection of columns, eluents and sample injection is pneumatically controlled. Only isocratic elution can be carried out. Anion exchange analytical column contains macroporus particles with 9.0 μm diameter, porus size 100 Å and total capacity of 78 μeq and can be operated in the pH range of 2–11. The particles are composed of a highly crosslinked core of ethylvinylbenzene with 55% divinylbenzene. The anion exchange layer is functionalized with quaternary ammonium groups and it has a controlled thickness resulting in excellent mass transfer. Cation exchange analytical column contains macroporus particles with 8.0 μm and total capacity of 1560 μeq and can be operated in the pH range of 0–14. The particles are composed of a highly crosslinked (55%) ethylvinylbenzene core and the cation exchange layer is functionalized with hydrophilic carboxylic acid. The chromatographic peaks were electronically integrated and recorded using an HP 3395 integrator.

For pH measurements a pH meter (Metrohm Herisau) equipped with a glass combination electrode was used. A filtration–vacuum system (Millipore) with type HVLP Millipore filters (diameter 47 mm, pore size 0.45 μm) was used for degassing the mobile phase and removing particles.

2.2. Reagents and standards

All solutions were prepared in HPLC-grade water (specific resistance > 17.8 MΩ cm) obtained by Milli-Q water purification system (Millipore) and all chemicals were of analytical reagent grade unless otherwise stated.

S-Carboxymethyl-L-cysteine was purchased from Sigma (product code C 7757) and its exact purity was determined using the European Pharmacopoeia procedure (potentiometric non-aqueous titration [2]). Pure solid S-CMC was stored protected from light in the refrigerator. A 1000 μg/ml standard stock solution was prepared in water and stored protected from light in the refrigerator. Working standard S-CMC solutions in the range of 20–400 μg/ml were daily prepared in water and were protected from light. The analyzed C-CMC pharmaceutical formulations (syrups) were obtained from local commercial sources.

For the selection and optimization of the mobile phase 1.00 M aqueous stock solutions of trifluoroacetic acid (TFA) (Sigma, >99%, spectrophotometric grade), NaOH (Sigma ACS, anhydrous pellets >98%), NaCl (Sigma ACS, >99%) and Na_2CO_3 (Sigma ACS, >99%) were prepared. More dilute working solutions were prepared daily in water. The optimized mobile phase for routine work was 0.25 mM TFA.

For the evaluation of the selectivity of the method a mixed solution of the acidic amino acids: S-CMC, L-aspartic acid (Sigma, >99%) and L-glutamic acid (Sigma, >99%) at concentration of 100 μg/ml was prepared in water. Binary mixtures of S-CMC with the amino acids: L-lysine, L-cysteine, L-glycine, L-methionine, L-histidine, L-asparagine, L-proline, L-tryptophan, L-threonine, L-serine, L-isoleucine and L-leucine, at concentration of 100 μg/ml were also prepared in water. A synthetic mixture containing S-CMC with all the aforementioned amino acids was also prepared for selectivity evaluation.

2.3. Sample preparation

Pharmaceutical formulations (syrups) of S-CMC were simply diluted with the mobile phase (0.25 mM TFA) at a concentration within the linear range of the method

(20–400 μg/ml). Due to the relatively high viscosity of syrups care must be taken for quantitative transfer of sample aliquot during the dilution.

2.4. Procedure

The chromatographic elution was performed at room controlled temperature (25 °C) in isocratic mode at 1.2 ml/min flow rate. Eluent solutions were filtered through a 0.45 μm membrane filter before usage. Flow path was rinsed for about 15 min, until baseline noise became less than 0.1 μS. Other instrumental parameters were: air pressure 5 psi and conductivity range 100 μS.

3. Results and discussion

3.1. Selection of ion-exchange column and mobile phase

S-CMC undergoes three stepwise ionization equilibria (Fig. 1), thus the electric charge of the migrating species can be controlled by the pH of its environment (mobile phase). The electric charge of S-CMC species strongly influences its selectivity coefficient (retention) as well as its response factor to the conductimetric detector.

Preliminary experiments were carried out applying the suppressed mode of ion-chromatography. Eluent suppression is in the main a means of improving signal-to-noise ratio by replacing the anions or the cations of the eluent with an equivalent number of hydroxide anions or hydronium cations, respectively. Therefore, the eluent is converted, in most cases, to deionized water and the eluite species appear as bands of their corresponding bases or acids. However, if the corresponding bases or acids appear small dissociation constant the eluite species would not be detected [19]. This was the case with S-CMC and none chromatographic peak was recorded. The pH of the mobile phase after passing through the suppressor was equal to the isoelectric point of S-CMC, resulting in the loss of conductivity of the eluted molecules. Therefore, the unsuppressed mode was necessarily followed, and the suppressor was disconnected from the chromatographic system.

3.2. Anion exchange column

Mobile phases of acidic (0.1–0.8 mM trifluoroacetic acid), neutral (0.1–0.5 mM NaCl) and basic (1.0 mM NaOH or Na_2CO_3) pH were examined using the AS-14 Dionex analytical column for anions.

Using the neutral (pH 7) mobile phase of NaCl, S-CMC migrates under the form III (Fig. 1) with a total charge of (−1). Prolonged retention time was appeared for S-CMC with very broad chromatographic peak and thus of low sensitivity (area/concentration). This is attributed to the low affinity of chlorides to the anion exchange material and therefore to their low eluting capability [19]. Therefore, mobile phase of NaCl appeared to be inappropriate for the elution of S-CMC.

Using the basic mobile phases (NaOH and Na_2CO_3) with pH about 10.5, S-CMC migrates under the form(s) III and IV and three peaks were obtained (Fig. 2A and C, respectively). The first narrow one was positive with relatively low retention time and since S-CMC appears smaller equivalent conductance than that of the eluent ions (OH^-, CO_3^{2-} or HCO_3^-) must be due to the counter-cations of S-CMC anionic species (such positive peaks close to the void volume of the system are common under the non-suppressed mode [19]). The second (negative) peak did not appear linear correlation between the peak area and the quantity of S-CMC and further, it was obtained for all amino acids tested with constant retention time regardless of the nature of the amino acid. It was also observed in chromatograms of blank injections (Fig. 2B). Therefore, this peak should be considered to be a system peak, which is caused by the pH disturbance of mobile phase produced by sample injection [20]. The third broad (negative) chromatographic peak is attributed to S-CMC (indirect detection). For NaOH mobile phase, the third (negative) peak, although of low sensitivity (area/concentration) may be utilized to construct a calibration curve. However, for Na_2CO_3 mobile phase, peak area appeared to have poor linearity versus S-CMC concentration.

Using acidic mobile phases (TFA) with pH approximately equal to 4, S-CMC migrates under the form II (with zero net charge) and form III resulting in one positive peak (Fig. 3). The retention time decreased considerably with increasing the eluent concentration. This peak can be utilized for the construction of a calibration curve.

Table 1 summarizes the chromatographic characteristics of the S-CMC peaks and the analytical characteristics of the calibrations curves obtained with the anion-exchange column in conjunction with the relatively more suitable eluents (NaOH and TFA). The resolution capability of each of the aforementioned mobile phases was tested using a mixture of the acidic amino acids (S-CMC, aspartic acid and glutamic acid). The resolution of S-CMC peak with aspartic acid peak (the closest using TFA eluents) is also included in Table 1.

In a further resolution study, binary mixtures of S-CMC with representative acidic, basic and neutral amino acids, as well as a mixture with the selected amino acids, were analyzed. In the case of the acidic phases (TFA), the neutral and basic amino acids exhibited total zero or positive charge and therefore they were eluted within the void volume. In the case of alkaline mobile phase (NaOH 1.0 mM), all the amino acids produced two negative peaks, like S-CMC, in addition to a first positive peak attributed to the counter-cation. The second (negative) peak was characterized by constant retention time for all amino acids and is considered to be a system peak, while the third one is attributed to the corresponding amino acid. As it is concluded from the results shown in Table 2, the resolution of S-CMC with all the amino acids tested is satisfactory.

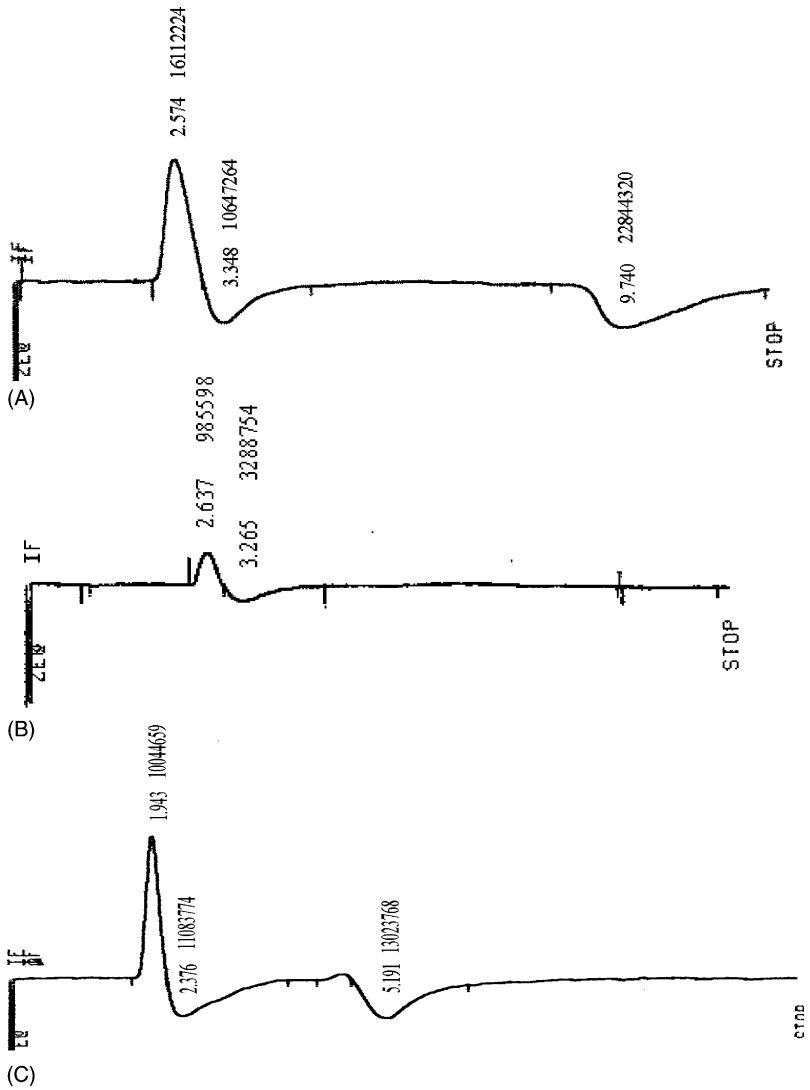

Fig. 2. Chromatograms using anion-exchange column (AS-14) and basic mobile phase. (A) S-CMC (250 μg/ml): aqueous 1 mM NaOH solution (t_R = 2.57 min (positive) due to counter cation, t_R = 3.35 min (negative) system peak, t_R = 9.74 min (negative) due to S-CMC). (B) Blank: aqueous 1 mM NaOH solution (t_R = 3.26 min (negative) system peak). (C) S-CMC (250 μg/ml): aqueous 1 mM Na_2CO_3 solution (t_R = 1.94 min (positive) due to counter cation, t_R = 2.38 min (negative) system peak, t_R = 5.19 min (negative) due to S-CMC). Integrated peak areas in μV s (detector full scale 100 μS).

Table 1
Chromatographic characteristics of S-CMC peaks and analytical characteristics of calibration curves using the anion exchange column

Characteristic	Mobile phase		
	NaOH 1.0 mM	TFA 0.4 mM	TFA 0.8 mM
Retention time (t_R) (min)	10.0	17.0	8.7
Peak width (min)	1.6	1.0	0.6
Retention factor/void time (min)[a]	6.0 (1.4)	12.1 (1.3)	5.7 (1.3)
Asymmetry factor	1.3	1.4	1.1
Theoretical plates (N)	5.0×10^2	2.5×10^3	1.7×10^3
Resolution S-CMC/aspartic acid	4.4	6.1	7.2
Slope of calibration curve (μV s ml μg^{-1})	$(8.43 \pm 0.12) \times 10^4$	$(9.48 \pm 0.16) \times 10^4$	$(6.34 \pm 0.11) \times 10^4$
Correlation coefficient (r) (50–400 μg/ml)	0.99990	0.9997	0.997
R.S.D. (100 μg/ml, n = 3) (%)	9.5	2.2	2.1
Detection limit[b]/absolute LOD	38 (μg/ml)/0.95 μg	6.6 (μg/ml)/0.16 μg	7.4 (μg/ml)/0.18 μg
Quantitation limit[b]/absolute LOQ	115 (μg/ml)/2.9 μg	20 (μg/ml)/0.50 μg	22 (μg/ml)/0.55 μg

[a] Equal to the determined retention time of unretained species (Na^+).
[b] Injection volume 25 μl.

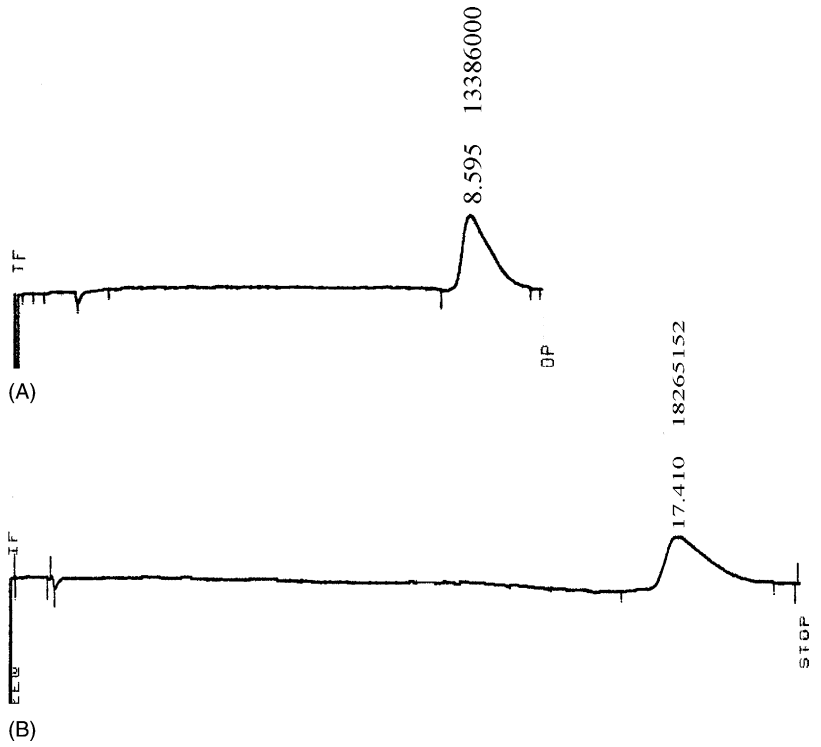

Fig. 3. Chromatogram of S-CMC (200 μg/ml) using anion exchange column (AS-14) and acidic mobile phases (aqueous TFA). Positive peak due to species III of S-CMC: (A) 0.8 mM TFA (t_R = 8.6 min); (B) 0.4 mM TFA (t_R = 17.4 min). Integrated peak areas in μV s (detector full scale 100 μS).

As it is revealed from Figs. 2 and 3, and Table 1 the best results with anion exchange column were obtained using a mobile phase of TFA 0.40 mM (regarding sensitivity and detectability) or TFA 0.8 mM (regarding analysis time and peak symmetry).

3.3. Cation exchange column

The cation exchange column (CS-14) was used in conjunction with acidic mobile phases (aqueous TFA solutions 0.25–1.0 mM, with pH in the range 4–3). Using these mobile phases S-CMC migrates as species II and III and one sharp positive peak was obtained (Fig. 4). The retention time increased with the increase of the eluent concentration (due to the increase of the positively charged fraction of S-CMC and thus to the increase of retention strength). The sensitivity (area/concentration) decreased considerably with the eluent concentration (due to the increase of the baseline conductivity and the increase of eluent acidity which results in increase of type III of S-CMC with zero net charge). Trials with mobile phases with pH less than pK_2 were not conducted because species II with zero net charge would predominate.

Table 2
Resolution of S-CMC from common amino acids using anion-exchange column (AS-14) with 1.0 mM NaOH mobile phase

Amino acid	t_R (min)	Width (min)	$R_{S\text{-}CMC/X}$
Glycine	5.6	0.7	3.8
Histidine	6.1	0.8	3.3
Asparagine	6.2	0.8	3.2
Serine	6.4	1.1	2.7
Threonine	6.5	1.0	2.7
Proline	7.0	0.9	2.4
Lysine	8.0	0.7	1.7
Cysteine	8.1	0.6	1.7
S-CMC	10.0	1.6	–
Methionine	11.8	2.0	1.0
Isoleucine	16.8	2.5	3.3
Leucine	17.0	2.5	3.4
Glutamic	18.6	2.5	4.2
Aspartic	19.0	2.5	4.4
Tryptophan	19.5	2.1	5.1

The selection of the optimum eluent concentration was based on the chromatographic characteristics of S-CMC peaks and the analytical characteristics of calibration curves in the range 50–400 μg/ml of S-CMC obtained with various TFA concentrations (Table 3). The resolution capability of each mobile phase was tested using a mixture of the acidic amino acids (S-CMC, aspartic acid and glutamic acid). The resolution of S-CMC peak with glutamic acid peak (the closest one) is also included in Table 3. As it is concluded from Fig. 4 and Table 3 the best results with cation exchange column were obtained using a mobile phase of TFA 0.25 mM (regarding short analysis time, sensitivity, detectability and resolution).

Comparing the results from the usage of the two exchange columns, the optimum performance (mainly sensitivity and analysis time) was obtained with the cation exchange

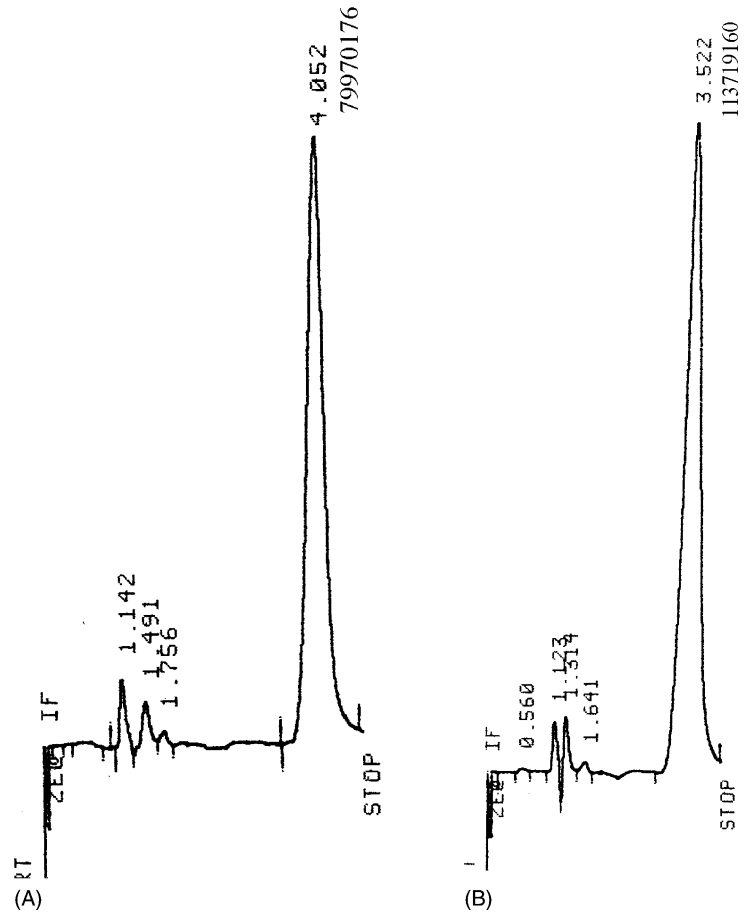

Fig. 4. Chromatogram of S-CMC (200 μg/ml) using cation exchange column (CS-14) and acidic mobile phases (aqueous TFA). Positive peak due to species III of S-CMC: (A) 0.50 mM TFA (t_R = 4.1 min); (B) 0.25 mM TFA (t_R = 3.5 min). Integrated peak areas in μV s (detector full scale 100 μS).

column. Therefore, the proposed method for the routine determination of S-CMC in pharmaceuticals is based on the use of a 0.25 mM TFA mobile phase with cation exchange column and non-suppressed conductimetric detection.

3.4. Application to pharmaceutical formulations

The proposed ion-chromatographic method was applied for the determination of S-CMC in intensely colored

Table 3
Chromatographic characteristics of S-CMC peaks and analytical characteristics of calibration curves using the cation exchange column

Characteristic	Mobile phase		
	TFA 0.25 mM	TFA 0.50 mM	TFA 1.00 mM
Retention time (t_R) (min)	3.5	4.0	4.5
Peak width (min)	0.5	0.5	0.5
Retention factor/void time (min)[a]	1.5 (1.4)	1.6 (1.5)	2.0 (1.5)
Asymmetry factor	1.1	1.1	1.1
Theoretical plates (N)	7.5×10^2	9.9×10^2	1.2×10^3
Resolution S-CMC/glutamic acid	2.6	1.4	0.1
Slope of calibration curve (μV s ml μg^{-1})	$(53.91 \pm 0.57) \times 10^4$	$(30.5 \pm 1.2) \times 10^4$	$(2.01 \pm 0.04) \times 10^4$
Correlation coefficient (r) (50–400 μg/ml)	0.99994	0.998	0.9996
R.S.D. (100 μg/ml, $n = 3$) (%)	1.5	1.7	2.0
Detection limit[b]/absolute LOD	5.6 (μg/ml)/0.14 μg	6.6 (μg/ml)/0.16 μg	7.6 (μg/ml)/0.19 μg
Quantitation limit[b]/absolute LOQ	17 (μg/ml)/0.42 μg	20 (μg/ml)/0.50 μg	23 (μg/ml)/0.58 μg

[a] Equal to the determined retention time of unretained species (Cl^-).
[b] Injection volume 25 μl.

Table 4
Assay of content and recovery results of S-CMC commercial formulations

Formulation (syrup)/claimed content	Content found (mg/ml) ± S.D. ($N = 3$)	Mean recovery (%)[a]
Mucothiol adults (Lavifarm) (50 mg/ml)	51.2 ± 3.7 (R.S.D. = 7.2%)	100.9
Mucothiol enfants (Lavifarm) (20 mg/ml)	19.6 ± 0.7 (R.S.D. = 3.6%)	97.5
Chilvax (Proel) (50 mg/ml)	49.0 ± 0.9 (R.S.D. = 1.8%)	104.9
Pulmoclase (UCB) (50 mg/ml)	48.4 ± 0.9 (R.S.D. = 1.8%)	96.0

[a] From three recovery experiments at different spiking levels.

commercial formulations (syrups). The only required sample treatment was the appropriate dilution with the mobile phase (0.25 mM TFA) in order to have S-CMC in the concentration range of 20–400 μg/ml. In the sample chromatograms only the peak of S-CMC was obtained (Fig. 5). Both calibration curve and standard addition procedures can be used with very similar results. The results shown in Table 4 reveal that all the commercial formulations conformed with the common pharmacopoeia requirement for a content within the range of 95–105% of the labeled content. The relatively low between determinations precision for the first formulation (R.S.D. 7.2%) was due to its high viscosity.

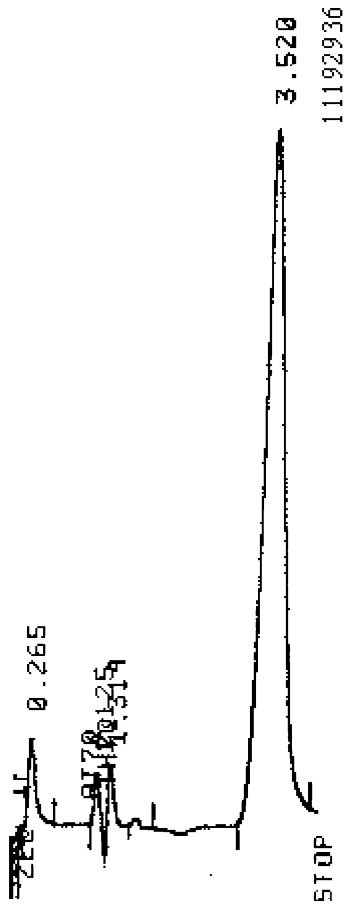

Fig. 5. Chromatogram of a working sample solution (S-CMC 200 μg/ml) of a commercial formulation (Mucothiol adults) using cation exchange column (CS-14) and mobile phase of 0.25 mM TFA. Only S-CMC peak is obtained ($t_R = 3.5$ min). Integrated peak areas in μV s (detector full scale 100 μS).

The accuracy of the method was evaluated by recovery experiments by spiking diluted syrup samples. The recoveries shown in Table 4 (96.0–104.9%) reveal sufficient accuracy. Further study of the matrix (excipients) effect on the determination was carried out by dilution experiments (determination of S-CMC content in commercial formulations using a varying dilution factor D ($V_{initial}/V_{final}$) at four different levels. The correlation curves of the concentration found (in the diluted solution) versus D were very linear ($r > 0.999$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curves of content found versus D were very linear with statistically (proven by t-test) zero slope. These results reveal the absence of any constant or proportional determinate error from the excipients.

4. Conclusions

The mucolytic and expectorant drug carbocisteine (S-CMC), with an acidic amino acid structure, can be determined using non-suppressed ion-chromatographic system with conductimetric detection using anion or cation exchange column and TFA eluent. Best performance, regarding sensitivity, detectability, analysis time and chromatographic characteristics, was obtained with cation exchange column and a mobile phase of 0.25 mM TFA. The separation from other acidic amino acids was sufficient and the calibration curve was linear in the range of 17–400 μg/ml. The proposed method can be applied for content assay of intensely colored pharmaceutical formulations (syrups) without any pretreatment step (only dilution with mobile phase) and interference from the matrix. The proposed method has the advantage of the low cost instrumentation and the quick and simple procedure, which are very important factors for routine pharmaceutical analysis of formulations. The obtained limit of detection, is quite greater than other published methods [16] and therefore the ion-chromatographic method is inappropriate for the determination of carbocysteine in biological fluids. However, this relatively low detectability is not important for assays of pharmaceutical formulations.

As concluded from the literature, this is the first work dealing with ion-chromatographic determination of amino acids or derivatives using direct conductimetric detection and can be applied to the assay of other drugs with similar (acidic amino acid) structure.

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