Journal of Chromatography, 507 (1990) 451–458
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1667

High-performance liquid chromatographic determination of aliphatic thiols with aroylacrylic acids as fluorogenic precolumn derivatization reagents

RITA GATTI, VANNI CAVRINI* and PAOLA ROVERI
Department of Pharmaceutical Sciences, Via Belmeloro 6, 40126 Bologna (Italy)
and
SERGIO PINZAUTI
Department of Pharmaceutical Sciences, Via G. Capponi 9, 50121 Florence (Italy)

SUMMARY

The use of the methyl ester of 4-(6-methoxynaphthalen-2-yl)-4-oxo-2-butenoic acid as a fluorogenic labelling reagent for the high-performance liquid chromatography (HPLC) of biologically important thiols (glutathione, cysteine, acetylcysteine, homocysteine, cysteamine, sodium 2-mercaptoethanesulphonate and thiola) was investigated. The compound reacts selectively and rapidly (10 min at ambient temperature and pH 7.5) with the thiols to give fluorescent adducts that can be separated by reversed-phase HPLC and detected fluorimetrically (λ_{em} = 450 nm; λ_{em} = 310 nm). Applications to the determination of L-cysteine and mesna in pharmaceutical formulations are described.

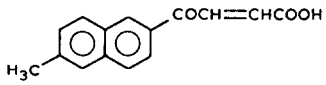
INTRODUCTION

High-performance liquid chromatography (HPLC) in conjunction with a pre- or post-column chemical derivatization constitues an effective technique for the sensitive and selective determination of aliphatic thiols of biological importance[1–3]. Maleimides[4,5], bimanes[6,7] and 7-fluoro-2,1,3-benzoxadiazoles[8,9] proved to be useful reagents for the fluorogenic labelling of thiols in HPLC analyses with fluorimetric detection.

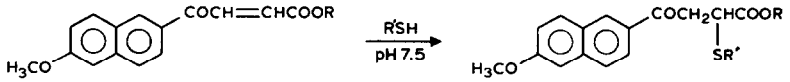
To develop additional selective flurogenic reagents for thiols, our attention was directed to compounds having a naphthoylacrylic structure[10] and, recently, 4-(6-methylnaphthalen-2-yl)-4-oxo-2-olutenoic acid (I) has been proposed as useful precolumn derivatization reagent for the HPLC analysis of thiol drugs[11]. This reagent, devoid of native fluorescence, displayed good selectivity toward thiol compounds, giving fluorescent derivatives that were separated by HPLC. The sensitivity achieved, however, was not very high, although it was adequate for the analysis of pharmaceutical formulations, and restricted chromatographic conditions were required. In order to enhance the sensitivity, the methoxy analogue of I was chosen as reagent potential-

0021-9673/90/$03.50

ly able to give derivatized thiols with high fluorescence quantum yields. This paper describes the applications of 4-(6-methoxynaphthalen-2-yl)-4-oxo-2-butenoic acid (**II**) and its methyl ester (**III**) as precolumn fluorogenic labelling reagents in the HPLC analysis of some biologically active thiols.



(**I**)



(**II**) R = H
(**III**) R = CH_3
non-fluorescent

(**IIa**) R = H
(**IIIa**) R = CH_3
fluorescent

EXPERIMENTAL

Chemicals

L-Cysteine was purchased from Carlo Erba (Milan, Italy) and N-acetylcysteine, N-(mercaptopropionyl)glycine (thiola), homocysteine, reduced glutathione, sodium 2-mercaptoethanesulphonate (MESNa), penicillamine, cysteine and cysteamine were from Fluka (Buchs, Switzerland). Captopril was kindly supplied by Squibb (Rome, Italy).

Methanol, acetonitrile and tetrahydrofuran (THF) were of HPLC grade from Carlo Erba and water was deionized and doubly distilled. All other chemicals were of analytical-reagent grade. A pH 7.5 borate buffer solution was prepared according to the Italian Pharmacopoeia[12].

As internal standards, 4-(2-naphthyl)-4-oxobutanoic acid[13] and 4-(6-methoxy-2-naphthyl)-4-oxobutanoic acid[14,15] were used.

Apparatus

The liquid chromatograph consisted of a Varian 2010 pump and a Varian 2070 fluorescence detector, operating at λ_{em} = 450 nm with λ_{ex} = 310 nm, connected to a Varian 4720 integrator. Manual injections were carried out using a Rheodyne Model 7125 injection valve (50-μl sample loop). IR spectra were taken in a Nujol mull on a Perkin-Elmer 298 IR spectrophotometer. UV spectra were recorded using a Jasco Uvidec 610 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian EM 390 spectrometer at 90 MHz using tetramethylsilane as internal standard.

Synthesis of reagents II and III

4-(6-Methoxynaphthalen-2-yl)-4-oxo-2-butenoic acid (**II**) was synthesized from 6-methoxy-2-acetylnaphthalene (Fluka) and glyoxylic acid (Fluka) as described previously[14]; m.p. = 167–169°C (acetic acid); IR (cm^{-1}), 1705,1655,1615,1180,840; ^{1}H NMR (acetone-d_6), δ 3.95 (3H, s, CH_3O–), 6.85 and 8.05 (2H, q, J = 15.0 Hz, *trans*

–CH=CH–), 7.15–8.6 (6H, m, Ar–H) A solution of the acid **II** ($5.2 \cdot 10^{-4}$ *M*) for analytical applications was prepared in 0.3 *M* phosphate buffer solution (pH 7.4).

The methyl ester **III** was prepared by heating under reflux for 1.0 h a solution of the parent acid (**II**) (0.5 g) and methanol (2.5 ml) in 25 ml of anhydrous benzene in the presence of sulphuric acid (2–3 drops). Water (20 ml) was added and subsequent extractions with 5% sodium hydrogencarbonate (10 ml) and water (20 ml) were performed. The benzene layer was dried over anhydrous sodium sulphate and evaporated *in vacuo*. The residue was purified by flash chromatography on a silica gel column using ethyl acetate–light petroleum (b.p 40–70°C) (4:6) as the mobile phase. A pale yellow compound was isolated; m.p 116–120°C; calculated for $C_{16}H_{14}O_4$,C 71.10,H 5.22; found, C 71.14,H 5.47%; IR (cm^{-1}), 1715, 1665, 1620, 1310, 1165, 850, 840, 825; ^1H NMR ([$^2H_6$]acetone), δ 3.85 (3H, s, –$COOCH_3$), 3.98 (3H, s, CH_3O–), 6,85 and 8.15 (2H, q, J = 15 Hz, *trans* –CH=CH–),7.20–8.80 (6H,m,ArH); UV (ethanol), λ_{max} = 332 nm ($\varepsilon = 1.24 \cdot 10^4$).

A solution of reagent **III** ($5.2 \cdot 10^{-4}$ *M*) was prepared by dissolving 3.5 mg of the compound in 10 ml of THF and diluting to 25 ml with borate buffer solution (pH 7.5).

*Synthesis of N-acetylcysteine adduct with reagent **III***

The methyl ester **III** (150 mg; 0.5 mmol) was dissolved in 30 ml of THF, then 20 ml of borate buffer solution (pH 7.5) and 90.5 mg (0.5 mmol) of N-acetylcysteine were added. The reacton solution was stirred at ambient temperature for 30 min, acidified with 2 *M* hydrochloric acid and then extracted with diethyl ether (2 × 30 ml). The combined extracts were dried over anhydrous sodium sulphate and then evaporated *in vacuo* to give a product homogeneous by thin-layer chromatography (silica gel) using chloroform–methanol–acetic acid (9:1:0.5, v/v/v) as eluent with detection at 254 and 366 nm; m.p. 112–118°C (decomp.); calculated for $C_{21}H_{23}NO_7S$, C 58.05, H 5.33, N 3.22; found, C 57.90, H 5.41, N 3.05%; ^1H NMR (C^2HCl_3), δ 2.0 (3H, s, CH_3CON), 3.75 (3H, s, $COOCH_3$), 3.95 (3H, s, CH_3O), 3.0–3.7 (5H, m, CH_2S + CHS + CH_2CO), 4.80 (1H, m, CHN), 7.10–8.80 (6H, m, Ar–H); UV (ethanol), λ_{max} = 315 nm ($\varepsilon = 1.334 \cdot 10^4$).

Chromatographic conditions

The HPLC separations were performed on a Spherisorb RP-8 (5-μm) column (150 × 4 mm I.D.) or a Hypersil RP-8 (5-μm) column (150 × 4 mm I.D.). For routine analyses of cysteine and MESNa, a mobile phase consisting of methanol–0.05 *M* triethylammonium phosphate (pH 3.0) (53:47) at a flow-rate of 1.0 ml/min was used.

Calibration graphs

Standard solutions of L-cysteine (0.66–2.65 μg/ml) were prepared in EDTA (disodium salt) solution (1 mg/ml); similarly, standard solutions of MESNa (1.34–4.46 μg/ml) were prepared in EDTA (disodium salt) solution (15 μg/ml).

A 1.0-ml volume of thiol standard solution was reacted with 0.3 ml of the reagent **III** solution at ambient temperature for 20 min, then 0.5 ml of 0.3 *M* phosphoric acid solution and 3.0 ml of the appropriate internal standard solution were added. The resulting solution was diluted to 10 ml with water and 50-μl aliquots were

injected into the chromatograph. The peak-height ratio of the thiol adduct to the internal standard was plotted against the corresponding thiol concentration to obtain the calibration graphs. As internal standards, 4-(naphthalen-2-yl)-4-oxobutanoic acid solution (0.24 mg/ml) was used for cysteine determination and 4-(6-methoxynaphthalen-2-yl)-4-oxobutanoic acid solution (4 μg/ml) for MESNa determination.

Sample preparation

Powders. An amount of powder equivalent to about 0.20 mg of cysteine was dissolved in 200 ml of EDTA solution (1 mg/ml).

Tablets. An amount of powdered tablet equivalent to about 7.7 mg of cysteine was treated with 250 ml of EDTA solution (1 mg/ml) under sonication for 5 min. After filtration through paper, a 3-ml aliquot of the clarified solution was diluted to 100 ml with EDTA solution (1 mg/ml).

Solutions. An aliquot of the commercial formulation was diluted with EDTA solution (15 μg/ml) to provide a sample solution containing about 3.2 μg/ml of thiol compound.

Assay procedure. A 1.0-ml aliquot of the sample solutions was subjected to the derivatization procedure with reagent **III** as described under *Calibration graphs.* The determination of the content of thiol compound in each sample was performed by comparison with an appropriate standard solution.

RESULTS AND DISCUSSION

The naphthoylacrylic acid **II** and the corresponding methyl ester **III** were synthesized by standard methods and, according to the NMR spectra, the *E* (*trans*) isomers were obtained. The reaction between **III** and N-acetylcysteine was carried out on a preparative scale and the analytical data for the adduct obtained were consistent with the proposed general structure **IIIa**, according to a Michael-type reaction involving thiol nucleophiles[16].

The fluorescence quantum yield of the N-acetylcysteine adduct in ethanol–triethylammonium phosphate (pH 3.0) (1:1, v/v) was found to be 0.15 by comparison with that of quinine sulphate (0.55 in 0.1 *M* sulphuric acid). This value is significantly higher than that of the corresponding adduct obtained from reagent **I**[10] and meets the requirements for sensitive HPLC analyses.

Chromatography

According to previous experience[11], the naphthoylacrylic acid **II** was first used for the thiol derivatization at pH 7.4 followed by reversed-phase HPLC separation. A representative chromatogram is shown in Fig. 1. As can be seen, biologically active thiols derivatized with **II** can be separated and identified but reagent peaks due to impurities are responsible for some interference. The complete elimination of these fluorescent impurities was attempted by recrystallization and chromatographic procedures, but failed. In addition, when the organic modifier content in the mobile phase was lowered to improve the resolution, splitting of the glutathione peak was observed, probably owing to the separation of the two diastereoisomer adducts from the derivatization reaction. These data led us to prepare the methyl ester **III**, which was obtained in high purity and therefore was chosen for the present study. A typical

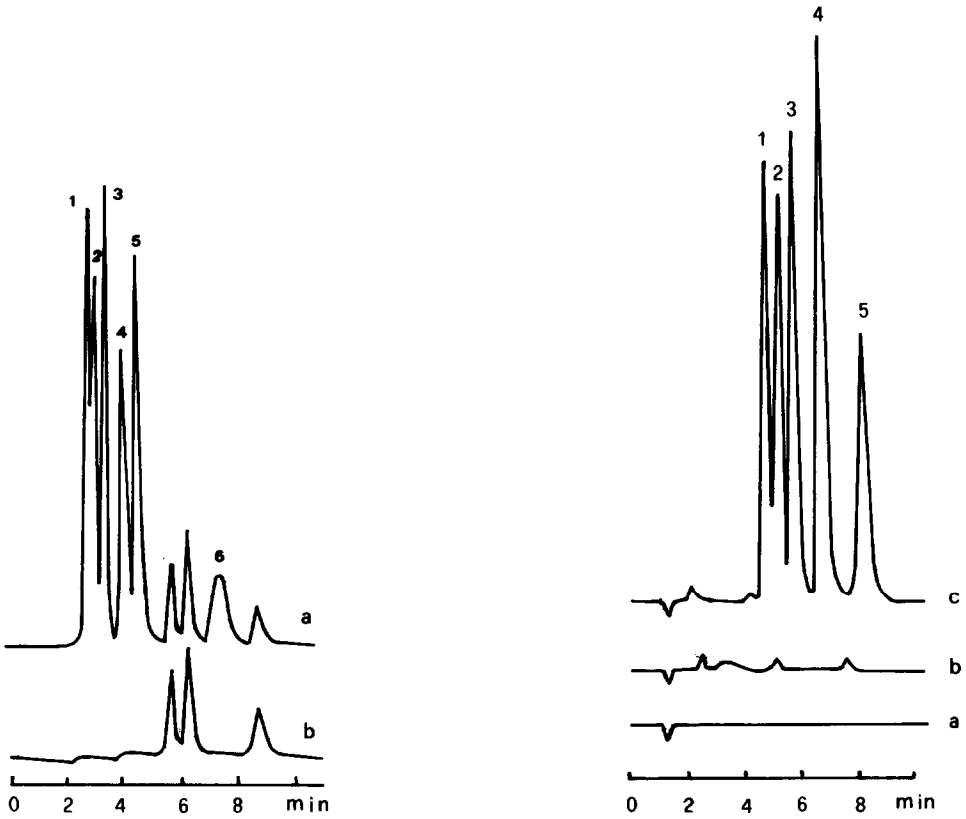


Fig. 1. HPLC of (a) adducts **IIa** from (1) glutathione, (2) L-cysteine, (3) homocysteine, (4) acetylcysteine, (5) thiola and (6) captopril; (b) reagent **II**. Column, Spherisorb RP-8 (5 μm); mobile phase, ethanol–0.05 *M* triethylammonium phosphate (pH 3.0) (40:60) at 1 ml/min. Fluorescence detector: λ_{em} = 450 nm; λ_{ex} = 310 nm.

Fig. 2. HPLC of (a) reagent **III**; (b) reagent **III** after 6 days at 4°C; (c) adducts **IIIa** from (1) glutathione, (2) L-cysteine, (3) MESNa, (4) homocysteine and (5) acetylcysteine. Column, Hypersyl RP-8 (5 μm); mobile phase, methanol–0.05 *M* TEA phosphate (pH 3.0) (53:47) at 1.2 ml/min. Detection as in Fig. 1.

separation of some thiols derivatized with the ester **III** is illustrated in Fig. 2. As shown, interfering reagent peaks were absent and the reagent solution proved to be stable for 3–4 days at 4°C. Using the ester reagent **III**, moreover, more lipophilic thiol adducts were obtained which provided single symmetric peaks under various chromatographic conditions.

The derivatization reaction course was studied with N-acetylcysteine. Using a molar ratio of reagent to analyte of >6, the reaction was completed within 10 min at ambient temperature and pH 7.5 and proved to be quantitative by comparison with an authentic specimen of the adduct. Using reversed-phase HPLC, methanol, acetonitrile and ethanol were found to be equally suitable as organic modifiers in the mobile phase. This represents an improvement with respect to the use of the methyl analogue **I**, which preferentially required ethanol.

The effect of the mobile phase pH on resolution and sensitivity for some biologically active thiols was evaluated and the results can be summarized as follows: (a) thriethylammonium (TEA) phosphate solutions at pH 3.0 and 4.0 were both suitable, whereas at pH 7.0 poorly reproducible separations were observed. (b) The sensitivities achieved at pH 3.0 and 4.0 were comparable but different for the various thiols examined. The highest sensitivity was obtained for glutathione, cysteine and homocysteine. The detection limit (signal-to-noise ratio = 3) was about 0.01 μg/ml. (c) Complete separation between glutathione and cysteine was obtained using a mobile phase consisting of methanol–0.05 *M* TEA phosphate (pH 4.0) (53:47, v/v) or methanol–0.01 *M* KH_2PO_4 (pH 3.0) (53:47, v/v). (d) Shorter retention times were obtained using acetonitrile instead of methanol. A suitable mobile phase was acetonitrile–0.05 *M* TEA phosphate (pH 3.0) (32:68, v/v) (Fig. 3). However, for the separation of more liphophilic thiols such as N-acetylcysteine, captopril, mercaptopropionylglycine and penicillamine, a higher acetonitrile content (42%) in the mobile phase was required.

Analysis of pharmaceutical formulations

The proposed HPLC method, based on precolumn derivatization with **III** and fluorimetric detection, was applied to the determination of L-cysteine and MESNa in commercial pharmaceutical formulations. L-Cysteine is an aliphatic thiol amino acid used as a dietary supplement and in the treatment of leg ulcers and MESNa is used as a mucolytic agent and also to prevent the urotoxic effects of cyclophosphamide or ifosfamide[17]. Under the described chromatographic conditions (Fig. 4), linear calibration graphs were obtained using fluorescent naphthoylpropionic acids (*i.e.*, hy-

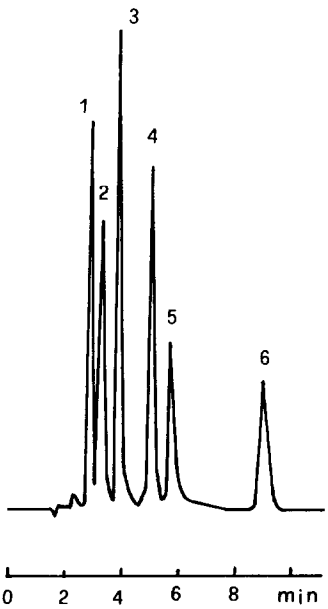


Fig. 3. Typical HPLC separation of adducts **IIIa** from (1) glutathione, (2) L-cysteine, (3) homocysteine, (4) cysteamine, (5) MESNa and (6) acetylcysteine. Column, Spherisorb RP-8 (5 μm); mobile phase, acetonitrile–0.05 *M* TEA phosphate (pH 3.0) (32:68). Detection as in Fig. 1.

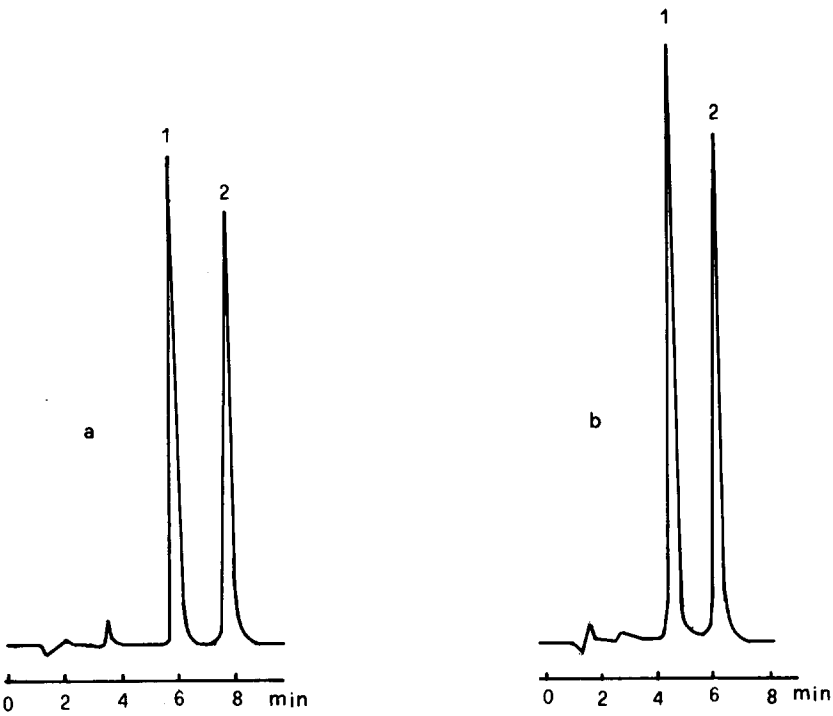


Fig. 4. Typical HPLC separations for quantitative applications: (a) (1) MESNa and (2) internal standard; (b) (1) L-cysteine and (2) internal standard. Column, Spherisorb RP-8 (5 μm); mobile phase, methanol –0.05*M* TEA phosphate (pH 3.0) (53:47) at 1 ml/min. Detection as in Fig. 1.

drogenated naphthoylacrylic acids) as internal standards. A typical calibration graph of the peak-height ratio of derivatized L-cysteine to internal standard (y) *versus* the drug concentration (x) was $y = 1.175x - 0.00276$ ($r = 0.9980$; $n = 6$). For MESNa the calibration graph was $y = 0.368x + 0.0110$ ($r = 0.9956$; $n = 6$). The precision of each method was evaluated by replicate analyses ($n = 8$) of a single standard solution. The relative standard deviation (R.S.D.) of the peak-height ratio of drug to internal standard was 0.6% and 0.85% for cysteine and MESNa, respectively.

TABLE I

ASSAY RESULTS FOR THE HPLC DETERMINATION OF L-CYSTEINE AND MESNa IN COMMERCIAL PHARMACEUTICAL FORMULATIONS

Drug	*Formulation*[a]	*Found (%)*[b]	*R.S.D. (%)*
L-Cysteine	Powder	100.07	0.73
	Tablet	99.50	1.80
MESNa	Solution	100.43	0.65

[a] Other ingredients: powder: neomycin sulphate, bacitracin Zn, glycine, DL-threonine, starch and magnesium oxide; tablet: D,L-methionine, L-cystine, protein hydrolysate, calcium pantothenate, vitamin B_2 phosphate, vitamin B_6, biotin and vitamin E; solution: EDTA disodium salt.

[b] Average of five determinations and expressed as a percentage of the claimed content.

Commercial formulations of L-cysteine and MESNa were analysed. The results obtained are summarized in Table I and were in close agreement with the claimed content. The other ingredients, including amino acids, of the analysed formulations did not interfere. The accuracy of the proposed HPLC method was verified by analysing samples (powders and tablets) spiked with known amounts of the drug; quantitative recoveries were obtained in each instance.

In conclusion, compound **III** proved to be a useful fluorogenic labelling reagent for the HPLC analysis of aliphatic thiols. The compound displayed a good reactivity and selectivity toward the thiol group, giving highly fluorescent derivatives that can be separated by reversed-phase HPLC. The proposed HPLC method exibited good sensitivity and its applications to the determination of bioactive thiols in biological samples are under study.

ACKNOWLEDGEMENTS

We are grateful to Dr. R. Ballardini for the fluorescence quantum yield determinations and to Miss P. Piccardoni for skillful technical assistance. This work was supported by the Ministero della Pubblica Istruzione (40%), Italy.

REFERENCES

1 D. Perret and S.R. Rudge, *J. Pharm. Biomed. Anal.*, 3 (1985) 3.
2 N.D. Danielson, M.A. Targove and B.E. Miller, *J. Chromatogr. Sci.*, 26 (1988) 362.
3 M.A. Martin, B. Lin and B. Del Castillo, *J. Pharm. Biomed. Anal.*, 6 (1988) 573.
4 M. Johansson and D. Westerlund, *J. Chromatogr.*, 385 (1987) 343.
5 K. Nakashima, C. Umekowa, H. Yoshida, S. Nakatsuji and S. Akiyama, *J. Chromatogr.*, 414 (1987) 11.
6 R.M. Maiorino, G.I. Weber and H.V. Aposhian, *J. Chromatogr.*, 374 (1986) 297.
7 W.R.G. Baeyens, G. van der Weken and P. de Moerloose, *Anal. Chim. Acta*, 205 (1988) 43.
8 T. Toyo'ka and K. Imai, *Anal. Chem.*, 56 (1984) 2461.
9 T. Toyo'ka, T. Suzuki, Y. Saito, S. Uzu and K. Imai, *Analyst (London)*, 114 (1989) 413; and references cited therein.
10 V. Cavrini, R. Gatti, P. Roveri and M.R. Cesaroni, *Analyst (London)*, 113 (1988) 1447.
11 V. Cavrini, R. Gatti, P. Roveri and A. Di Pietra, *Chromatographia*, 27 (1989) 185.
12 *Farmacopea Ufficiale della Repubblica Italiana*, Vol. I, Istituto Poligrafico e Zecca Dello Stato, Rome, 9th ed., 1985, p. 749.
13 A. Eirin, F. Fernandez, G. Gomez, C. Lopez, A. Santos, J. M. Colleja, D. Dela Iglesia and E. Cano, *Arch. Pharm. (Weinheim)*, 320 (1987) 1110.
14 V. Cavrini, P. Roveri, R. Gatti, C. Ferruzzi, A. M. Panico and M. S. Pappalardo, *Farmaco, Ed. Sci.*, 37 (1982) 171.
15 M. Ghosal, *J. Org. Chem.*, 25 (1960) 1856.
16 J. March, *Advanced Organic Chemistry*, Wiley, New York, 3rd ed., 1985, p. 670.
17 E. F. Reynolds (Editor), *Martindale, The Extra Pharmacopoeia*, Pharmaceutical Press, London, 29th ed., 1989.