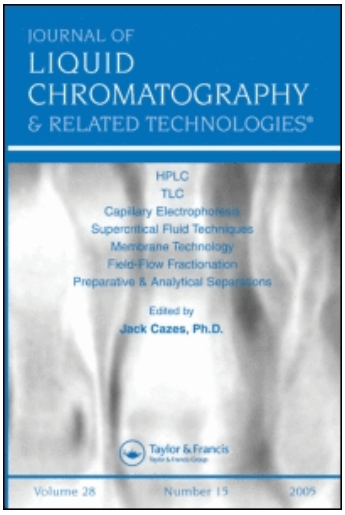

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Chromatographic Determination of Thiols After Pre-column Derivatization with *o*-Phthalaldehyde and Isoleucine

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ABSTRACT

The reaction of primary amines with excess *o*-phthalaldehyde (OPA) and thiol yields unique isoindole derivatives that are readily separated by reversed-phase liquid chromatography. In a previous work, a spectrophotometric procedure was proposed for the assay of *N*-acetylcysteine by derivatization with OPA and isoleucine at pH 9.5, with satisfactory results. The chromatographic determination of this and other low molecular-weight thiols, after isoindole formation with isoleucine, using mobile phases of acetonitrile–water at pH 3 and spectrophotometric detection, is now examined. From the assayed thiols (thioglycolic acid, 3-mercaptopropionic acid, tiopronin, *N*-acetylcysteine, *N*-acetylpenicillamine,

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glutathione, captopril, cysteamine, cysteine, and penicillamine), the latter three did not seem to form isoindole derivatives measurable at 335 nm. The factors affecting the formation and stability of the derivatives were examined. Glutathione, tiopronin, and captopril produced double peaks, whereas *N*-acetylcysteine, *N*-acetylpenicillamine, 3-mercaptopropionic acid, and thioglycolic acid gave rise to single peaks at reasonably low times, using a mobile phase containing 40% (v/v) acetonitrile at pH 3. The procedure was applied to the chromatographic determination of *N*-acetylcysteine in pharmaceutical formulations with good results.

Key Words: Thiols; Isoindole formation; *o*-Phthalaldehyde; Reversed-phase liquid chromatography.

INTRODUCTION

The thiol group is widely distributed in living cells, as part of proteins or in independent molecules. This group is also found in many drugs. The analysis of thiol-containing compounds is thus of interest in several fields, as biochemistry, clinical control, and pharmaceutical analysis. Derivatization of thiols is usually needed to achieve more stable compounds with better detectability and separation characteristics. The derivatization reagents require two features: a functional group reactive towards the thiol group, and a detectable group upon reaction with a high response. Several reagents have been reviewed with respect to their reactivity, selectivity, spectroscopic properties, and applicability to high-performance liquid chromatography of thiol-containing compounds.[1]

One of the most popular reagents in chromatographic analysis used for the derivatization of primary amino functions, such as amino acids and polyamines, is *o*-phthalaldehyde (OPA). It forms 1-alkylthio-2-alkyl-substituted isoindoles in the presence of a thiol (Fig. 1).[2] The isoindole formation with aminoethanol, tryptophan, and taurine has been also applied in both pre-[3] and post-[4,5]column derivatization of several thiols (*N*-acetylacysteine,

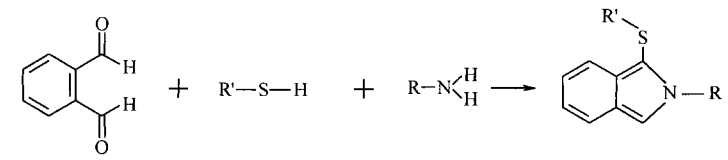

Figure 1. Isoindole formation.

cysteine, glutathione, 2-mercaptoethanesulphonic acid, 2-mercaptoethanol, 3-mercaptopropionic acid, mercaptosuccinic acid, methanethiol, monothioglycerol, thioglycolic acid, and thiolactic acid).

The thiol structure affects the formation rate and stability of the isoindole derivatives. For this reason, an extensive group of thiols has been examined in order to achieve rapid and quantitative formation, and adequate stability of the derivatives for the analysis of primary amino acids.[6] Among the studied thiols, *N*-acetylcysteine and 3-mercaptopropionic acid have shown the best characteristics. They form highly stable derivatives with amino acids, and do not require a strict control of the reaction time.[7,8]

In previous work, we proposed a simple spectrophotometric procedure for the assay of *N*-acetylcysteine by derivatization with OPA and isoleucine at pH 9.5.[9,10] In this work, we check the performance of isoindole formation in the presence of isoleucine, for the chromatographic determination of *N*-acetylcysteine and other low molecular-weight thiols (*N*-acetylpenicillamine, captopril, glutathione, 3-mercaptopropionic acid, thioglycolic acid, and tiopronin). The procedure is applied to the determination of *N*-acetylcysteine in pharmaceutical formulations.

EXPERIMENTAL

Reagents and Columns

Aqueous 10^{-3} M stock solutions of the following thiols were prepared: *N*-acetylcysteine (Fluka, Buchs, Switzerland), *N*-acetylpenicillamine, captopril, cysteamine, glutathione, and tiopronin (Sigma, St. Louis), thioglycolic acid, 3-mercaptopropionic acid, and L-penicillamine (Aldrich, Steinheim, Germany), and L-cysteine (Guinama, Valencia, Spain) (see Fig. 2 for chemical structures).

The thiols were derivatized with OPA (99%, Fluka) and isoleucine (Guinama). A 2.5×10^{-3} M OPA solution was prepared by dissolving the reagent in a small amount of ethanol (Merck, Darmstadt, Germany), and dilution with 0.1 M boric/borate buffer at pH 9.5, obtained from boric acid (99.5%, Probus, Barcelona, Spain) and sodium hydroxide (AnalaR, Poole, Great Britain). Isoleucine was dissolved in a few drops of 1 M HCl (Panreac, Barcelona) and diluted to get a 1.25×10^{-2} M solution. All reagents were of analytical grade. Nanopure water was used to prepare all solutions (Barnstead, Sybron, Boston, MA), which were protected from light by covering them with aluminum foil and kept at 4°C.

The aqueous–organic mobile phases were prepared with acetonitrile (HPLC grade, Scharlab, Barcelona) and 1×10^{-2} M citric acid monohydrate

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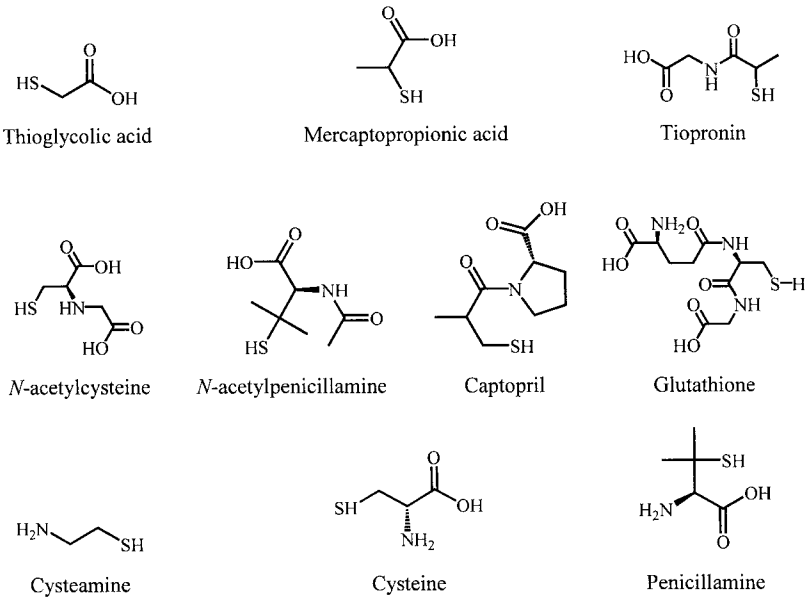

Figure 2. Chemical structures of the studied thiols.

(analytical grade, Panreac, Barcelona). The mobile phase pH was adjusted with 0.1 M sodium hydroxide in the range 3–5. Mobile phases and the solutions of the OPA–isoleucine derivatives were filtered through 0.45 μm membranes of 47 and 17 mm diameter, respectively (Micron Separations, Westboro, MA).

A Kromasil C18 column (5 μm particle size, 150 × 4.6 mm i.d., Análisis Vínicos, Ciudad Real, Spain) was used, which was protected with a Nucleosil ODS pre-column (30 × 4 mm i.d., Scharlab).

Apparatus

An HP 8452A diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA), provided with a 1-cm optical path quartz cell, was used for the direct measurement of absorbance. Chromatographic separations were carried out with an Agilent system, equipped with an automatic sampler, an isocratic pump (Model HP 1100, Palo Alto, CA), and a UV–VIS detector (Model HP 1050), connected to a PC through an integrator (Model HP 2296A). The signals were processed with the PEAK-96 software (Agilent, Avondale, PA).

Procedures

Derivatization

The spectrophotometric and chromatographic analysis of thiols were carried out after derivatization with 10 mL of the OPA/buffer mixture and 2 mL of isoleucine, and dilution to 25 mL with water. The solutions were stored in darkness during 5–40 min to get the maximal signal, the waiting time depending on the thiol nature (the derivatives of *N*-acetylcysteine and 3-mercaptopropionic acid were formed in only 1–5 min). Maximal absorbance was achieved at 335 nm, which was selected as detection wavelength in the chromatographic analysis.

Analysis of Pharmaceutical Formulations of *N*-Acetylcysteine

Formulations marketed as tablets, powder, and syrup, were analyzed. For the analyses, three effervescent tablets were weighed, ground to fine powder, and homogenized. Five portions were taken and weighed, dissolved, and diluted with water to an adequate concentration, to be injected into the chromatograph. The solutions were stirred to eliminate CO_2. For the powder, the dissolved contents of one sachet, and for the syrup an aliquot of 1 mL, were transferred to a volumetric flask, and diluted with water. Five powder sachets and five syrup aliquots were processed, independently, in the same way. The excipients were soluble in water, except for Fluimucil (powder), which had to be filtered before injection. The derivatization procedure described above was then followed.

RESULTS AND DISCUSSION

Formation and Chromatographic Separation of Isoindoles

The derivatization reaction for thiols requires the selection of an adequate amine. Nakamura et al.[11] proposed the use of tryptophan for this purpose. However, we found that the blank solution (a mixture of OPA and tryptophan) was highly colored (yellow to red depending on the concentration of OPA), and its absorbance increased with time.[9] In addition, the absorbance of tryptophan isoindoles decreased gradually, indicating that the derivatives were unstable. In contrast, isoleucine showed a low reactivity towards OPA in the absence of thiols when compared to other amino acids.[9] Also, as shown below, the isoleucine isoindoles for different thiols are stable enough to be useful in chromatography.

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The suitability of isoindole formation to monitor thiols of different nature was first examined. The study included 10 thiols showing diverse functional groups, being the absence or presence of an amine group (primary, secondary, or tertiary) the most relevant feature (Fig. 2). The thiols were reacted with OPA and isoleucine at pH 9.5, which is the recommended pH for these determinations.[12] The final concentration of thiols was 1×10^{-4} M, except for thioglycolic acid (2×10^{-5} M), which formed a derivative with a higher molar absorptivity than the other studied thiols. The concentration for both OPA and isoleucine was 1×10^{-3} M in all cases.

After a reaction time of about 20 min, the absorption spectra of the thiol–OPA–isoleucine mixtures were obtained, measuring them against a water blank. The formation of an absorption band with maximum at 330–340 nm, which is characteristic of isoindoles, was expected. Among the assayed thiols, those showing a primary amine in the molecule (cysteamine, cysteine, and penicillamine) did not produce the prominent band. Therefore, the study was further continued with the other seven thiols (thioglycolic acid, 3-mercaptopropionic acid, tiopronin, *N*-acetylcysteine, *N*-acetylpenicillamine, glutathione, and captopril).

The chromatographic separation of isoindoles was performed with acetonitrile–water mobile phases at the pH values 3.0, 3.5, 4.0, and 5.0. Figure 3 shows the chromatogram of two mixtures of the isoindoles, using 40% (v/v) acetonitrile at pH 3. *N*-Acetylcysteine, *N*-acetylpenicillamine, thioglycolic acid, and 3-mercaptopropionic acid gave rise to a single peak at different retention times (6.7, 10.1, 16.9, and 24.0 min, respectively), whereas the peaks of glutathione, tiopronin, and captopril, were split into a double peak at the assayed experimental conditions (1.3/2.0, 9.2/9.6, and 31.3/32.5 min, respectively). These peaks may correspond to the diastereomers. The resolution of such enantiomeric thiols-containing compounds (e.g., captopril), after applying a derivatization procedure, has been reported.[13]

The retention of the different derivatives correlates rather satisfactorily with the polarity of the thiols, measured as octanol–water partition coefficients ($\log P_{o/w}$, calculated values are given): glutathione (−5.41), *N*-acetylcysteine (−0.66), tiopronin (0.21), *N*-acetylpenicillamine (−0.66), thioglycolic acid (0.03), 3-mercaptopropionic acid (0.52), and captopril (0.84).

Using 40% acetonitrile mobile phases, the retention time of the glutathione isoindole (the least retained) was almost constant with pH, being close to 2 min. For the other thiols, the retention depended strongly on pH, showing a large drop at pH > 4. At pH 5 the peaks eluted close to the dead time. Two examples of this dependence are the *N*-acetylcysteine isoindole (which eluted at 6.9, 2.3, and 1.9 min at pH 3, 4, and 5, respectively), and the captopril isoindole (the most retained, eluting at 32.4, 10.2, and 2.4 min,

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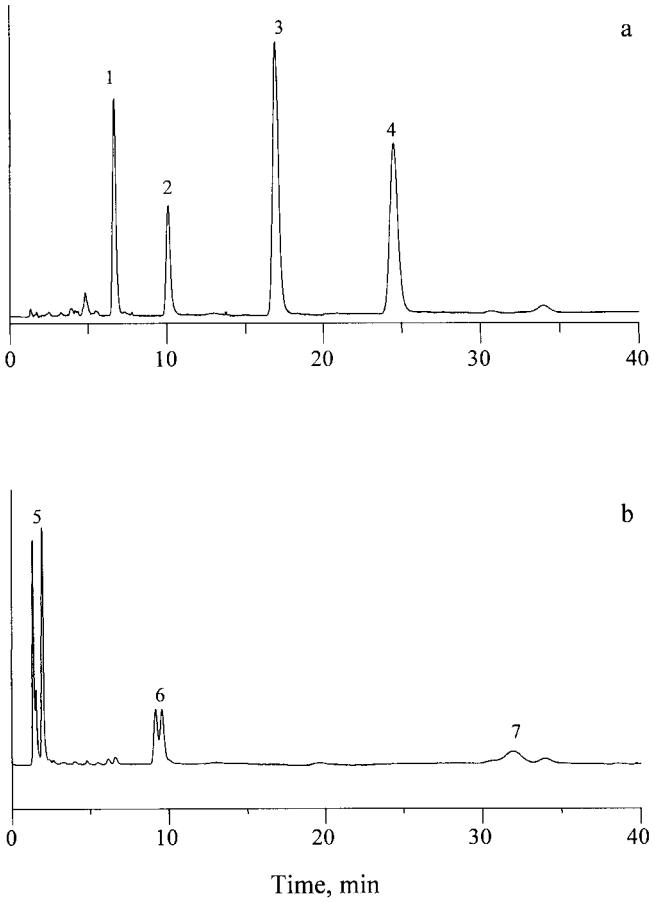

Figure 3. Chromatograms of isoindole-forming thiols using OPA and isoleucine. Compounds: 1, *N*-Acetylcysteine; 2, *N*-acetylpenicillamine; 3, thioglycolic acid; 4, 3-mercaptopropionic acid; 5, glutathione; 6, tiopronin; and 7, captopril. Mobile phase: 40% acetonitrile at pH 3.

at the same pH values). It can be concluded that the pH of the mobile phase should be fixed in the 3–4 range.

At the selected separation conditions (40% acetonitrile at pH 3), the thiols were resolved satisfactorily, including *N*-acetylpenicillamine and tiopronin, which showed close retention times. Achievement of more practical retention times for mixtures containing the seven assayed thiols would require gradient

elution. The individual analysis of the most retained thiols (thioglycolic acid, 3-mercaptopropionic acid, and captopril) should be performed preferably with a stronger mobile phase (with a higher acetonitrile content). It should be noted, however, that the screening of the whole set of studied thiols lacks interest.

Factors Affecting the Formation of Isoindoles

The examined factors were the formation time, stability, OPA/thiol, and isoleucine/thiol concentration ratios. Parallel studies were made, on the one hand, measuring directly (without separation) the absorbance of the derivatives solutions, and on the other, the heights of the chromatographic peaks obtained with a 40% acetonitrile mobile phase at pH 3. The derivatives solutions were protected from light from its formation to its detection.

First, the change in absorbance of isoindoles (obtained with a diode array spectrophotometer) was monitored at varying reaction time. For this study, 100 mL of each derivative solution was prepared, and aliquots were taken at different times to follow the signal evolution. The solution was kept in darkness during the whole experience. The preparation of the derivatives and conditioning of the spectrophotometer cell required about 2 min. The signal was measured as soon as possible and the monitoring was extended for a period of 3–5 hr [Fig. 4(a)]. The instrument was a single beam spectrophotomer. Since the absorbance of the OPA–isoleucine blank was observed to increase considerably with time (it was stabilized only after 3 hr from mixing the reagents to $A = 0.1$ for 1×10^{-3} M OPA and isoleucine), the depicted signals were achieved as the difference between two values: the absorbance of the thiol–OPA–isoleucine solution and the OPA–isoleucine blank, both measured against water. The changing residual absorbance of the blank made the perfect subtraction of the absorbance of both solutions difficult. However, the formation time of isoindoles was apparently below 30 min, and the signals were stable at least during the measured time (3–6 hr).

The stability of the isoindoles was also examined through chromatographic monitoring. For this purpose, 20 μL of a solution of each thiol isoindole was injected in minimal intervals of 20–60 min during 4–6 hr. During this period, aliquots of the solutions were kept in darkness in the chromatographic vials. Taking into account that the spectrophotometric study suggested that the formation of some isoindoles needed 20–40 min to reach the stability, their injection was made after this formation time. The abscise axis in Fig. 4(b) corresponds to the injection time; the retention time should be, thus, added to consider the whole formation time (it should be recalled that the thiol derivatives eluted in the 1–30 min range with the assayed mobile phase). It may be

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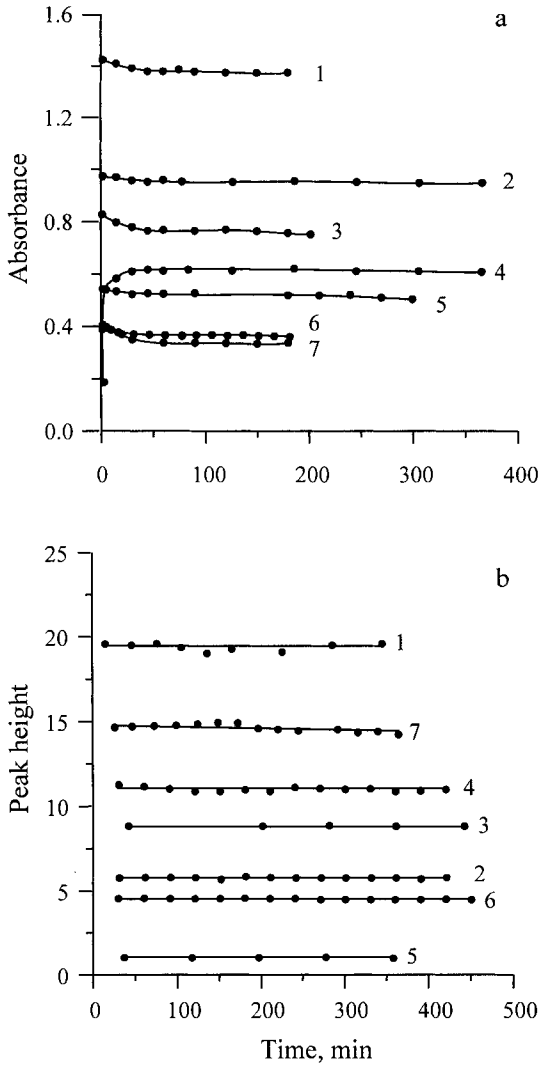

Figure 4. Formation and stability of isoindoles of several thiols, using 1×10^{-3} M OPA and isoleucine and: (a) direct measurement of the absorbance, or (b) peak height measurement after chromatographic separation. The concentration of thiols in (a) was 1×10^{-4} M, except for thioglycolic acid (2×10^{-5} M); and in (b) 5×10^{-5} M, except for captopril and glutathion (1×10^{-4} M). Compounds: 1, 3-mercaptopropionic acid; 2, thioglycolic acid; 3, captopril; 4, glutathione; 5, *N*-acetylcysteine; 6, *N*-acetylpenicillamine; and 7, tiopronin.

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observed that the signals of isoindoles, obtained once the reagents (OPA and isoleucine) are separated, were completely stable.

The effect of the OPA/thiol and isoleucine/thiol concentration ratios on the isoindole signal was studied using *N*-acetylcysteine as probe compound. This thiol shows very favorable characteristics with respect to the isoindole formation rate and stability. Figures 5 and 6 show the signal variation at increasing reagent/thiol molar ratio for OPA and isoleucine, respectively. The signal was measured again either directly, or after the chromatographic separation. In the spectrophotometric and chromatographic studies, *N*-acetylcysteine concentration was 3.3×10^{-5} and 2.6×10^{-5} M, respectively.

In the first series, the isoleucine concentration was kept constant at 1×10^{-3} M and the OPA concentration was modified in the OPA/thiol molar ratio range 0.25–50. In the second series, the OPA concentration was 6×10^{-4} M and isoleucine was varied in the range isoleucine/thiol 0.5–60. Figures 5(a) and 6(a) depict the change in the absorbance of thiol–OPA–isoleucine and OPA–isoleucine (blank) solutions. The increase in the blank signal at higher concentration of the reagents is remarkable, especially for OPA. An incomplete subtraction of the blank signal may be the reason of the absorbance decrease observed in Fig. 5 at the higher OPA concentrations. It should be considered that both solutions (thiol–OPA–isoleucine and OPA–isoleucine) were prepared and measured independently, and that the most concentrated OPA solutions are more susceptible to error. Again, this problem was eliminated with the chromatographic separation.

Finally, a calibration curve was prepared for each thiol after its derivatization with OPA–isoleucine, using in all cases a 1×10^{-3} M solution of both OPA and isoleucine. The thiol concentration covered in the calibrates were in the ranges $1–4 \times 10^{-5}$ to $0.5–2.3 \times 10^{-4}$ M. The responses were linear at least in the assayed ranges for all thiols, with typical intercepts of $A = 0.01$ in the spectrophotometric measurement. The molar absorptivities are given in Table 1 for each thiol isoindole, together with the correlation coefficient of the linear fittings, which were satisfactory for both spectrophotometric and chromatographic measurements.

Analysis of Pharmaceutical Formulations

The chromatographic determination of thiols previous derivatization with OPA and isoleucine was applied to the control of pharmaceutical formulations containing *N*-acetylcysteine. This compound is a mucolytic agent widely used in the treatment of respiratory disorders. It is also useful against hepatotoxicity produced by acetaminophen overdosage, and has been found to be effective in

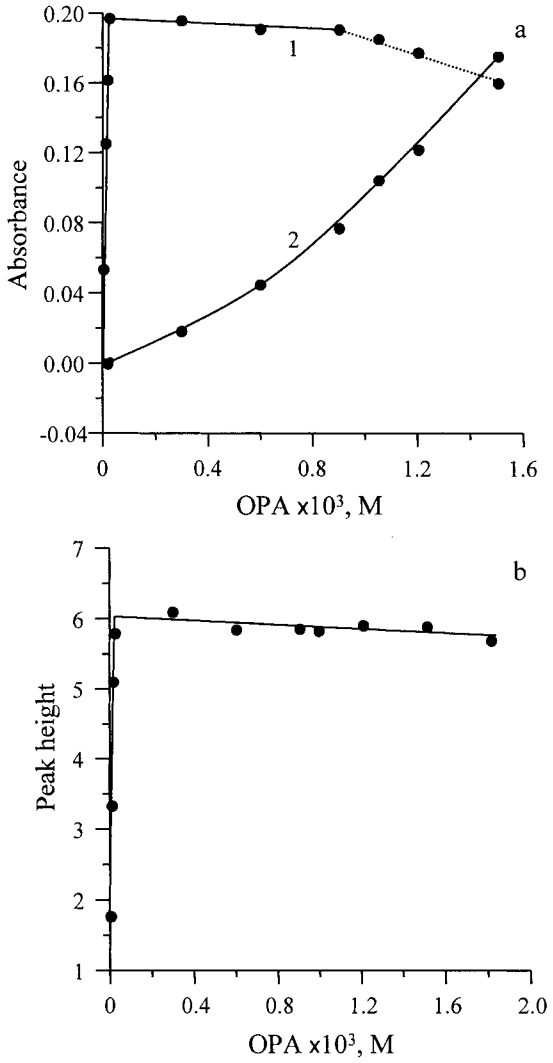

Figure 5. Influence of OPA concentration on the: (a) spectrophotometric, and (b) chromatographic signals. The concentration of *N*-acetylcysteine and isoleucine were 3.3×10^{-5} and 1×10^{-3} M, respectively. The lines correspond to: (1) isoindole (OPA–isoleucine–*N*-acetylcysteine), and (2) blank (OPA–isoleucine).

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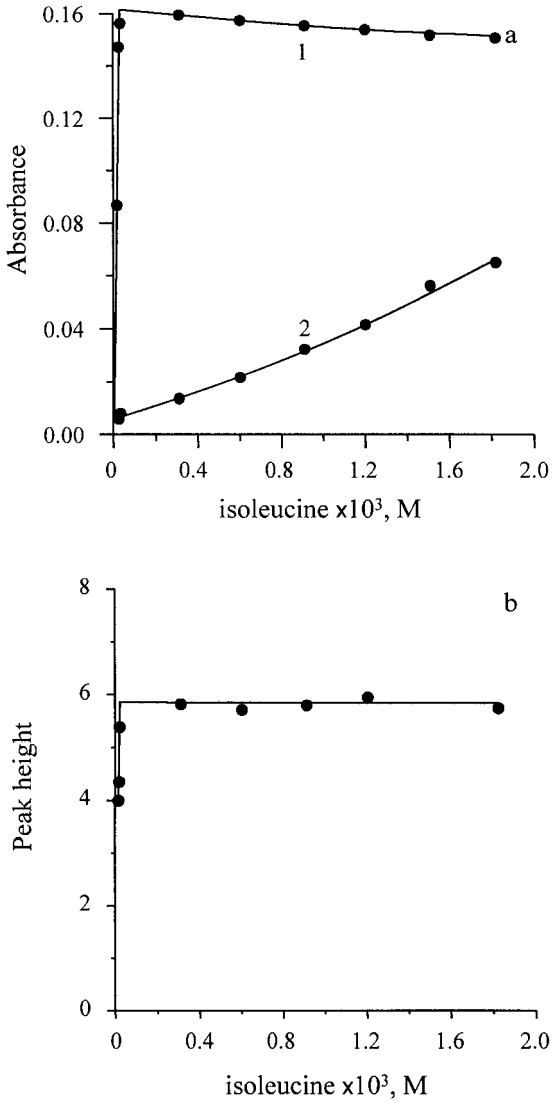

Figure 6. Influence of isoleucine concentration on the: (a) spectrophotometric, and (b) chromatographic signals. The concentration of *N*-acetylcysteine and OPA were 2.6×10^{-5} and 6×10^{-4} M, respectively. The lines correspond to: (1) isoindole (OPA–isoleucine–*N*-acetylcysteine), and (2) blank (OPA–isoleucine).

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Table 1. Molar absorptivities (ε) of OPA–isoleucine isoindoles.

Thiol	ε ($M^{-1}\,cm^{-1}$)	r
N-Acetylcysteine	6,005	0.9999
N-Acetylpenicillamine	1,110	0.9997
Captopril	1,560	0.9999
Glutathione	6,044	0.9998
3-Mercaptopropionic acid	18,070	0.9994
Thioglycolic acid	22,440	0.9999
Tiopronin	7,240	0.9999

the prevention of cardiotoxicity by doxorubicin and haemorrhagic cystitis from oxazaphosphorines.[14,15]

The usual amounts of *N*-acetylcysteine in formulations makes the use of spectrophotometry more appropriate than fluorimetry for detection. Calibration curves were constructed in the concentration range 2.2×10^{-6}–1.0×10^{-4} M, quantifying the areas of the chromatographic peaks of aqueous solutions of the thiol. The variability of the parameters (intercept and slope) of independent calibration straight-lines was studied. During 3 days, five

Table 2. Day-to-day calibration parameters obtained with *N*-acetylcysteine standards.

Day	Intercept	Slope	r
1[a]	0.038 ± 0.014	984 ± 9	0.9989
2[a]	0.106 ± 0.032	990 ± 3	0.9985
3[a]	0.041 ± 0.024	972 ± 7	0.9984
4[b]	−0.14	900	0.9996
	−0.13	886	0.997
5[b]	−0.20	837	0.9996
	−0.19	845	0.9996
6[b]	−0.062	755	0.997
	−0.005	723	0.997
7[c]	0.011	594	0.988
	0.046	589	0.976

[a]Series obtained in different days using each day a new OPA solution. Mean values from five calibration sets are given.
[b]Series obtained in different consecutive days using the same OPA solution prepared just before running the first calibration.
[c]Series obtained with the same OPA solution as in[b] but run 3 days after series 6[b].

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Table 3. Within-day accuracy and precision for *N*-acetylcysteine assay.

Added concentration (M)	Measured concentration (M)	Accuracy (%)	Precision (%)
2.086×10^{-5}	$(2.03 \pm 0.03) \times 10^{-5}$	−2.7	1.5
3.129×10^{-5}	$(3.04 \pm 0.03) \times 10^{-5}$	−2.8	1.0
5.214×10^{-5}	$(5.04 \pm 0.05) \times 10^{-5}$	−3.3	1.0
8.343×10^{-5}	$(8.23 \pm 0.07) \times 10^{-5}$	−1.4	0.9
1.043×10^{-4}	$(1.064 \pm 0.012) \times 10^{-4}$	2.0	1.1

calibration sets were measured using OPA solutions prepared every day. Table 2 (series 1–3) shows the results. It may be observed that, for the slope, the day-to-day (984 ± 9) and the within-day (see Table 2) variabilities were similar, with a relative standard deviation (RSD) of 0.9%. In contrast, series 4–7 in Table 2 correspond to calibration curves obtained in four different days using the same OPA solution, which was prepared in the first day. Although the OPA reagent was protected from light, it suffered decomposition, which is evident in the day-to-day diminution of the slope of the calibration straight-line.

Table 4. Analysis of formulations containing *N*-acetylcysteine.

Formulation (manufacturer)	Composition	Declared (mg)	Found[a] (mg)
ACC (Hexal, Mexico, D.F.)	Unspecified excipient	100	100.7 ± 2.7
Acetilcisteína (Bexal, Madrid, Spain)	Ascorbic and citric acids, lactose, manitol, sodium hydrogencarbonate, trisodium citrate, sodium saccharine, cychlamate, and flavor agents	600	650 ± 18
Fluimucil oral (Zambon, Barcelona, Spain)	Aspartame, sorbitol, β-carotene and flavor agents	200	207.8 ± 4.6
Flumil (Pharmazam, Barcelona)	Methyl *p*-hydroxybenzoate, sodium benzoate, sodium hydroxide, sodium carboxymethylcellulose, sodium saccharine, and flavor agents	20	19.77 ± 0.09

[a]Average values from five analyses.

The within-day accuracy and precision of the procedure was obtained for five different known concentrations of the thiol, which were injected five fold every day (Table 3). The precision showed an RSD of up to 1.5%.

The limit of quantification (LQ) for *N*-acetylcysteine, defined as the analyte concentration producing a peak area equal to 10*s* (*s*, standard deviation of the analyte peak area at low concentration), was evaluated by measuring five

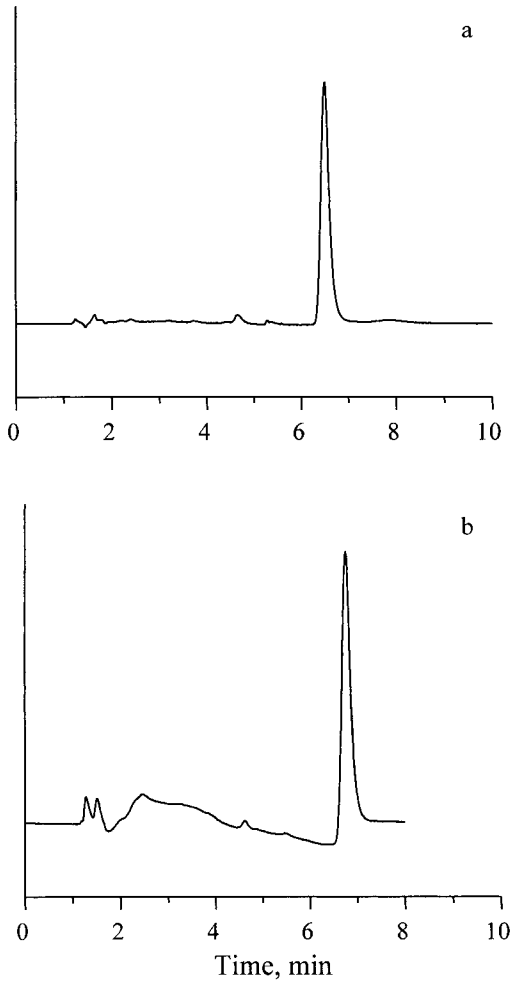

Figure 7. Chromatograms of two formulations containing *N*-acetylcysteine: (a) Fluimucil, and (b) Flumil. Mobile phase: 40% acetonitrile at pH 3.

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peaks for replicated injections (five fold) of a 5.0×10^{-6} M solution of the thiol, with LQ = 1.1×10^{-6} M.

Five samples of each of the following formulations were analyzed: ACC and Acetilcisteína (tablets), Fluimucil oral (powder), and Flumil (syrup). Table 4 gives the values declared by the manufacturers and the values found. The label claim percentages were: ACC (100.7%), Acetilcisteína (108.3%), Fluimucil oral (103.9%), and Flumil (98.9%). The reproducibilities (RSD) in these analyses were: ACC (2.7%), Acetilcisteína (2.8%), Fluimucil oral (2.2%), and Flumil (0.5%). Figure 7 shows chromatograms of two formulations. No interference was observed from the excipients or other accompanying compounds. Therefore, the proposed procedure is adequate for the determination of *N*-acetylcysteine, at least in these kinds of samples.

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