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Quantitative analysis of *N*-acetylcysteine and its pharmacopeial impurities in a pharmaceutical formulation by liquid chromatography–UV detection–mass spectrometry

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Abstract

A new method for the simultaneous determination of *N*-acetylcysteine and its pharmacopeial impurities, cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine in an effervescent tablet has been developed. The method is based on on-line LC–UV–MS using a pneumatically-assisted electrospray interface (ionspray). The stability of the thiol moieties of the analytes was ensured by the acidic pH of the LC mobile phase. Quantitation of *N*-acetylcysteine was performed with UV detection to avoid ion-source overloading effect due to its higher concentration, whereas the impurities could be easily separated and quantified in MS. The method was validated in terms of stability, linearity, precision and accuracy.

Keywords: Pharmaceutical analysis; Validation; Acetylcysteine; Cysteine; Cystine; Diacetylcystine; Diacetylcysteine; Amino acids

1. Introduction

N-Acetylcysteine is an important mucolytic agent used to reduce the viscosity of pulmonary secretions in respiratory diseases and when administered intravenously it is an effective antidote in the treatment of paracetamol poisoning [1–3]. The impurities of *N*-acetylcysteine limited by the European Pharmacopeia requirements are cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine (Fig. 1) [4].

Quantitative analysis of therapeutic compounds and their impurities in pharmaceutical formulations requires sensitive and selective methods to allow the determination of concentrations in the ng/ml range. Numerous methods have been developed for *N*-acetylcysteine determination in aqueous solution or in biological fluids. Several liquid chromatographic (LC) methods involve spectrophotometric detection with pre- or post-column derivatization [5–14], polarographic detection [15], chemiluminescence detection [16], amperometric detection [17–20], potentiometric detection [21,22], chlorocoulometric detection [23] and colorimetric detection [24–26]. Capillary electrophoresis (CE) methods have also been developed using ultraviolet detection for *N*-acetylcysteine determination in pharmaceutical formulations [27] or in bulk form [28]. Determination of *N*-acetylcysteine conjugates by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [29] and gas

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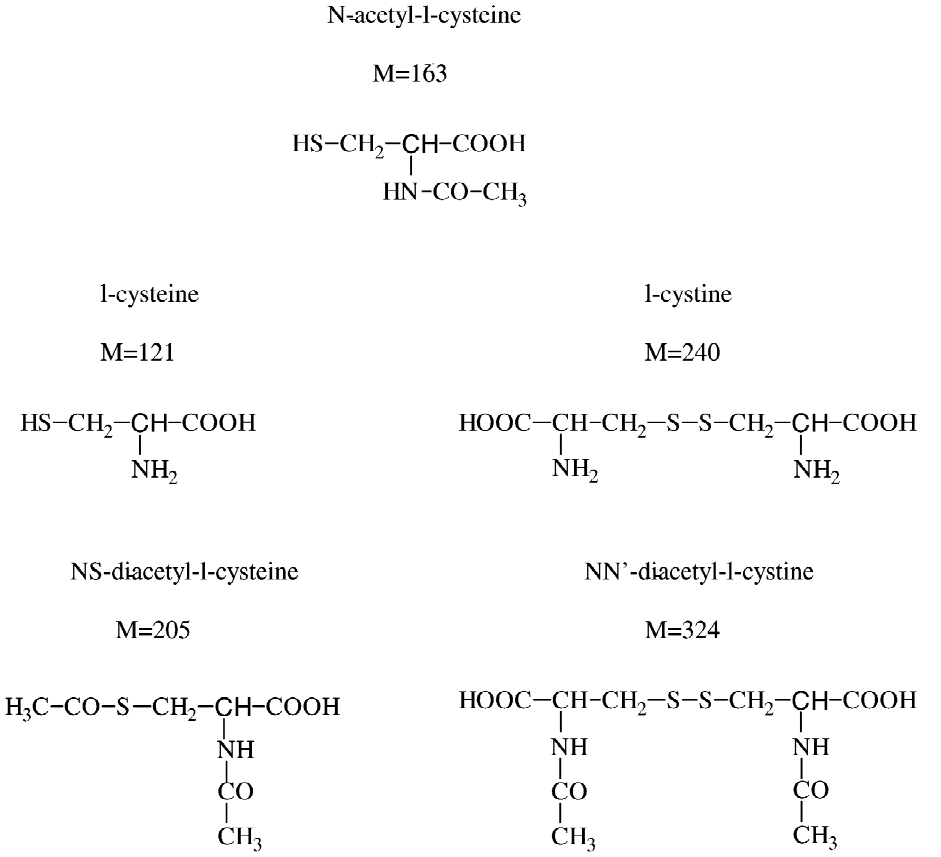

Fig. 1. Chemical structures of *N*-acetyl-L-cysteine, L-cysteine, L-cystine, *N,N′*-diacetyl-L-cystine and *N,S*-diacetyl-L-cysteine.

chromatography–mass spectrometry (GC–MS) [30,31], or off-line identification by fast atom bombardment (FAB) MS [28] have also been reported in literature. However very few methods involving on-line LC or CE separation and MS detection have been mentioned for *N*-acetylcysteine determination in literature compared to the huge amount of other detection methods described. Nevertheless, in the last few years, LC coupled to MS detection has been widely used for both identification and quantitation at low concentrations in bulk form and in biological fluids [32–41]. This technique provides highly selective and sensitive detection of analytes in a complex matrix. The LC–MS interfaces based on atmospheric pressure ionization (API) and represented by electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are mostly employed. ESI produces negligible fragmentation of the compounds compared to APCI and is particularly suitable for polar molecules analysis. A previous paper reported a comparison between APCI and ESI mode for *N*-acetylcysteine determination [35]. A higher detection sensitivity was observed with ESI but a split of the LC eluent was necessary in order to perform LC–MS coupling. Nowadays, the use of a pneumatic nebulization (pneumatically assisted electrospray or ionspray) makes the LC–ESI-MS coupling easier because it tolerates eluent flow-rates up to 200 μl/min without splitting [42].

This paper presents a new method for the determination of *N*-acetylcysteine and its four Pharmacopeial impurities, in presence of excipients of a pharmaceutical formulation. The method was developed to perform the quality control of effervescent tablets, a new oral formulation containing 200 mg of *N*-acetylcysteine together with excipients. The procedure involves the coupling of LC to UV and subsequent MS detection with an ionspray interface.

The use of successive UV and MS detection provides double identification and double selectivity thanks to retention time and mass measurement. Because of its higher concentration, *N*-acetylcysteine was quantified in UV detection while the impurities could be separated and quantified in MS detection. The tolerated limit for each impurity in the effervescent tablets was 2% whereas 0.5% of each impurity are tolerated for *N*-acetylcysteine in bulk form [4]. The MS detection of the impurities was first optimized without analytical column, using flow injection analysis (FIA). Secondly, the LC–UV–MS coupling was performed and the selectivity of the method was investigated by analyzing the excipients in the selected experimental conditions. The method was then validated in two steps: first, validation of *N*-acetylcysteine determination in UV detection and second, validation of the impurities determination in MS detection. Finally, the LC–UV–MS method was successfully applied to the single-run determination of *N*-acetylcysteine and its impurities in effervescent tablets.

2. Experimental

2.1. Chemicals

N-Acetyl-L-cysteine, L-cysteine, L-cystine, *N*,*N*′-diacetyl-L-cystine and *N*,*S*-diacetyl-L-cysteine were supplied by SMB Technology (Marche-en-Famenne, Belgium). Formic acid (98–100%), 37% hydrochloric acid and 25% ammonia were of analytical grade from Merck (Darmstadt, Germany). Methanol, acetonitrile and water were of HPLC grade from Merck. Nitrogen (99.999%) was produced by an on-side N_2 generator.

Standard solutions of the analytes except cystine were prepared by dissolving the in the LC mobile phase. Cystine was first dissolved in 1 *M* hydrochloric acid before dilution to the desired concentration with the LC mobile phase. The pulverized effervescent tablets were dissolved in the LC mobile phase as well.

2.2. Instrumentation and methods

The LC system was a Model 1100 series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostated column compartment and an autosampler, all from Hewlett-Packard (Palo Alto, CA, USA). The LC separations were performed at 25°C on a C_{18} Platinum EPS column (5 μm, 150 mm×2.1 mm I.D.) from Alltech Associates (Deerfield, IL, USA). The mobile phase was a mixture of acetonitrile and ammonia–formic acid (7/12 m*M*) buffer at pH 3.0 (2:98, v/v). The mobile phase was degassed for 15 min in an ultrasonic bath before use. The flow-rate was 0.3 ml/min. The sample injection volume was 10 μl.

UV and MS detection were carried out using a mass-selective detector from Hewlett-Packard equipped with an ionspray interface. The heated drying gas (N_2) temperature was 350°C and its flow-rate was 10 l/min. The nebulizer gas (air) pressure was 50 p.s.i. (1 p.s.i.=6894.76 Pa). The capillary voltage was 4000 V. The cone voltage was 50 V for cysteine and cystine detection but was changed to 40 V for *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine detection. The mass spectrometer was set to generate and to select positive pseudo-molecular ions at *m*/*z* 164.0 for *N*-acetylcysteine, *m*/*z* 122.0 for cysteine, *m*/*z* 241.0 for cystine, *m*/*z* 325.1 for *N*,*N*′-diacetylcystine and *m*/*z* 206.0 for *N*,*S*-diacetylcysteine. The UV detector was set at 210 nm.

3. Results and discussion

3.1. Method development

The LC separation of *N*-acetylcysteine, its impurities and the excipients was performed with the same LC column and mobile phase as developed in the literature [35] for *N*-acetylcysteine determination by LC–ESI-MS–MS. Using those conditions, cysteine and cystine coeluted and could not be quantified in UV whereas the impurities were separated within 5 min (Fig. 2).

N-Acetylcysteine was identified by MS (*m*/*z* 164.0) but the UV absorbance was used for quantitation. Indeed, at the concentration range used for *N*-acetylcysteine, an overloading of the MS ion-source was observed so that a recovery of 70% was obtained for *N*-acetylcysteine using MS quantitation

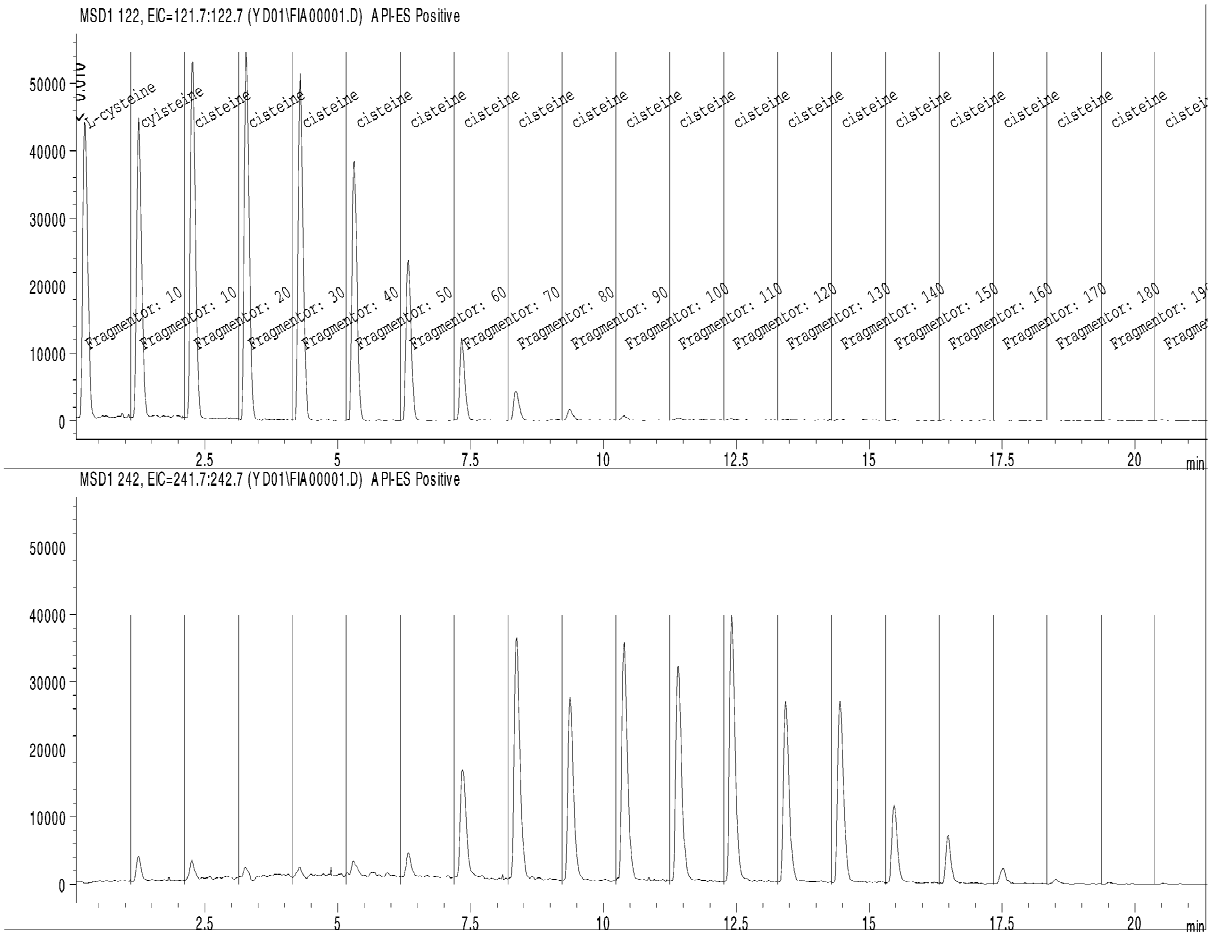

Fig. 2. Up: SIM ion chromatogram of cysteine (m/z 122) obtained after several successive injection of a cysteine standard solution (100 μg/ml) in FIA–UV–MS (SIM) at different cone voltage values. Down: SIM ion chromatogram of cystine (m/z 241) obtained simultaneously.

instead of 100% with UV. On the other hand, all impurities could be quantified in MS with a recovery of 100%. The UV detection of *N*-acetylcysteine at 210 nm is illustrated in Fig. 3.

On the other hand, the MS detection of the impurities was optimized in the positive ion mode by FIA of each analytes in standard solutions using a flow-rate of 0.3 ml/min. The single ion monitoring (SIM) mode was used for MS detection. Parameters such as capillary voltage, cone voltage, drying gas flow-rate and temperature and nebulizing gas pressure were investigated. Capillary and cone voltages from 1000 to 6000 V and from 10 to 200 V were tested, respectively. The best detection sensitivity was obtained with a capillary and cone voltage of 4000 and 40–50 V, respectively. At higher values of one or the other voltage, dimeric compounds were observed in the mass spectra of monomeric compounds (Fig. 2). As their intensity increased with the voltage value, it seems that dimerization of thiols is favored in the ion-source at a higher voltage. On the other hand, drying gas temperatures from 200 to 350°C were tested. As the LC mobile phase was mainly composed of aqueous phase (98%), the upper temperature value was selected in order to allow a proper evaporation of the liquid phase before MS. It was also needed to increase the drying gas flow-rate up to 10 l/min to stabilize the spray and to avoid

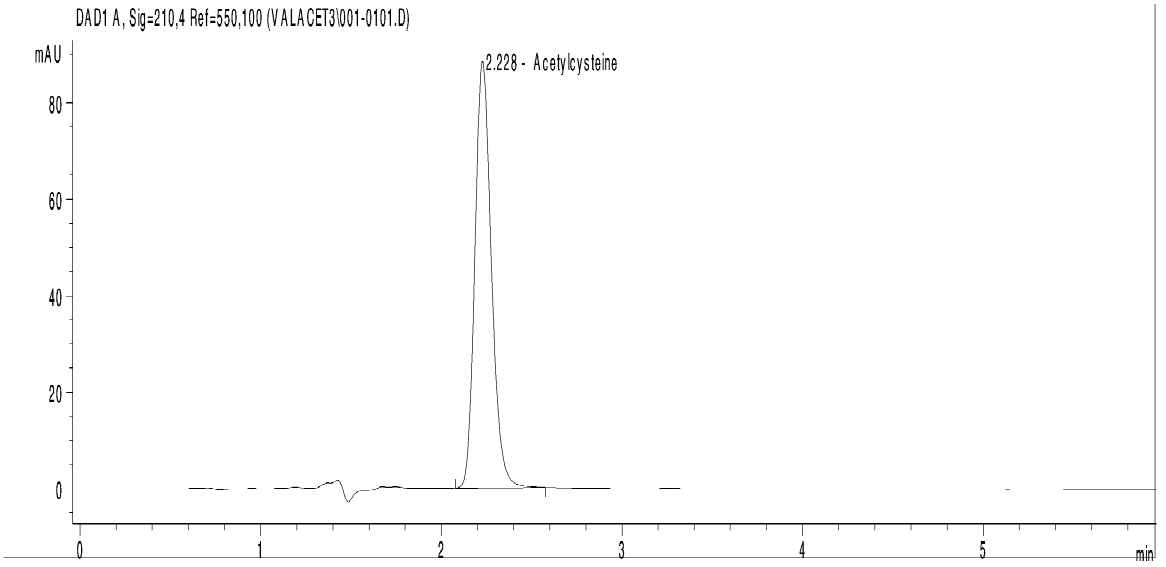

Fig. 3. UV chromatogram of *N*-acetylcysteine in standard solution (40 μg/ml) obtained by LC–UV–MS.

spikes in the reconstructed ion chromatogram. No detrimental effect on detection sensitivity was observed. The nebulizing gas pressure was investigated from 20 to 60 p.s.i. Probably for the same reasons as above, an improvement in detection sensitivity could be observed by increasing the gas pressure and 50 p.s.i. was selected as the optimum value. As expected, the drying and nebulizing gas were essential to ensure evaporation and stabilization of the spray when an aqueous mobile phase was introduced in MS. The temperature of the drying gas could be used to enhance the evaporation until it did not lead to the thermodegradation of the analytes. As several compounds had to be quantified in a single run, a compromise between optima values was selected. The reconstructed ion chromatogram of the four impurities, obtained after LC–UV–MS analysis of their standard solution, is shown in Fig. 4.

Then, the LC–UV–MS analysis of a standard solution containing the excipients was performed and compared to the analysis of *N*-acetylcysteine and the impurities under the same UV and MS conditions. No interference was observed.

N-Acetylcysteine and the impurities were finally determined in the effervescent tablets by LC–UV–MS (SIM). Fig. 5 illustrates the UV detection of *N*-acetylcysteine and the reconstructed ion chromatogram of the impurities. The use of subsequent UV and MS detection allowed the identification and the quantitation of coeluting cysteine and cystine. Moreover, *N*-acetylcysteine and the impurities could be quantified in a single run in spite of their highly different concentration levels.

3.2. Validation

3.2.1. Analytes stability

Thiol compounds are sensitive to oxidation into disulfides, particularly in solution at neutral or alkaline pH [35,43]. The stability of *N*-acetylcysteine was then investigated in standard solutions and in the reconstituted pharmaceutical form by comparing the concentrations of the same solutions before and after 1 h at daylight exposure. The solutions used in this study were 40 μg/ml aqueous solution of *N*-acetylcysteine prepared in the LC mobile phase as well as the same concentrations prepared in the reconstituted pharmaceutical form. In order to improve the stability of the thiol, the LC mobile phase was set at the acidic pH of 3.0 by mean of an ammonia/formic acid buffer. Indeed, as it is shown by the Student's *t*-test (calculated *t* values inferior to the theoretical value), the concentrations measured were not significantly different after 1 h at daylight

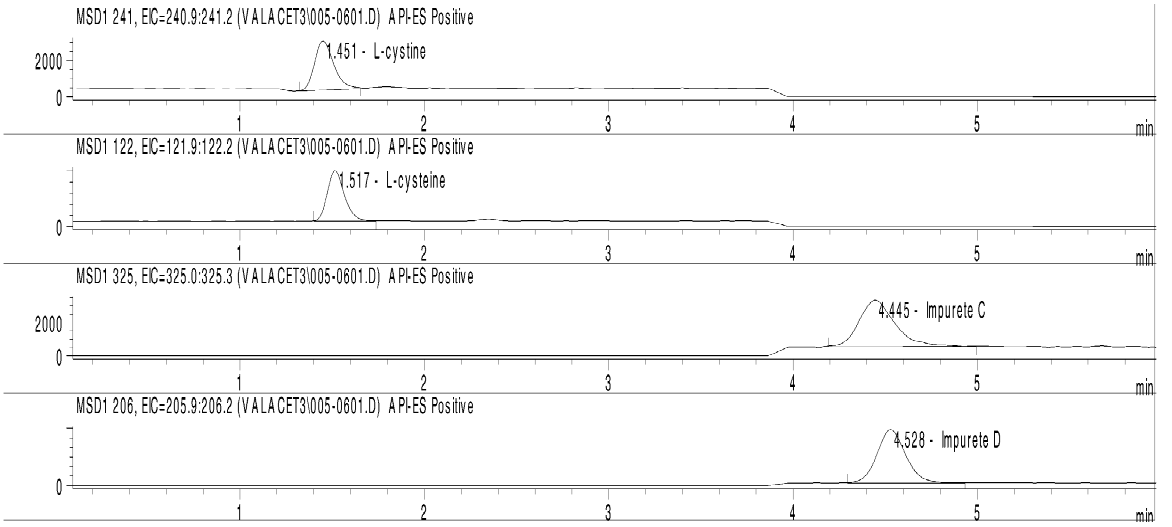

Fig. 4. SIM ion chromatogram of cysteine (pharmacopeial impurity A, m/z 122,0), cystine (pharmacopeial impurity B, m/z 241.0), *N*,*N*′-diacetylcystine (pharmacopeial impurity C, m/z 206.0) and *N*,*S*-diacetylcysteine (pharmacopeial impurity D, m/z 325.1) obtained after LC–UV–MS analysis of their standard solution at 800 ng/ml, which corresponds to 2% of impurities in the pharmaceutical formulation. The change of the baseline at 4 min is due to the change of the cone voltage passing from 50 to 40 V for the detection of impurities C and D.

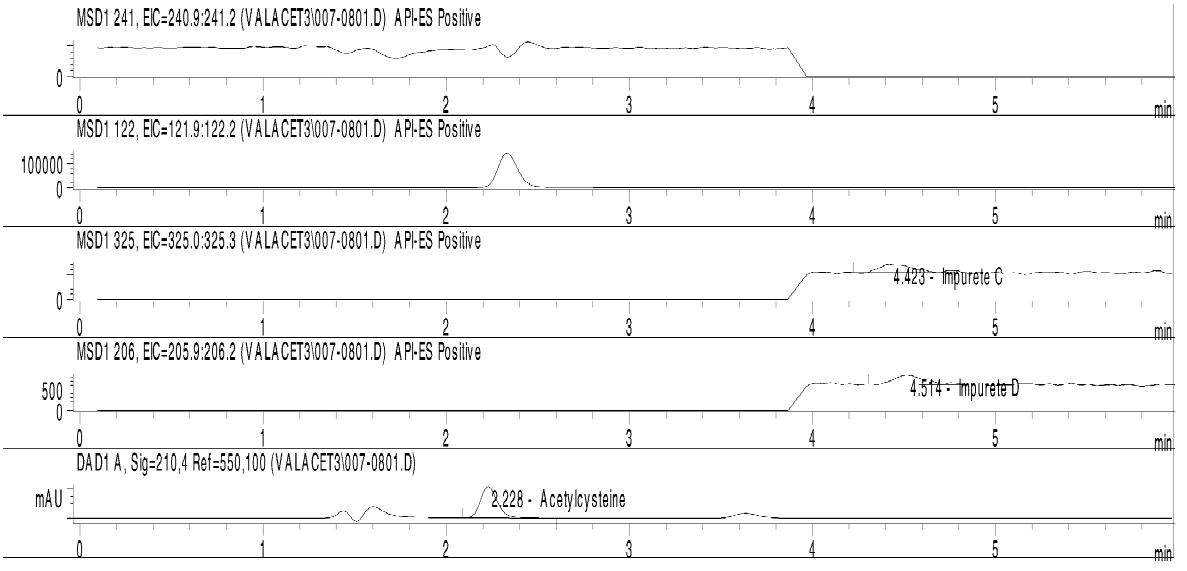

Fig. 5. SIM ion chromatogram of the impurities and UV chromatogram of *N*-acetylcysteine obtained after LC–UV–MS analysis of an effervescent tablet (the mean weight from 10 tablets at 200 mg of *N*-acetylcysteine was dissolved and diluted in the LC mobile phase so that *N*-acetylcysteine was 40 μg/ml). Only *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine were observed as impurities (<1%).

exposure, which is the time required to prepare reconstituted pharmaceutical samples (Table 1).

3.2.2. Selectivity

The selectivity of the method was demonstrated by the analysis of the excipients in standard solution and their detection in UV–MS (SIM). No interference from the excipients was observed at the retention time of *N*-acetylcysteine in UV detection, neither in the reconstructed ion chromatogram of the impurities in MS detection.

3.2.3. Linearity

Linear regression analysis of *N*-acetylcysteine (Table 1) and the pharmacopeial impurities (cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine) (Table 2) was carried out by plotting peak area (y) versus analyte concentration (x), in the 20–60 μg/ml concentration range and the 200–1200 ng/ml concentration range, respectively. The calibration curves were constructed at five concentration levels and three independent determinations ($n=3$) were performed at each concentration. The linearity of the responses as a function of the concentration was confirmed by an analysis of the variance (ANOVA) [44,45].

3.2.4. Limits of detection and quantitation

The limits of detection (LODs) and quantitation (LOQs) for *N*-acetylcysteine and the impurities were calculated from regression lines [46]. The LODs were found to be 0.68 μg/ml, 76.19 ng/ml, 70.94 ng/ml, 89.51 ng/ml, 78.58 ng/ml for *N*-acetylcysteine, cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine, respectively. The LOQs were found to be 2.28 μg/ml, 230.87 ng/ml, 214.96 ng/ml, 271.24 ng/ml, 238.13 ng/ml for *N*-

Table 1
Validation results for *N*-acetylcysteine (UV detection)

	N-Acetyl-L-cysteine
Stability: 40 μg/ml, 1 h, 20±2°C	2.20
Student's *t*-test (0.95; n_1+n_2-2) ($n=6$)	
Standard solution (calculated *t* value)	1.27
Reconstituted pharmaceutical form (calculated *t* value)	1.74
Linearity:	
Calibration range (μg/ml)	20–60
Calibration points	5
Equation	$y=20.0190x+22.7177$
Coefficient of determination (r^2)	0.9997
Accuracy (k[a]=1, n[b]=6):	
Mean recovery (%) at 20 μg/ml	100.16
Mean recovery (%) at 40 μg/ml	100.19
Mean recovery (%) at 60 μg/ml	100.44
Mean recovery±CI (%)	100.24±0.34
Repeatability ($k=3$, $n=6$; RSD, %):	
20 μg/ml	1.35
40 μg/ml	0.76
60 μg/ml	0.66
Intermediate precision ($k=3$, $n=18$; RSD, %):	
20 μg/ml	1.64
40 μg/ml	0.99
60 μg/ml	0.97

[a] k=number of days.
[b] n=number of injections.

Table 2
Validation results for *N*-acetylcysteine impurities (MS detection)

	L-Cysteine	L-Cystine	*N*,*S*-Diacetylcystine	*N*,*N*′-Diacetylcysteine
Linearity:				
Calibration range (ng/ml)	200–1200	200–1200	200–1200	200–1200
Calibration points	5	5	5	5
Equation	$y=28.1006x-1292.78$	$y=36.7534x-937.739$	$y=75.8395x-1060.80$	$y=253.058x-9373.14$
Coefficient of determination (r^2)	0.9961	0.9966	0.9959	0.9946
Accuracy ($k=1$, $n=3$):				
Mean recovery (%) at 200 μg/ml	106.06	98.42	101.32	104.6
Mean recovery (%) at 600 μg/ml	98.24	98.13	103.14	100.46
Mean recovery (%) at 1200 μg/ml	97.04	100.81	97.84	96.14
Mean recovery±CI (%)	101.08±3.29	98.26±2.33	99.54±2.50	99.18±2.84
Repeatability ($k=3$, $n=3$; RSD, %):				
200 ng/ml	2.38	4.57	4.67	1.81
600 ng/ml	1.98	4.30	2.64	1.26
1200 ng/ml	2.54	3.37	2.86	1.69
Intermediate precision ($k=3$, $n=9$; RSD, %):				
200 ng/ml	3.95	4.57	4.67	2.62
600 ng/ml	5.32	5.40	5.38	2.20
1200 ng/ml	2.99	3.37	3.40	2.96

acetylcysteine, cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine, respectively.

3.2.5. Precision

The precision of the method was determined by measuring repeatability and intermediate precision for each analyte at three different concentrations, ranging from 20 to 60 μg/ml and 200 to 1200 ng/ml for *N*-acetylcysteine and the impurities, respectively. The mean values for repeatability were 0.89, 2.41, 4.31, 3.33, 1.62% for *N*-acetylcysteine, cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine, respectively. The mean values for intermediate precision were 1.14, 4.71, 4.83, 4.14, 2.94% for *N*-acetylcysteine, cysteine, cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine, respectively.

3.2.6. Accuracy

The overall accuracy of the procedure was assessed by plotting the graph of the amount of each analyte found versus the amount spiked in the reconstituted pharmaceutical form, at six concentration levels ($n=6$) ranging from 20 to 60 μg/ml and 200 to 1200 ng/ml for *N*-acetylcysteine and the impurities, respectively. The analysis of the variance ($P>0.05$) showed that the slope of the lines was not significantly different from unity and that the lines passed through the origin. The accuracy can also be defined as mean recovery %±confidence interval ($P>0.05$). As can be seen in Table 1 for *N*-acetylcysteine and Table 2 for the impurities, the LC–UV–MS procedure can be considered accurate and linear within the concentration range investigated for each compound.

4. Conclusions

A fast, sensitive, accurate and precise method based on LC–UV–MS has been developed for the determination of *N*-acetylcysteine and its four pharmacopeial impurities in effervescent tablets prepared at 200 mg of *N*-acetylcysteine per tablet. Thanks to subsequent UV and MS detection, coeluting analytes could be identified and quantified in a single run and interference from excipients could be avoided. Limits of quantitation of 2.28 μg/ml could be obtained for *N*-acetylcysteine and between 214.96 and 271.24 ng/ml for its impurities. The method was

validated in UV detection for *N*-acetylcysteine, using pure standard solutions, and in MS detection for the impurities, using standard solutions of the impurities in mixture. Finally, the method was successfully applied to the quality control of effervescent tablets.

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