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Determination of the cysteine derivatives N-acetylcysteine, S-carboxymethylcysteine and methylcysteine in pharmaceuticals by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method was developed for the determination of three mucolytic agents, N-acetylcysteine (NACS), S-carboxymethylcysteine (SCMCS) and methylcysteine (MCS). Chromatography was performed on an ODS column using an isocratic mobile phase consisting of acetonitrile, methanol and sodium hexanesulfonic acid buffer (pH 2.9) at a flow-rate of 1.0 ml/min, with UV detection at 220 nm. Methionine was used as an internal standard. Good quantitative results were obtained, the recoveries from synthetic mixtures being >98.2% with R.S.D. <1%. Linearity over a concentration range of over one order magnitude was obtained; the correlation coefficients were >0.9997 at concentrations of 0.05, 0.1, 1.0, 2.0 and 3.0 mg/ml. The addition of methanol and acetonitrile to the mobile phase improved the peak shape and retention of the compounds of interest. The selectivity of the method was demonstrated by separating four similar thiol compounds and determination of the analytes in pharmaceuticals.

1. Introduction

The cysteine derivatives N-acetylcysteine (NACS), S-carboxymethylcysteine (SCMCS) and methylcysteine (MCS), amino acid-like compounds, are members of the group of mucolytic agents. They are used to reduce the viscosity of pulmonary secretions [1], to improve chronic bronchitis by reducing coughing and sputum [2], as nebulizers [3] and as aids suppressing coughing in anti-influenza preparations. They can also protect against hepatoxicity of high doses of acetaminophen [4–6].

Numerous methods have been studied for their determination. NACS has been determined by colour development with iron(III) in the presence of 1,10-phenantholine [7], potentiometrical titration of the thiol group with mercury (II) nitrate [8,9] and spectrophotometry [10]. These methods were not specific and were unable to distinguish among thiol-containing compounds. Various advanced methods for NACS have been tested, with postcolumn [11,12] and precolumn [13,14] derivatization with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole, N-[4-(6-dimethylamino-2-benzofuranyl)phenyl] [maleimide and 2,4-dinitro-1-fluorobenzene, and spectrofluorimetry. A gas chromatographic (GC) method for SCMSC, of high volatility, involved time-consuming sample preparation because it required derivatization with N,O-bis(trimethyl)-trifluoroacetamide–1% trimethylchlorosilane [15], and HPLC with buffered eluents [15–18]. The sample preparation time using GC assay was

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about 3–4 h. No method has been reported for MCS.

This paper describes an HPLC method using sodium hexanesulfonic acid, which can eliminate the interference from other organic thiol-containing compounds, and allows NACS, SCMCS and MCS to be distinguished from each other. It also represents a distinct challenge because of the lack of a UV chromophore in the molecule. The UV detection mode at low wavelength limits the mobile phase modifier that can be used to enhance selectivity and peak shape. For the recovery study, synthetic mixtures were made up with lactose and potato starch. The aim of this work was to establish a routine method and deal with the above problem to provide an assay procedure for the determination of NACS and its analogues in pharmaceuticals, synthetic mixtures or bulk drugs.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol, acetonitrile and phosphoric acid and analytical-reagent grade sodium hydrogen sulfite were purchased from Merk (Darmstadt, Germany) and sodium hexanesulfonic acid, standard NACS and MCS and the internal standard methionine from Sigma (St. Louis, MO, USA) and SCMCS was donated by Isochem (Philippelbon, Gennevillers, France). Various production forms were purchased commercially. Excipients, lactose and potato starch, used in synthetic formulations, were of reagent grade. Water was of HPLC grade, obtained by passage through a 0.22-μm membrane filter (Waters Milli-Q SP reagent water system).

2.2. Apparatus

A Model 600 E liquid chromatographic dual-pump system controller, Model 700 Satellite WISP autosampler with a loop injection valve, Model 486E tunable UV detector and 745B data module integrator (Waters, Bedford, MA, USA) were employed. The mobile phase was pumped through a reversed-phase column (μBondapak ODS, 30 cm × 3.9 mm I.D.; 10 μm; Waters) with an isocratic flow-rate of 1 ml/min. The detector was set at 220 nm. Chromatography was performed at room temperature. Injections of 10 μl of all solutions at concentrations of 0.05–3.0 mg/ml, synthetic formulations and commercial products were made.

2.3. Mobile phase

A series of mobile phases of 5 m*M* sodium hexanesulfonic acid (pH 2.9) with different percentages of organic modifier were prepared in order to study the effect of solvent strength. Another mobile phase composition of 5 m*M* sodium hexanesulfonic acid (pH 2.9)–acetonitrile–methanol (98.6:0.8:0.6, v/v/v) was prepared, the pH being adjusted with phosphoric acid. This solution was filtered through a membrane of 0.45 μm, degassed under a stream of helium and used to establish the best separation and peak shape.

2.4. Internal standard stock standard solution

Methionine (250 mg) was dissolved in 40 ml of 0.05% sodium hydrogensulfite solution and adjusted to pH 3 with phosphoric acid. The flask was shaken in an ultrasonic bath and diluted to 50 ml with methanol.

2.5. Standard solutions

To prepare each standard solution, the internal standard stock standard solution was added to an accurately weighed 50-mg amount of NACS, SCMCS and MCS, dissolved in 90 ml of 0.05% sodium hydrogen sulfite solution and adjusted to pH 3 with phosphoric acid. The flask was shaken in an ultrasonic bath and the contents were diluted to 100 ml with methanol.

2.6. Sample preparation

To prepare a sample solution, 10 ml of internal standard stock standard solution were added to an accurately weighed homogeneous tablet,

capsule contents or granule dosage equivalent to 50 mg of NACS, SCMCS and MCS and the volume was adjusted to 100.0 by adding 80 ml of 0.05% sodium hydrogensulfite solution and then methanol.

2.7. Solution for linearity response

Five concentrations of NACS, SCMCS and MCS ranging from 0.05 to 3.0 mg/ml, with internal standard stock standard solution added, were prepared. Solutions of each concentration were chromatographed five times.

2.8. Solutions for recovery study of NACS, SCMCS and MCS in synthetic mixtures

Solutions of five synthetic formulations of NACS, SCMCS and MCS were prepared by accurately weighing 50.0-mg amounts. Each solution was made up to 100.0 ml with 0.05% sodium hydrogen sulfite solution, 10 ml of internal standard stock standard solution and methanol, and was chromatographed five times.

3. Results and discussion

The linearity of the peak-area ratio of cysteine derivatives versus the internal standard was verified by injection of five solutions containing NACS, SCMCS and MCS at concentrations of 0.05, 0.1, 1.0, 2.0 and 3.0 mg/ml. A straight line with a good correlation coefficient was obtained for five injections and good R.S.D., as shown in Table 1. The data in Table 1 demonstrate the linearity of the response for standard solutions using the proposed method.

Reproducibilities for these cysteine derivatives, both on the same day and day-to-day, were evaluated. The R.S.D. on the basis of peak-area ratio for five standard injections on the same day for NACS, SCMCS and MCS was between 0.11% and 0.38% for an amount of 10 μg; the day-to-day R.S.D. was 0.43% for same amount. As expected, the same-day R.S.D.s were much lower because they were not affected by as many variables. The mean deviations for NACS, SCMCS and MCS was 0.01, 0.02 and 0.07, respectively. Based on the recovery range, the low R.S.D. and mean deviation, the precision was good.

The results for standard addition recovery studies of NACS, SCMCS and MCS in five synthetic formulations are given in Table 2 and range from 98.8% to 99.2%. Because the average recovery was greater than 98.8% and the R.S.D. was 0.02–0.16%, there was obviously no interference due to the excipients. This indicated that the proposed HPLC method is relatively unaffected by the sample matrix.

Typical chromatograms of NACS, SCMCS and MCS commercial dosage forms are shown in Fig. 1B, C and D. The retention times were 4.1 min for SCMCS, 6.8 min for NACS, 5.2 min for MCS and 11 min. for the internal standard. When compared with that obtained for a standard mixture shown, in Fig. 1A, no additional peak elutes. The results for various pharmaceuticals are given in Table 3, and lie within the official requirement of 90–110% based on the declared concentration. The selectivity of the method was demonstrated by using different pH

Table 1
Linearity results for standard MCS MCS and SCMCS at concentrations of 0.05, 0.1, 1.0, 2.0 and 3.0 mg/ml

Compound	r	y	R.S.D. (%) ($n = 5$)
MCS	0.9998	$1.4955x - 0.0066$	0.05
NACS	0.9997	$2.2113x - 0.0278$	0.02
SCMCS	0.9999	$1.1294x - 0.0054$	0.08

Table 2
Recovery of NACS, SCMCS and MCS from synthetic formulations

Formulation	Component	Added (mg)	Mean found (mg)[a]	Mean recovery (%)[a]	R.S.D. (%)[a]
(A) Granule	NACS	20	19.80	99.0	0.09
	Lactose	440			
	Potato starch	540			
(B) Granule	NACS	40	39.52	98.8	0.16
	Lactose	420			
	Potato starch	520			
(C) Tablet	SCMCS	375	371.62	99.1	0.02
	Lactose	100			
	Potato starch	100			
(D) Tablet	SCMCS	250	248.0	99.2	0.08
	Lactose	100			
	Potato starch	100			
(E) Capsule	MCS	50	49.55	99.1	0.06
	Lactose	124			
	Potato starch	124			

[a] $n = 5$.

values and the four similar thiol compounds were completely resolved, as illustrated in Fig. 1. Excipients from commercial formulations did not affect the resolution.

An organic modifier can be used to control the retentions of the three systeine derivatives. Complete separation using a mobile phase with only an ion-pair reagent as buffer. It was possible to control the separation of the analytes by adding either acetonitrile or methanol alone to adjust the eluent strength, and badly tailing peaks were obtained. The retention and peaks shape were investigated with both methanol and acetonitrile added to the mobile phase. Fig. 2 shows chromatograms obtained as the acetonitrile concentration was increased at the expense of the buffer. With increase in acetonitrile to 0.8%, NACS, SCMCS, MCS and methionine were rapidly separated, and the peak shape improved dramatically.

Plots of retention volume vs. percentage of organic modifier are shown in Fig. 3. The variations in retention and peak shapes of the four analytes were investigated as methanol and acetonitrile were added to the mobile phase. An optimum percentage of acetonitrile was necessary to enhance the peak shape of NACS and the unsymmetrical tailing of methionine. Upto at least 0.7% of acetonitrile added the retention volume of NACS decreased, after which there was hardly any change according to Fig. 3. The plot for SCMCS hardly changed at different percentages of organic solvent.

The buffer concentration was found to be a key parameter in controlling the separation of NACS and its analogues. Peak tailing and long retention were observed in the absence of buffer, and improved with increasing buffer concentration. No further change in retention occurred at buffer concentrations higher than 7 m*M*. At a concentration of 10 m*M*, NACS was eluted after methionine and both retentions were longer than at 5 m*M*.

The dependence of the separation on pH is illustrated by the plot of retention volume vs. pH at constant buffer concentration in Fig. 4. pH values of the mobile phase less than 3.2 were necessary for optimum resolution. A slight increase in pH produced shorter retentions and incomplete resolution. As has been observed

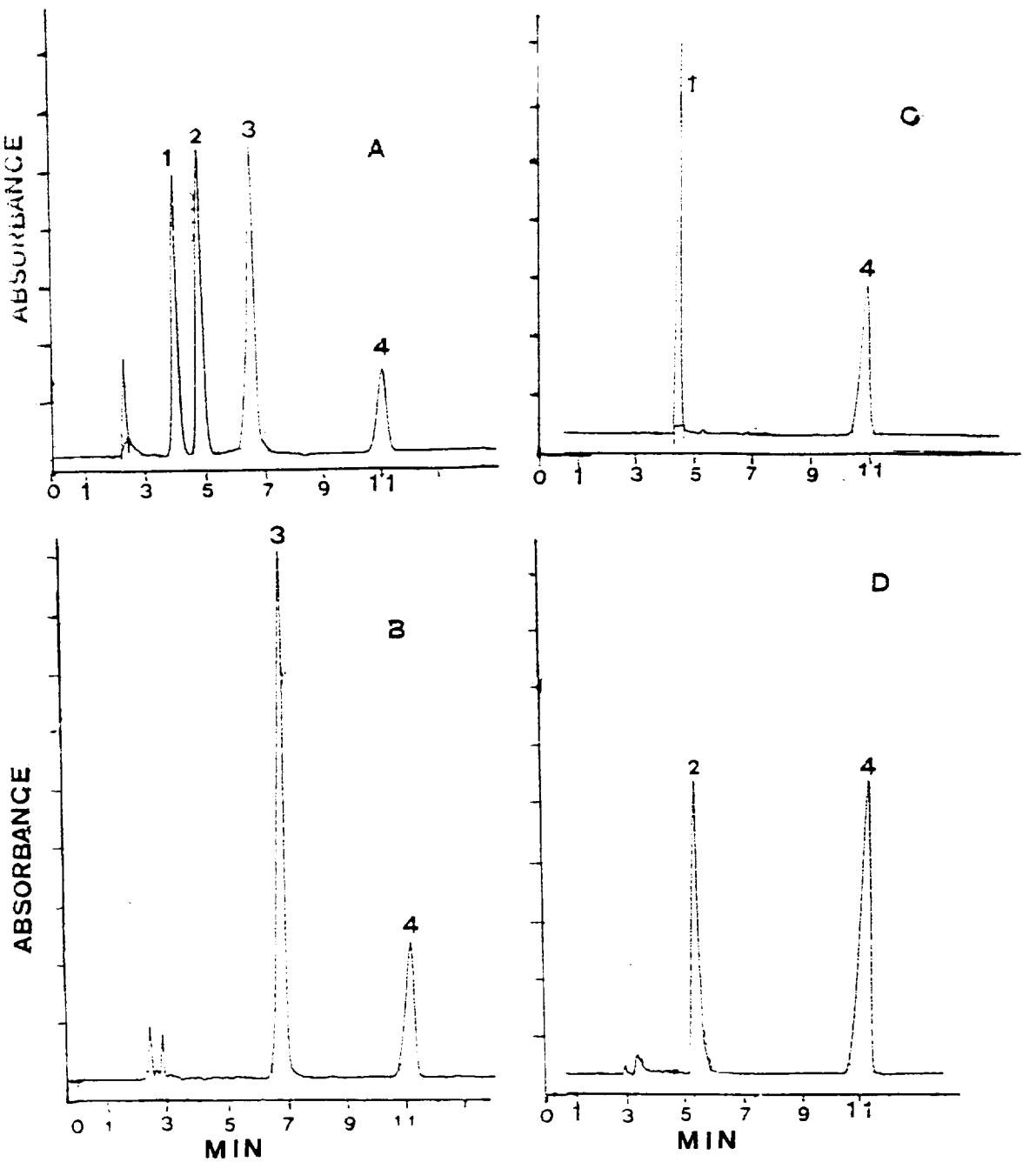

Fig. 1. Chromatograms of N-acetylcysteine, S-carboxymethylcysteine, and methylcysteine in commercial preparations. (A) 1 mg of standard and 0.5 mg/ml internal standard mixture; (B) 40 mg of NACS granule; (C) 50 mg of MCS capsule; (D) 375 mg of SCMCS table. Peaks: 1 = SCMCS; 2 = MCS; 3 = NACS; 4 = methionine (internal standard).

Table 3
Recoveries of NACS, SCMCS and MCS from various commercial formulations

Formulation	Component	Added (mg)	Found (mg)	Recovery (%)
(1) Granule	NACS (5 g)	100	99.40	99.4
(2) Granule	NACS (3 g)	200	197.80	98.9
(3) Tablet	SCMCS	375	372.5	99.8
(4) Capsule	MCS	50	49.55	99.1

with NACS and SCMCS, an increase in high pH produced same retention [11,13]. A better peaks shape was also observed at lower pH for NACS, SCMCS and MCS. As expected, pH prolonged the retention of the internal standard. At pH less than 2.5, three analytes were eluted fast than at pH 2.9 and methionine eluted at 15.2 min with a broad peak, as shown in Fig. 5. From the retention volumes of MCS and SCMCS, no significant change was obtained when the pH was 3.0–3.1. However, the retention volume for NACS was higher in the same pH range.

4. Conclusions

A mobile phase consisting of methanol, acetonitrile and sodium hexanesulfonic acid buffer with a μBondapack ODS reversed-phase column was shown to improve the separation of N-acetylcysteine, S-carboxymethylcysteine, methylcysteine and internal standard methionine. Addition of methanol or acetonitrile alone prolonged the retention time and resulted in

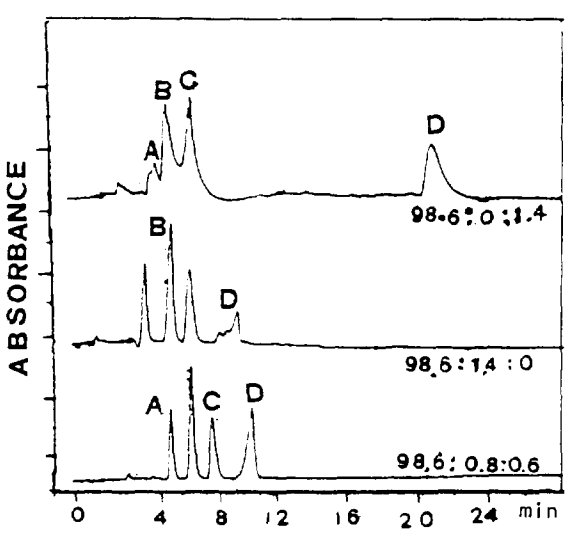

Fig. 2. Effect of methanol and acetonitrile on separation and peak shapes of MCS, NACS and SCMCS. Mobile phase 5 m*M* sodium hexanesulfonic acid (pH 2.9)–acetonitrile–methanol in the ratios indicated. A = SCMCS; B = NACS; C = MCS; D = methionine (in amounts of 2.5 μg).

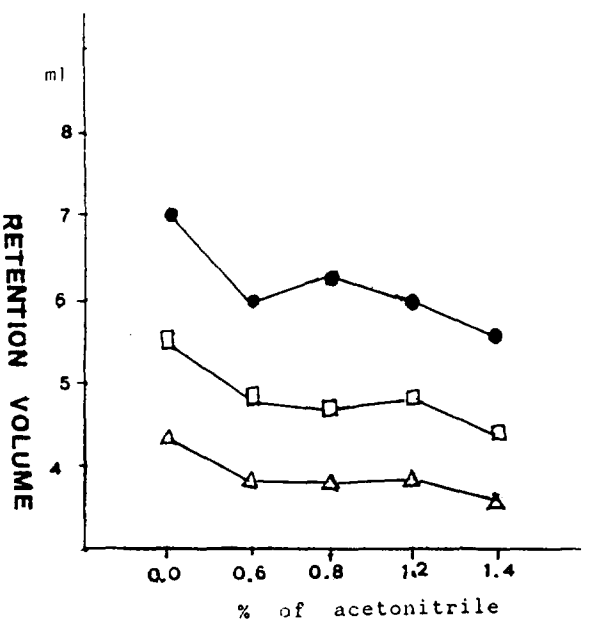

Fig. 3. Effect of solvent strength on retention volumes of (△) SCMCS, (□) MCS and (●) NACS. Mobile phase, 5 m*M* sodium hexanesulfonic acid (pH 2.9)–acetonitrile–methanol; column temperature, room temperature.

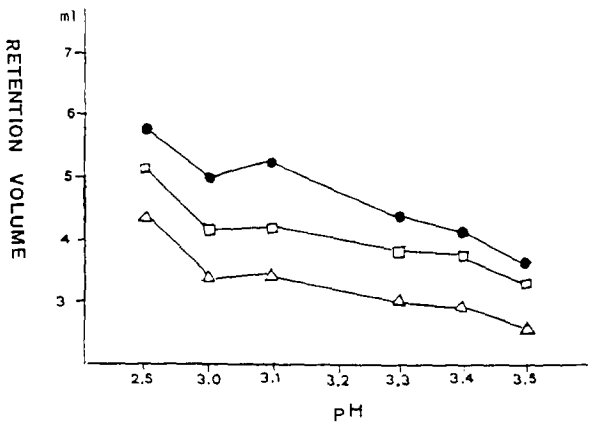

Fig. 4. Effect of pH on retention volumes of (△) SCMCS, (□) MCS, and (●) NACS. Mobile phase, 5 m*M* sodium hexanesulfonic acid (pH 2.9)–acetontrile–methanol (98.6:0.8:0.6); column temperature, room temperature.

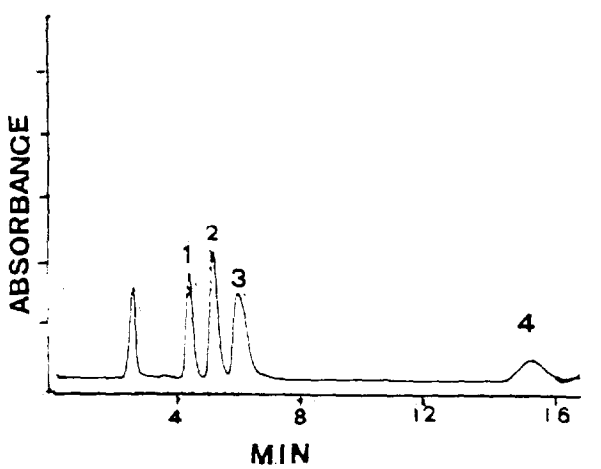

Fig. 5. Chromatogram of N-acetylcysteine, S-carboxymethylcysteine and methylcysteine obtained with 5 m*M* sodium hexanesulfonic acid (pH 2.45)–acetontrile–methanol (98.6:0.8:0.6), 0.5 mg/ml of standard and 0.5 mg/ml internal standard mixture. Peaks: 1 = SCMCS; 2 = MCS; 3 = NACS; 4 = methionine (internal standard).

peak tailing; however, acetonitrile reduced the peak tailing except for methionine. When the concentrations of methanol and acetonitrile are 0.6 and 0.8% (v/v), the peak shape and retention are greatly improved. The proposed method for NACS, SCMCS and MCS successfully separates the analytes within 12 min, provides good precision and is a sensitive and suitable quality control for pharmaceuticals or bulk drugs.

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