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Determination of L-lysine *N*-acetylcysteinate and its mono- and dimeric related compounds by liquid chromatography–mass spectrometry

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Abstract

Methods for the simultaneous determination of *N*-acetylcysteine, L-lysine, L-cysteine, L-cystine, *N*,*N*′-diacetylcystine and *N*,*S*-diacetylcysteine in aqueous solutions were developed. The method is based on the on-line coupling of liquid chromatography (LC) to tandem mass spectrometry (MS–MS) with an atmospheric pressure chemical ionisation (APCI) interface. The optimal LC phase system consisted of a C_{18} stationary phase and an acidic solution of formic acid in acetonitrile as the mobile phase, while D,L-phenylalanine was selected as an internal reference compound. The best transitions for MS–MS detection were selected after optimisation of the APCI ionisation yield in full scan. The optimisation was performed by loop injection. By coupling LC to MS–MS in the APCI+ (positive ion mode) as well as in the multiple reaction monitoring mode, satisfactory results were obtained with a mixture of the seven compounds, each at a concentration level of 1 μg/ml. For a further improvement of the detection limit of the dimeric compounds, electrospray ionisation was then investigated. By use of this interface a quantitation limit down to 10^{-8} *M* (3 ng/ml) could be achieved for both dimers. The developed method provides improved selectivity based on the combination of chromatography and mass spectrometry thus facilitating identification.

Keywords: Pharmaceutical analysis; Lysine acetylcysteinate

1. Introduction

Acetylcysteine is a mucolytic agent administered orally or by inhalation of a nebulised solution in the treatment of respiratory disorders associated with the production of excessive or viscous mucus [1–4]. Its usefulness against hepatotoxicity secondary to acetaminophen overdosage is also well known [5,6]. The metabolic pathway commonly proposed for *N*-acetylcysteine involves the production of cysteine, cystine and *N*,*N*′-diacetylcystine as well as the formation of labile disulfide complexes with tissue and plasma proteins [7]. *N*,*S*-Diacetylcysteine is also presented as a potential impurity of *N*-acetylcysteine synthesis [8] and can therefore be found in biological fluids.

Lysine acetylcysteinate is a new combination proposed in aerosol administration to improve the efficacy of the drug. The pharmacokinetic properties of this new association have still to be investigated in clinical assays. The methods previously reported for the determination of *N*-acetylcysteine in biological

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samples involve liquid chromatography (LC) coupled to UV spectrophotometry [9,10], fluorimetry [11,12] or electrochemical detection [13,14]. Some gas chromatographic methods have also been described [15,16].

In the last decade, hyphenated techniques such as LC coupled to mass spectrometric (MS) detection have been intensively developed and applied in bioanalysis [17–21]. The high selectivity and the sensitivity of MS detection methods associated with the resolution of LC provide decisive advantages to perform qualitative as well as quantitative analysis of a wide range of molecules at trace levels [22,23]. However, the coupling of both techniques is only possible using a suitable interface. Atmospheric pressure ionisation (API) interfaces, represented by atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI), are commonly used in LC–MS. APCI can manage LC flow-rates in the range of 1 ml/min without splitting the effluent. Moreover, it is particularly suitable for the ionisation of low-molecular-mass and non-volatile compounds [24,25]. ESI induces negligible fragmentation of the compounds compared to APCI. It is particularly suitable for the analysis of polar molecules. However, recommended flow-rates are generally lower than 50 μl/min [24–26].

In order to perform pharmacokinetic studies of L-lysine *N*-acetylcysteinate, it was first necessary to develop a method able to separate and identify this analyte as well as its related compounds at low concentrations in aqueous solutions. The method reported in the present paper involves LC with on-line MS detection using APCI as an interface. After optimisation of the LC separation with UV detection, on-line LC–MS–MS was performed using a highly specific multiple reaction monitoring mode (MRM) of analysis. The influence of various parameters such as the concentration of formic acid in the LC mobile phase, the injection volume, the APCI probe temperature or the curtain nitrogen flow-rate was investigated.

Considering the particular limitations for the detection of the dimers (L-cystine and *N*,*N*′-diacetylcystine), either due to their instability or to a weak ionisation efficiency, a modification of the method, involving electrospray ionisation, was developed for the compounds.

2. Experimental

2.1. Chemicals and reagents

N-Acetylcysteine and D,L-phenylalanine were obtained from Federa (Brussels, Belgium). L-Lysine (monohydrate) came from Synthelabo Benelux (Brussels, Belgium) whereas L-cysteine and L-cystine (dichlorohydrate) were products of Sigma (St. Louis, MO, USA). *N*,*S*-Diacetylcysteine and *N*,*N*′-diacetylcystine (disodium salt) were kindly offered by SMB Labs. (Brussels, Belgium).

Acetonitrile was of HPLC grade from Fisher Scientific (Leicestershire, UK). Water was of Milli-Q quality from Millipore (Bedford, MA, USA). Formic acid 98–100% and ammonia 25% were both of analytical grade from Merck (Darmstadt, Germany). Hydrochloric acid min. 37% was also of analytical grade obtained from Acros Organics (Geel, Belgium).

Nitrogen (99.999%) and argon were purchased from Air Liquide (Milmort, Belgium).

2.2. Instrumentation and methods

2.2.1. LC–UV

The LC system consisted of a Model HP-1100 liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted column compartment (25°C), an autosampler and a diode array detector, all from Hewlett-Packard (Palo Alto, CA, USA). Different LC analytical columns were used: a C_8 and a C_{18} Platinum EPS (Extended Polar Selectivity) column (5 μm, 150 μm×4.6 mm I.D.) from Alltech Associates (Deerfield, IL, USA) and two LiChroCart columns (5 μm, 125 mm×4 mm I.D.), one containing LiChrospher 100 RP-18, the other one with a LiChrospher 100 CN stationary phase from Merck. The mobile phase consisted of a mixture of acetonitrile and diluted formic acid (1.8 m*M*) of pH 3.0 (2:98, v/v). This mixture was degassed for 10 min in an ultrasonic bath before use. The flow-rate was 1.0 ml/min. UV detection was achieved at 197 nm, corresponding to maximum absorbance for lysine.

2.2.2. LC–APCI-MS–MS

The APCI measurements were performed under

the experimental conditions given above with the exception that a HP-1050 Model from Hewlett-Packard was used.

The mass spectrometer was a VG Quattro II from Fisons Instruments (Altringham, UK) equipped with MassLynx software. An APCI interface was used with source and probe temperatures maintained at 125 and 450°C, respectively. Corona discharge occurred at 3.5 kV and the volatised ions were accelerated by application of a 7 V cone voltage.

Argon was introduced into the collision cell (radio frequency, RF, only hexapole collision cell) at 10^{-3} mbar pressure and the MS–MS fragmentation of the pseudo-molecular ion occurred at a collision energy of 9 eV (laboratory scale). Nitrogen was used as drying and sheath gas at flow-rates of 300 and 125 l/h, respectively.

2.2.3. LC–ESI-MS

The LC system consisted of a Rheos 4000 binary pump (Flux Instruments, Karlskoga, Sweden), a vacuum degasser (degasys DG-13 10, Uni-flows, Tokyo, Japan) and a 20 μl calibrated injection loop (Rheodyne 7125, Cotati, CA, USA). A C_{18} Platinum EPS (5 μm, 150 mm×2.1 mm) analytical column from Alltech was used.

The mobile phase consisted of a mixture of acetonitrile and volatile buffer of pH 3.0 (2 m*M* formic acid/7 m*M* ammonia) in the ratio 2:98 (v/v). The flow-rate was set at 0.3 ml/min. A AC-20 Model splitter (ratio 1:20) from LC Packings (Amsterdam, Netherlands) was used after the HPLC column to divide the flow-rate before ESI-MS.

MS detection was achieved on a single quadrupole VG Platform instrument from Fisons Instruments, equipped with MassLab software. Source temperature was set at 80°C. Nitrogen was used as drying and sheath gas at flow-rates of 250 and 1.7 l/h, respectively.

2.3. Sample solutions

2.3.1. Stock solution

A stock standard solution containing *N*-acetylcysteine, L-cysteine, D,L-phenylalanine, L-cystine, L-lysine, *N*,*S*-diacetylcysteine and *N*,*N*′-diacetylcystine was prepared by dissolving appropriate amounts of each compound in 0.04 *M* HCl to give final concentrations of 0.5 mg/ml for each compound.

2.3.2. Standard solutions at 10 μg/ml and 1 μg/ml

The standard solutions were prepared by diluting the stock solution with a mixture of acetonitrile and a pH 3.0 hydrochloric acid solution (0.9 m*M*) (2:98, v/v) in order to obtain final concentrations of 1 μg/ml and 10 μg/ml for each compound.

3. Results and discussion

3.1. Selection of the liquid chromatographic conditions with UV detection

Different deactivated (C_8 and C_{18} Platinum EPS), or classical (C_{18} and CN) silica bonded phases were tested. As L-lysine *N*-acetylcysteinate and related compounds are mostly highly polar amino acids (Fig. 1a), no suitable retention was obtained on the cyano bonded phase and incomplete resolution was observed with the C_8 Platinum EPS stationary phase.

On the other hand, the compounds studied were entirely resolved on both classical and deactivated C_{18} stationary phases. However, L-lysine was only eluted after 30 min on classical C_{18} while the complete analysis of the analytes was achieved within 8 min on a C_{18} Platinum EPS stationary phase. Therefore, this phase was selected confirming its usefulness for the separation of polar molecules [27].

The composition of the mobile phase was also investigated. Studies of the influence of the mobile phase pH in the range from 2.5 to 6.0 have indicated that the best separation of the analytes was obtained at pH around 3. Only a very small percentage of organic modifier (2% acetonitrile) was necessary to obtain an adequate retention for these compounds. However, no salts were used here in order to avoid signal suppression in MS detection. In the same way, formic acid was chosen as ionisation promoting factor for these analytes which should be best detected in the positive ion mode. An acidic pH could be obtained by use of a 1.8 m*M* formic acid solution.

In order to avoid oxidation of the thiol group and

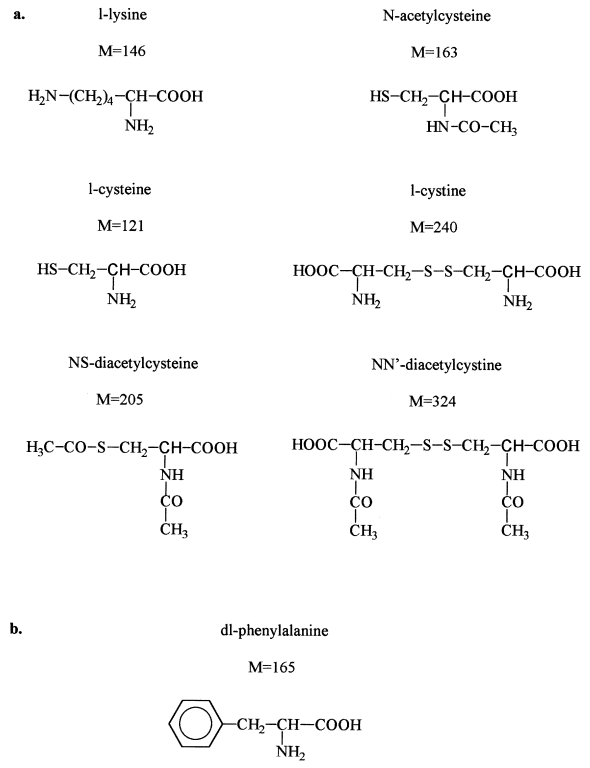

Fig. 1. (a) Structures of L-lysine *N*-acetylcysteine and its related compounds. (b) Structure of the internal reference, D,L-phenylalanine.

dimerisation of the cysteine moiety, the sample solution had to be acidified with diluted hydrochloric acid (0.9 m*M*). A satisfactory stability of the samples stored at 4°C was obtained for seven days under these acidic conditions. Fig. 2 illustrates the complete resolution within 8 min of the seven analytes except cysteine and cystine. The latter two compounds show a similar chromatographic behaviour but they could be completely resolved by MS detection. In addition, since L-lysine has very low UV absorbance properties, MS detection should also be useful to improve the detection sensitivity for this compound.

3.2. Optimisation of the mass spectrometric conditions

Each compound was first directly introduced in mass spectrometry (MS1) using APCI ionisation and parameters such as the source temperature, the corona discharge and the cone voltage were individually optimised. Considering the acidic conditions, the positive ion mode with detection of pseudo-molecular ions $[M+H]^+$ was selected. Fig. 3 illustrates the *N*-acetylcysteine mass spectrum.

MS–MS of each compound was then performed, producing fragmentation of the pseudo-molecular ions (i.e., parent ions). Parameters such as the argon pressure and the collision energy had to be optimised as well. Daughter ion spectra of each compound were recorded and filiations were confirmed by processing parent ion spectra of each fragment. Following those experiments, fragmentation reactions were selected which generated intense and high mass ionic species in order to improve the overall sensitivity (cf. Table 1).

3.3. Choice of a reference compound

D,L-Phenylalanine was selected as an internal reference (Fig. 1b). Due to its amino acid structure, it showed a chromatographic behaviour similar to that of the analytes. Owing to its phenyl group, it was eluted in a interference free region of the chromatogram. Moreover, a more selective detection was obtained for this compound in MS due to its relatively high molecular mass.

3.4. Coupling of LC to APCI-MS–MS

LC–APCI-MS–MS analysis were performed under the optimum conditions described in Section 2.2.2. After LC separation, the compounds were detected in the MRM mode. This detection is based on the transition involving a pseudo-molecular ion and a specific daughter ion. Individual transitions providing the best detection selectivity together with a high signal-to-noise ratio were followed for each compound.

Different parameters were investigated which could affect the sensitivity of the method. The formic acid concentration in the mobile phase plays a critical part in the ion formation mechanism in MS. A slight improvement in the symmetry of the chromatographic peaks could be observed with formic acid at the selected concentration (1.8 m*M*), compared with a mobile phase buffered with another acid. However, concentrations of formic acid higher

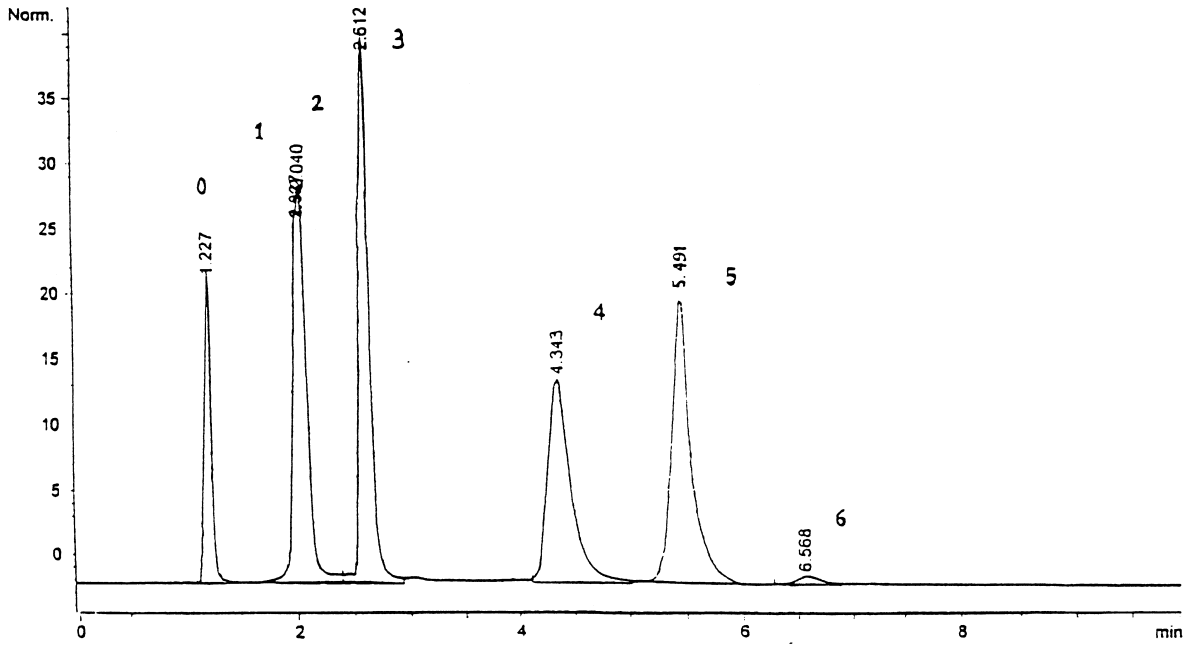

Fig. 2. HPLC–UV analysis of *N*-acetylcysteine and its related compounds: 1=L-cystine, 2=L-cysteine, 3=*N*-acetylcysteine, 4=*N*,*N*′-diacetylcystine, 5=*N*,*S*-diacetylcysteine, 6=L-lysine. Sample solution: 10 μg/ml. Detection wavelength: 197 nm. See text (Section 2.2.1) for other chromatographic conditions.

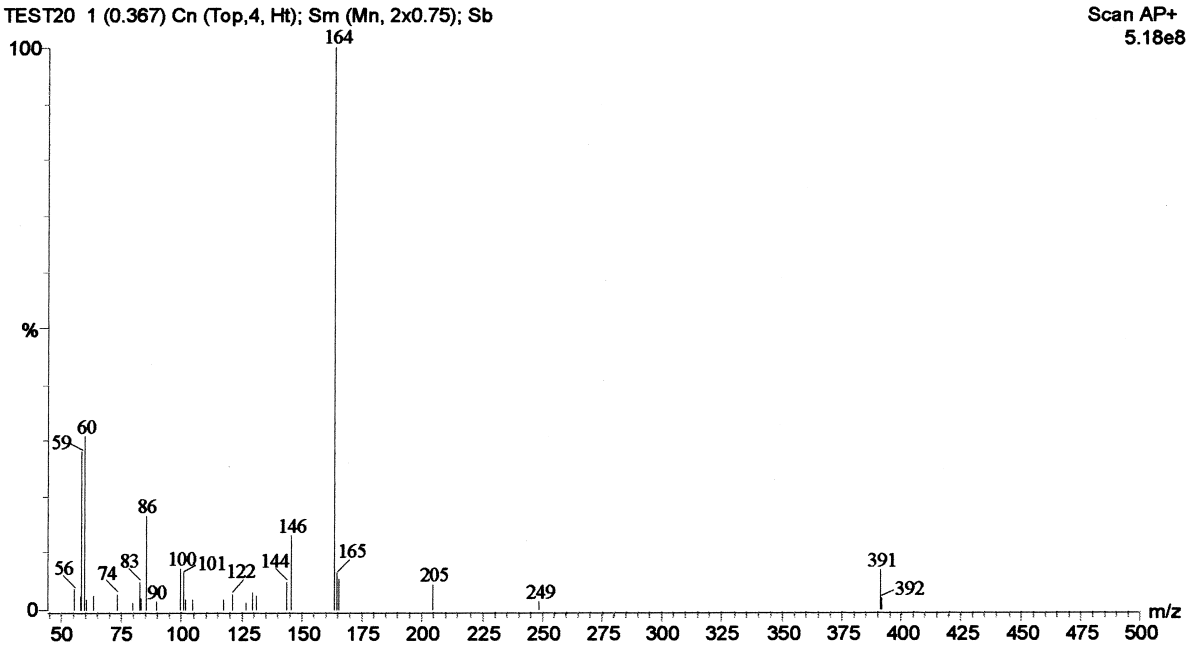

Fig. 3. Direct introduction of *N*-acetylcysteine (1 mg/ml) in positive APCI-MS: mass spectrum illustrating the pseudomolecular ion *m*/*z* 164 and its deacylated fragment *m*/*z* 122 (cysteine).

Table 1
Daughter ions and fragmentation reactions followed in MRM mode during LC–positive APCI-MS–MS analysis of the sample

Compounds	Molecular ion MH+	Daughter ion M′H+	Fragmentation reaction
N-Acetylcysteine	164	122	Deacetylation
L-Lysine	147	130	Dehydration
L-Cysteine	122	76	Decarboxylation
L-Cystine	241	152	Decarboxylation
N,*S*-Diacetylcysteine	206	164	Deacetylation
N,*N*′-Diacetylcystine	325	162	Disulfide bond breakage
D,L-Phenylalaine	166	120	Decarboxylation

Conditions: APCI probe temperature: 450°C. Corona discharge: 3.5 kV. Source temperature: 120°C. Cone voltage: 7 V. Argon pressure: 10^{-3} mbar. Collision energy: 9 eV. Drying gas (N_2): 300 l/h. Sheath gas (N_2): 125 l/h.

than 2 m*M* were detrimental to the detection sensitivity. The ionisation was found to vary significantly from a monomeric component to the corresponding dimer and the ionisation of the dimeric compounds seemed to be particularly delicate. The influence of the APCI probe temperature, set in a range from 400 to 600°C, was also investigated. Since the selected mobile phase was mostly aqueous and the analytes had limited volatility, a temperature of 450°C was necessary to produce sufficient vaporisation of the chromatographic effluent inside the source. However, thermal degradation of all compounds was observed at 600°C.

Different flow-rates of nitrogen used in the probe as drying gas (promoting solvent evaporation) and sheath gas (protecting the sample from thermal degradation) were also tested. An increase of the sheath gas flow-rate from 75 to 125 l/h resulted in better sensitivity whereas the drying gas flow-rate seemed to have no significant influence on detection and was set at an usual value of 300 l/h. This LC–APCI-MS–MS method has permitted the separation and identification of L-lysine *N*-acetylcysteinate and its related compounds at 1 μg/ml concentration. Fig. 4 illustrates the MRM ionic traces collected during such an analysis.

However, the 1 μg/ml concentration (about 10^{-5} *M*) corresponds in fact to the detection limit for the dimers (L-cystine and *N*,*N*′-diacetylcystine) while monomers can still be easily detected. This particular limitation could be due to the instability of the disulfide bond of the dimers during ionisation and/or to the weakness of their ionisation efficiency.

3.5. Electrospray ionisation

In order to improve the detection limit for the dimers, ESI was also investigated since this technique is known to achieve a softer ionisation process compared to APCI.

3.5.1. Adaptation of LC conditions

The LC flow-rate should in principle be limited to 50 μl/min with the electrospray interface in order to produce an adequate spraying of the sample into the ion source. The LC conditions were therefore adapted to the use of a 2.1 mm I.D. analytical column.

However, a split had still to be used between the LC column and the electrospray interface in order to reduce the flow-rate of the effluent down to 15 μl/min before entering the MS detector.

In addition, it was found that, with this narrow-bore LC column, it was necessary to use a sufficient buffer capacity in order to avoid detrimental effects of the sample composition on the LC separation. Commonly recommended buffer additives in LC–MS are volatile compounds such as trifluoroacetic acid (0.01%), acetic or formic acid (0.1%) and ammonia/acetic or formic acid (0–10 m*M*/0–20 m*M*) mixtures in the pH 3–10 range [28–31]. Referring to the possible acetylation of some ana-

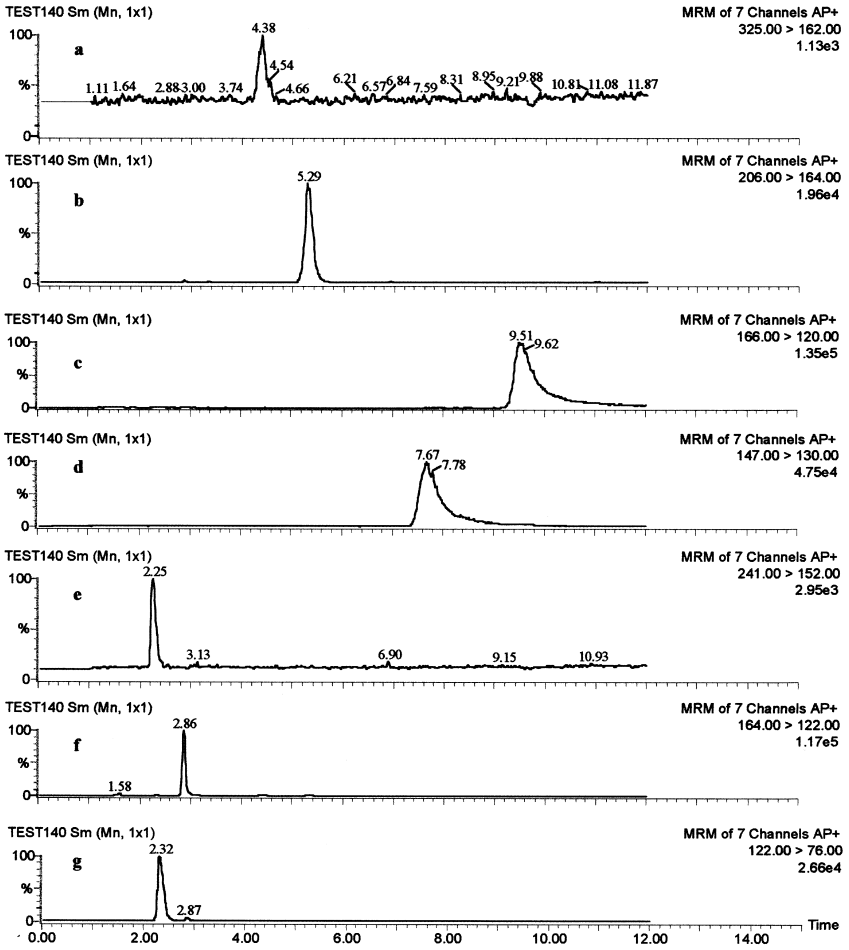

Fig. 4. MRM ion chromatograms obtained after LC–APCI-MS–MS analysis of L-lysine *N*-acetylcysteinate and its derivatives. Sample solution: 1 μg/ml. Other conditions are specified in Section 2.2.2. (a) *N*,*N*′-Diacetylcystine, (b) *N*,*S*-diacetylcysteine, (c) D,L-phenylalanine, (d) L-lysine (e) L-cystine, (f) *N*-acetylcysteine, (g) L-cysteine.

lytes and to the critical influence of formic acid on sensitivity, an ammonia/formic acid buffer (7 m*M*/2 m*M*) at pH 3.0 was finally selected.

3.5.2. Selection of ESI conditions and on-line coupling to LC

Optimal ionisation conditions for the compounds were selected after direct introduction of each of them in ESI-MS. Special attention was paid to the detection of *N*,*N*′-diacetylcystine.

Different cone voltage values were investigated to enhance the intensity of the pseudo-molecular ions.

A comparison of the spectra recorded both in positive and negative ion modes indicated that positive spectra are simpler with more easily identified fragment ions. Moreover, [M+36] adducts were observed in the negative ion mode due to the presence of chlorides in the sample. This could be confirmed by the presence of [M+38] isotopic ions with the expected isotopic ratio (MI[M+2]: 3/1). In positive ESI, filiations were found identical to those

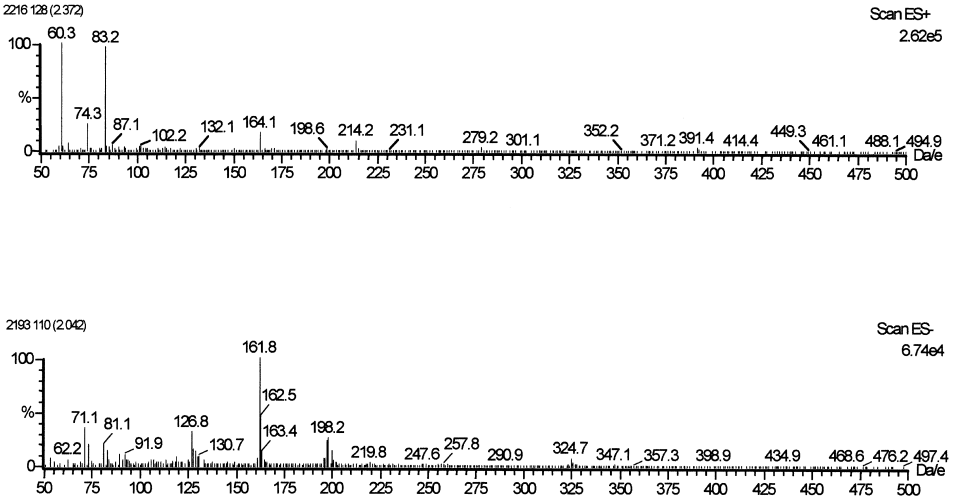

Fig. 5. Direct introduction of *N*-acetylcysteine at 0.1 mg/ml in ESI-MS (cone voltage=12 V): (a) positive ion mode, (b) negative ion mode.

observed with a positive APCI interface. Fig. 5 illustrates the mass spectra obtained by direct introduction of *N*-acetylcysteine in ESI-MS, either in positive (Fig. 5a) or negative (Fig. 5b) ion mode.

Because *N,N′*-diacetylcystine was only commercialised as disodium salt, the negative ion mode was more suitable for the detection of the pseudo-molecular ion. This also allowed to avoid the generation of bad looking overcrowded spectra due to the presence of multiple sodium adducts. The injection of *N,N′*-diacetylcystine at 10^{-6} and 10^{-8} *M* concentrations illustrates the sensitivity of detection reached in MS analysis (cf. Fig. 6).

The on-line coupling of LC to ESI-MS was finally performed using a flow-rate splitter. The seven compounds were analysed simultaneously in single ion reaction mode (SIR) improving the selectivity of detection (cf. Fig. 7).

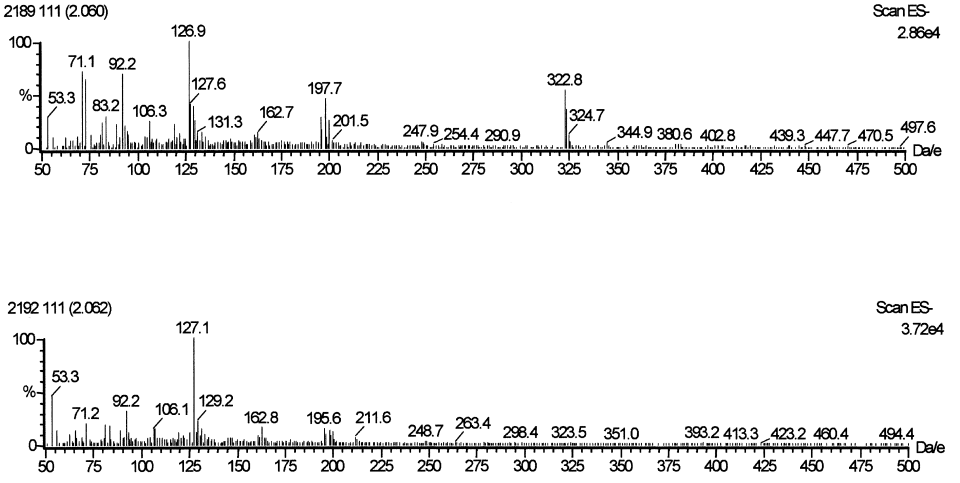

Fig. 6. Direct introduction of *N,N′*-acetylcysteine in ESI-MS in the negative ion mode (cone voltage=12 V): (a) concentration=10^{-6} *M*, (b) concentration=10^{-8} *M*.

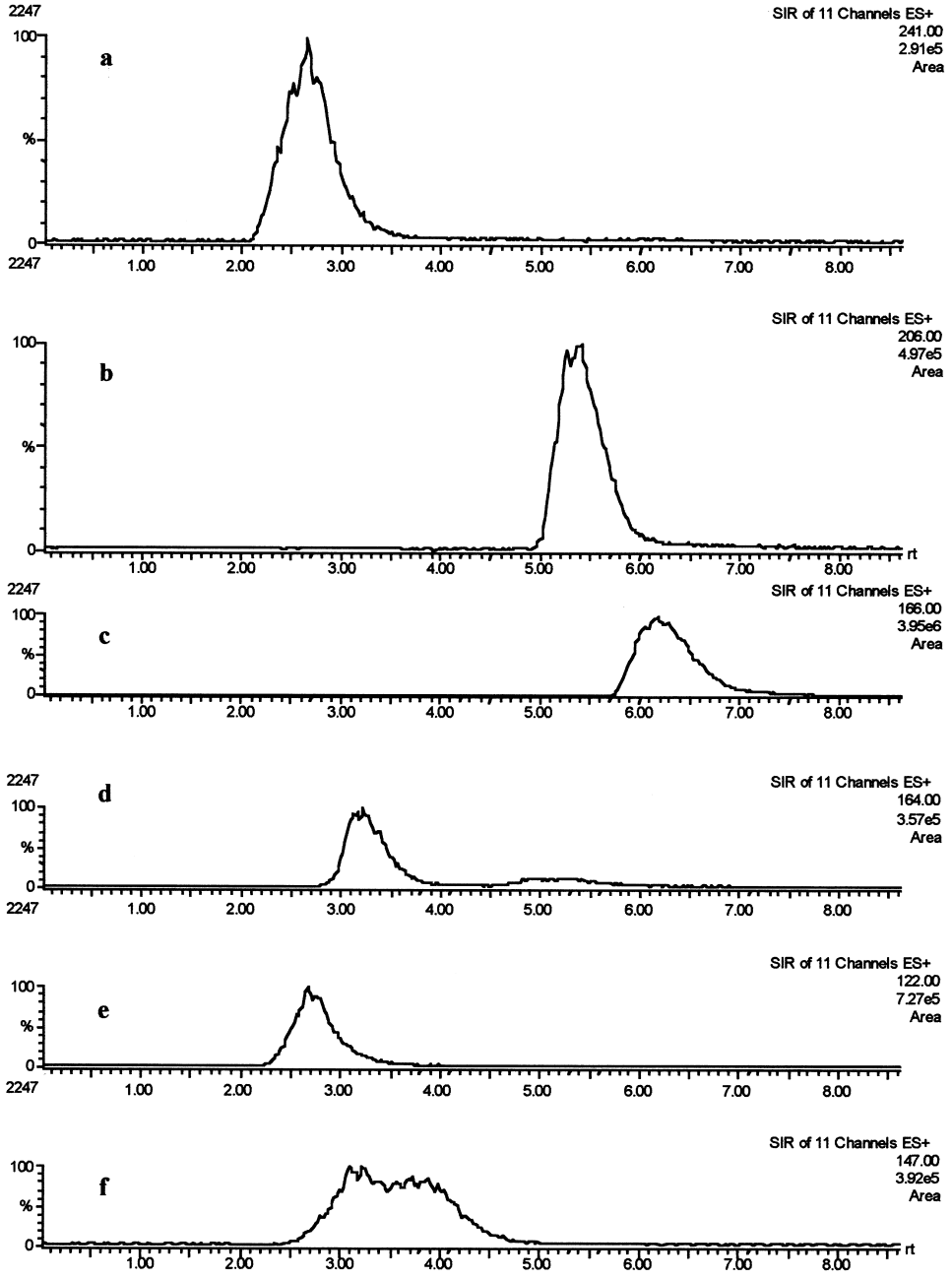

Fig. 7. SIR ion chromatograms obtained after LC–ESI-MS analysis of L-lysine *N*-acetylcysteinate and its derivatives using a buffered mobile phase. Sample solution: 0.1 μg/ml. Mobile phase: formic acid/ammonia (2 m*M*/7 m*M*) at pH 3.0. Other conditions are specified in Section 2.2.3. (a) L-Cystine, (b) *N*,*S*-diacetylcysteine, (c) D,L-phenylalanine, (d) *N*-acetylcysteine, (e) L-cysteine, (f) L-lysine.

4. Conclusions

A highly selective method based on direct coupling of LC to MS has been developed for the simultaneous determination of L-lysine *N*-acetylcysteinate and its related compounds. The seven compounds were separated and identified within 12 min using LC–APCI-MS–MS. Dimeric compounds presented a limited detectability either due to their lability or to the weakness of their ionisation efficiency. They were detected down to 1 μg/ml (10^{-5} *M*). The selectivity of the method, which is of crucial importance for further biological applications, was optimised as well.

ESI appeared to be an interesting alternative to APCI particularly for the dimers as it considerably improves their detectability, a 10^{-8} *M* detection limit being reached for *N*,*N*′-diacetylcystine.

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