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Alternative methods for the determination of trace amounts of 4-aminomorpholine in molsidomine and linsidomine

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Abstract

Different methods based on either ion chromatography with electrochemical detection or precolumn derivatization with *o*-phthaldialdehyde were developed to determine trace amounts of 4-aminomorpholine in molsidomine and linsidomine. Electrochemical detection combined with ion chromatographic separation revealed outstanding selectivity whereas derivatization with *o*-phthaldialdehyde turned out to be very sensitive using RP-HPLC and standard UV detection.

1. Introduction

The identification and determination of synthesis intermediates in drug substances is an important task in synthesis development, optimization and final quality control. Substituted hydrazines, e.g., 4-aminomorpholine are synthesis intermediates of pharmacologically active 3-dialkylaminosydnonimines used as anti-anginal agents. The scheme of their synthesis is presented in Fig. 1. Hydrazines may be present in trace amounts in the final product. The absence of π-bonding in these compounds has the consequence of low sensitivity for photometric detection. Conductivity detection in LC or separation by GC is not possible owing to the low conductivity of the hydrazines and thermal instability of the sydnonimines, respectively.

As all other potential by-products and degradation products of molsidomine and linsidomine hydrochloride have already been determined

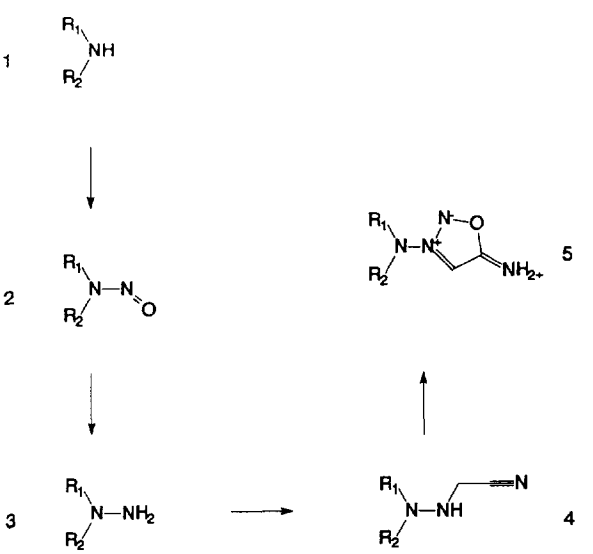

Fig. 1. Scheme of synthesis of 3-dialkylsydnonimines starting with the secondary amine (1) (e.g., morpholine). The hydrazine (3) (e.g., 4-aminomorpholine) is formed as a synthesis intermediate which is not isolated during synthesis.

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using a gradient HPLC method, we investigated the development of a suitable, very sensitive detection procedure for 4-aminomorpholine. Several structural closely related synthesis intermediates and potential degradation products had to be taken into account, demanding the highest selectivity (Fig. 2).

Many advances have been reported in the use of preinjection and postcolumn derivatization to generate photometrically or electrochemically active adducts [1]. Only the determination of morpholine in molsidomine using precolumn derivatization with dansyl chloride has been reported so far [2]. *o*-Phthaldialdehyde (OPA) is a well known derivatizing reagent for primary amines, amino alcohols, peptides and amino acids which fulfils the demands for highest sensitivity, as fluorescent products are formed [3]. It has not been used so far, to our knowledge, for the derivatization of hydrazines.

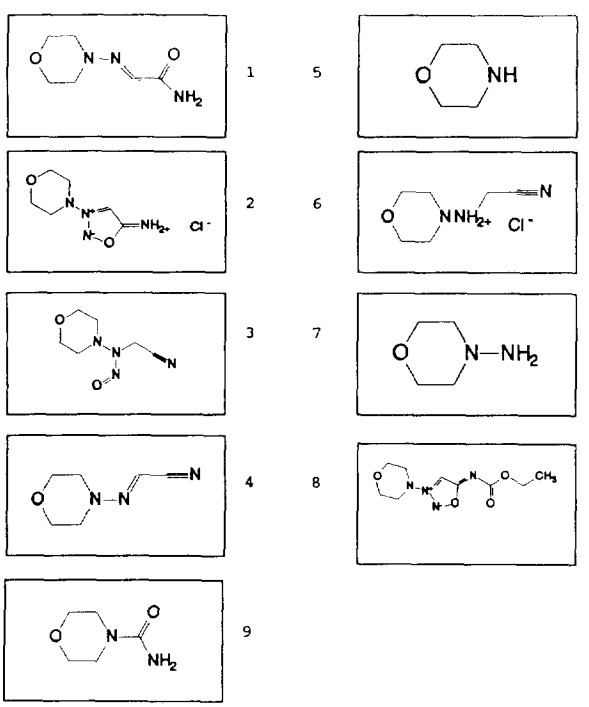


Fig. 2. Synthesis intermediates and some of the major decomposition products of molsidomine and linsidomine hydochloride. 1 = (4-Morpholinylimino)acetamide (SIN 1 C amide); 2 = 3-morpholinosydnonimine (linsidominehydrochloride); 3 = (4-morpholinylnitrosoamino)acetonitrile (SIN 1 A); 4 = (4-morpholinylimino)acetonitrile (SIN 1 C); 5 = morpholine; 6 = (4-morpholinylamino)acetonitrile (SIN 1 B); 7 = 4-aminomorpholine; 8 = molsidomine; 9 = 4-morpholinecarboxamide.

Precolumn derivatization of 4-aminomorpholine for separation by RP-HPLC and UV detection is discussed in this paper. Disturbances occurred when the method was transferred to linsidomine hydrochloride. Ion chromatographic separation followed by direct electrochemical detection was then performed. This mode of detection had already been applied to the determination of hydrazines [4]. We found that precolumn derivatization could not be transferred to other related hydrazines whereas electrochemical detection turned out to be very dependent on the pattern of substitution.

2. Experimental

2.1. Apparatus

Chromatographic analysis was performed using a HP 1090 LC system with an HP 79994A Chem Station (Hewlett-Packard, Waldbronn, Germany).

2.2. Precolumn derivatization

The chromatographic system was equipped with a photodiode-array detector set at 235 nm when precolumn derivatization was used. For derivatization, the injector programme was used. A 5-μl volume of the sample solution was sandwiched between two 5-μl volumes of derivatization reagent. The mixing volume was 10 μl and ten mixing cycles were applied. After a waiting time of 10 min, the whole sample was injected.

2.3. Electrochemical detection

An HP 1049A programmable electrochemical detector equipped with a solid silver/silver chloride reference electrode and a glassy carbon working electrode (Hewlett-Packard) was used. An HP 35900 dual-channel interface was used for digital conversion of the analogue signal

delivered by the electrochemical detector (Hewlett-Packard).

2.4. Chromatographic method

Precolumn derivatization

The column used was a Nucleosil 120 C_{18}, 3 μm (250 mm × 3 mm I.D.) stainless-steel column (Macherey–Nagel, Düren, Germany).

The solvents used were (A) 50 m*M* potassium dihydrogenphosphate, (B) methanol and (C) acetonitrile–water (80:20, v/v). The initial mobile phase was A–B–C (80:20:0, v/v/v). After injection, a stepwise gradient was applied as follows: (1) the initial mixture was maintained for 5 min; (2) B was increased to 80% (v/v) in 25 min with a linear gradient; (3) C was increased to 100% (v/v) with a linear gradient in 5 min; (4) the system was returned to the initial mobile phase in 5 min according to the following scheme:

0 min	80% A–	20% B–	0% C
5 min	80% A–	20% B–	0% C
30 min	20% A–	80% B–	0% C
35 min	0% A–	0% B–	100% C
40 min	80% A–	20% B–	0% C

The flow-rate was 0.5 ml/min.

Electrochemical detection

The column used was an OmniPac PCX-100 (10-32) (Dionex, Sunnyvale, CA, USA). The mobile phase was phosphate buffer (pH 4.0)–acetonitrile (75:25, v/v). The phosphate buffer was prepared by dissolving 6.9 g of sodium dihydrogenphosphate and 0.75 g of sodium chloride in 1000 ml of demineralized water. The pH of this solution was adjusted to 4.0 with orthophosphoric acid.

2.5. Chemicals

4-Aminomorpholine (analytical-reagent grade) was purchased from Fluka (Buchs, Switzerland). All other chemicals and solvents were obtained at the highest purity available from Riedel-de Haën (Seelze, Germany). Molsidomine, linsidomine hydrochloride and all potential impurities were manufactured at our plant and were fully characterized prior to use.

Water for HPLC analyses was demineralized water passed through a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.6. Derivatization reagent

The reagent for derivatization was prepared by dissolving 50 mg of *o*-phthaldialdehyde in 1 ml of methanol. The solution was diluted to 10 ml with a solution containing 2 g of sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in 100 ml of demineralized water, then 50 μl of 2-mercaptoethanol were added. After mixing thoroughly, the solution was filtered through a 0.45-μm filtration unit. The reagent was prepared freshly every day.

2.7. Precolumn derivatization

Preparation of standard solutions

Standards were prepared by dissolving 100.0 mg of 4-aminomorpholine reference standard in 100.0 ml of water–acetonitrile (80:20, v/v). A 100-μl volume of this solution and 100 μl of an internal standard solution containing 100 mg of 3-aminopropanol per 100 ml of water were mixed and diluted to 10.0 ml with water–acetonitrile (80:20, v/v) in a volumetric flask.

Sample preparation

A 100.0-mg amount of molsidomine was dissolved in 8 ml of water–acetonitrile (80:20) and 100 μl of the internal standard solution were added. The solution was diluted to 10.0 ml with demineralized water.

Selectivity and stability

The selectivity was tested by injecting solutions of the potential synthesis intermediates and/or degradation products SIN-1, SIN-1 A, SIN-1 B, SIN-1 C amide, morpholine and 4-morpholinecarboxamide at a concentration of 0.5% each, relative to molsidomine.

The stability of molsidomine with regard to the alkaline reagent was tested by dissolving the required amount of molsidomine in 0.1 *M* sodium hydroxide solution for the specified time (see chromatograms). The stability was also tested in 0.1 *M* hydrochloric acid. For testing the light

stability of molsidomine it was exposed to direct sunlight in a petri dish.

2.8. Electrochemical detection

Preparation of standard solutions

Standard solutions were prepared by dissolving 25.0 mg of 4-aminomorpholine reference standard, accurately weighed, in exactly 100.0 ml of demineralized water. A 100-μl amount of this solution was diluted to 10.0 ml with the mobile phase in a volumetric flask.

Sample preparation

A 250.0-mg amount of linsidomine hydrochloride, accurately weighed, was dissolved in 100.0 ml of the mobile phase in a volumetric flask.

Selectivity

The selectivity of the method was tested by injecting solutions of all synthesis intermediates and/or degradation products (4-morpholinecarboxamide, SIN-1 A, SIN-1 B, SIN-1 C, SIN-1 C amide and morpholine) using UV detection at 210 nm for the electrochemically inactive compounds and by increasing the potential to 1200 mV to obtain a signal for the electrochemically active compounds.

Hydrodynamic voltammograms were obtained with solutions of the reference standards of 4-aminomorpholine, *cis*-2,6-dimethylpiperidine and 3,3-dimethyl-4-aminothiomorpholine at a concentration of 0.25 mg per 100 ml each by increasing the potential stepwise from 0.3 to 1.2 V.

3. Results and discussion

3.1. Precolumn derivatization

A critical aspect of derivatizations, which dictates the usefulness of this analytical technique, is the selectivity and reproducibility with regard to the stability of the derivatives formed. The results presented here clearly show that problems of this kind could be overcome effectively. No interferences with any of the synthesis intermediates or decomposition products could be observed (Fig. 3).

The formation and stability of the derivatives were investigated by varying the time of derivatization. The peak height of the derivative of 3-aminopropanol decreased rapidly when the time of derivatization exceeded 2 min, whereas the amount of the derivative formed with 4-aminomorpholine could even be increased by lengthening the time of derivatization up to 8 min. A time of 1 min for derivatization was found to be suitable for obtaining sufficiently large peak areas for both compounds (Fig. 4).

The relative standard deviation of the results obtained in the test for precision was about 4% and the recoveries were in the range 90–110%. Linearity was satisfactory over the whole concentration range from 20 to 1000 ppm (Fig. 5).

It was also demonstrated that decomposition of molsidomine, which may take place in the alkaline medium of the derivatization reagent, does not disturb the determination of 4-aminomorpholine. No interfering peaks were observed after leaving a solution of molsidomine at pH 12 for 8 h (Fig. 6).

At a strongly acidic pH of 2, no 4-aminomorpholine was formed, which is in contradiction to earlier reports on the stability of molsidomine [5].

The derivatization product formed revealed no fluorescence, in contrast to the products formed with, e.g., primary amines, amino alcohols or amino acids which have been described so far. Identification is still in progress. The UV spectrum exhibited a shoulder at 220 nm and a maximum at 335 nm.

Absorption of the derivative of 4-aminomorpholine was found to be sufficiently high for the low limit of detection demanded. The limit of detection, defined by a signal-to-noise ratio of 3, was as low as 20 ppm, relative to molsidomine.

Linsidomine hydrochloride is the final synthesis intermediate of molsidomine but it is also used as an anti-anginal agent for injection solutions. As linsidomine hydrochloride obviously behaves as a primary amine and consequently reacts with *o*-phthaldialdehyde itself, the derivatization method could not be used for the

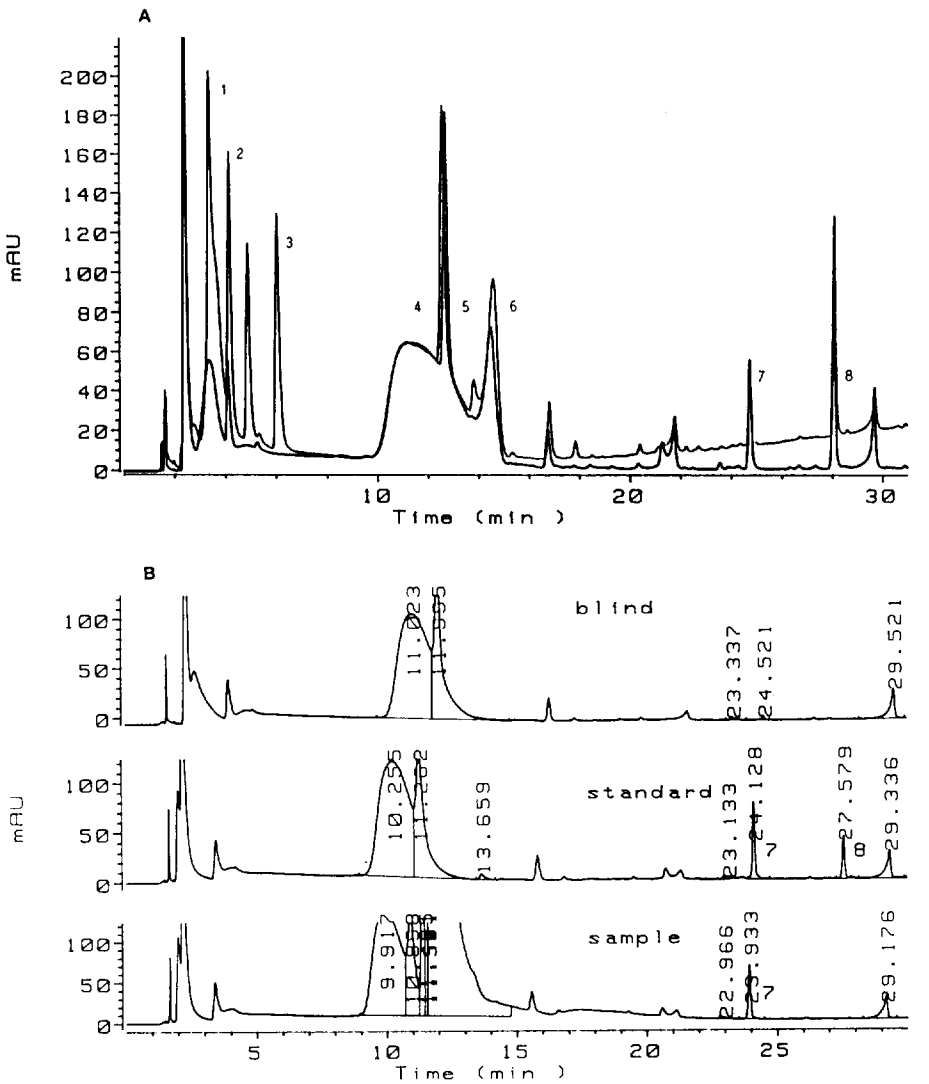


Fig. 3. Chromatograms demonstrating the selectivity of precolumn derivatization for molsidomine. (A) Overlay of a chromatogram obtained with a standard solution containing 0.1% of 4-aminomorpholine and the internal standard and a chromatogram of the potential byproducts without 3-aminopropanol. Peaks: 1 = SIN 1 C amide; 2 = SIN 1; 3 = SIN 1 A + C; 4–6 are from the derivatization reagent; 7 = 3-aminopropanol; 8 = 4-aminomorpholine. (B) Chromatogram of the solvent (top), the standard solution containing 3-aminopropanol and 4-aminomorpholine (middle) and a typical batch of molsidomine (bottom) (the content of 4-aminomorpholine is below the limit of detection). Retention time of molsidomine = 11 min.

detection of 4-aminomorpholine in this substance as a great excess of *o*-phthaldialdehyde would be necessary. Further, when we tried to transfer the method to related hydrazines which are of importance for the synthesis of new anti-anginal compounds, we found that substitution of the hydrazine also plays an important role, as the structurally analogous 4-amino-*cis*-2,6-dimethylpiperidine could not be derivatized satisfactorily.

Steric hindrance by the two methyl groups at positions 2 and 6 influences the reactivity to a great extent, as could also be confirmed by

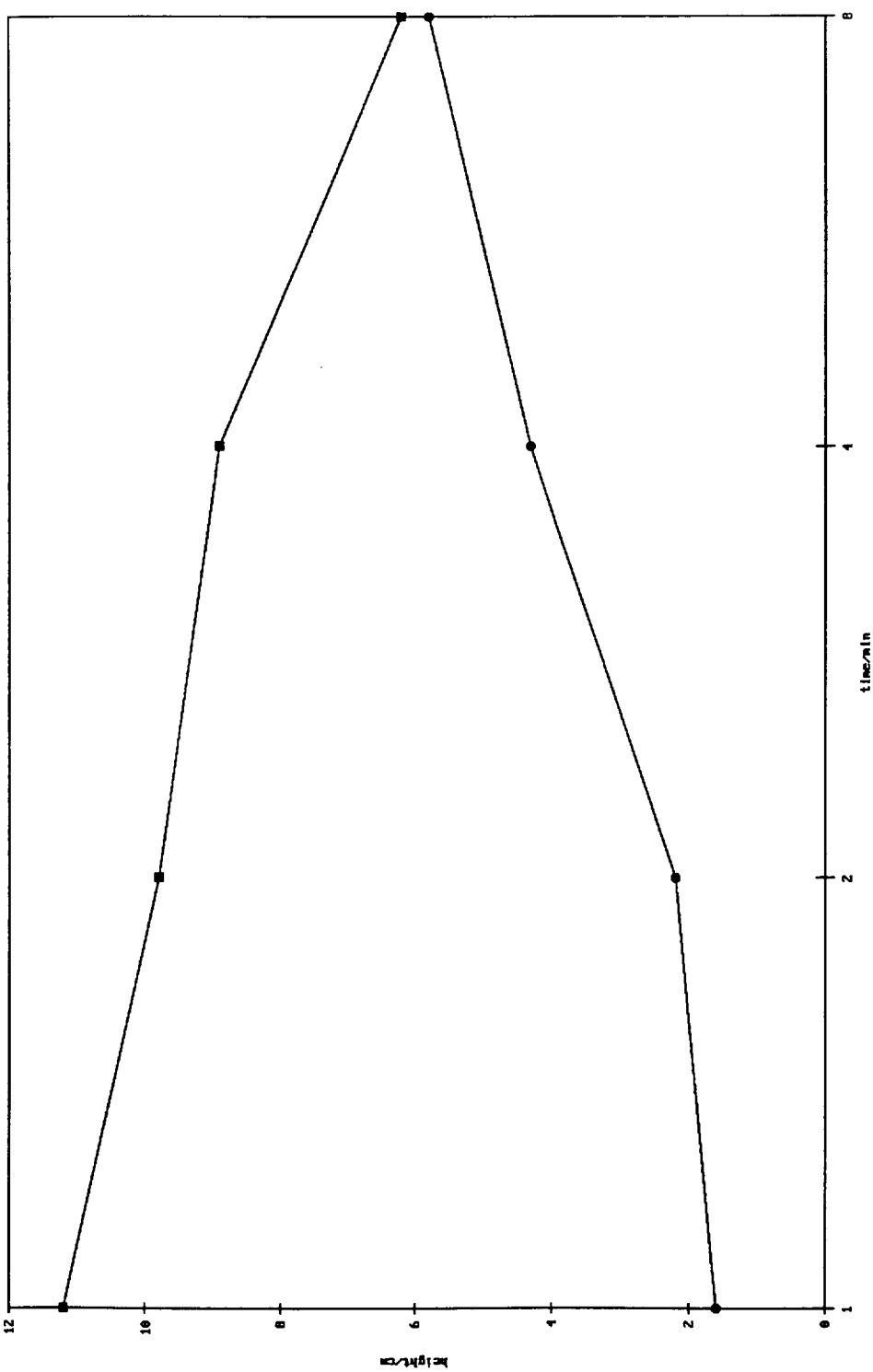
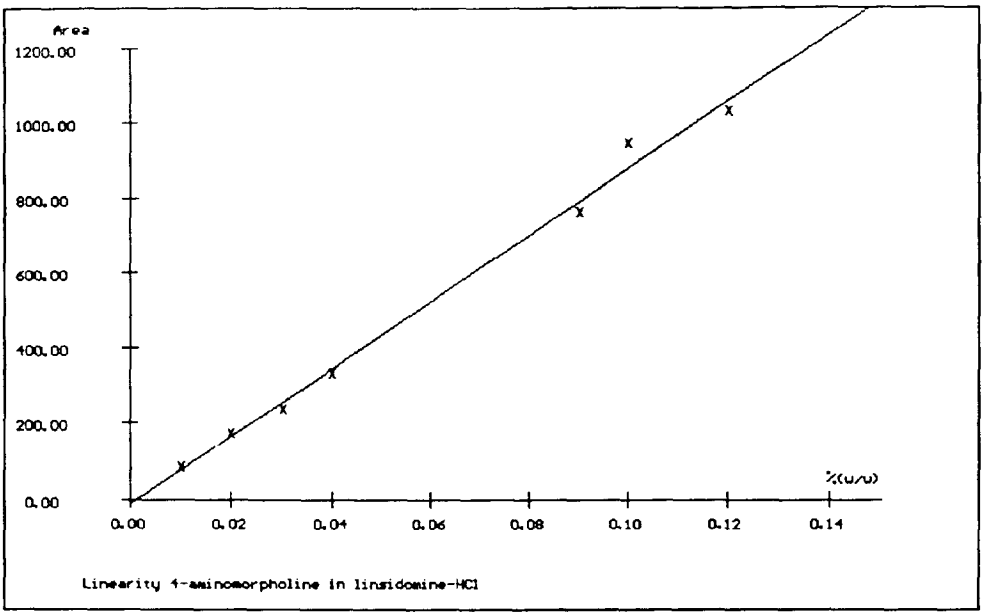


Fig. 4. Diagram showing dependence of peak areas on derivatization time for (●) 4-aminomorpholine and (■) internal standard 3-aminopropanol.



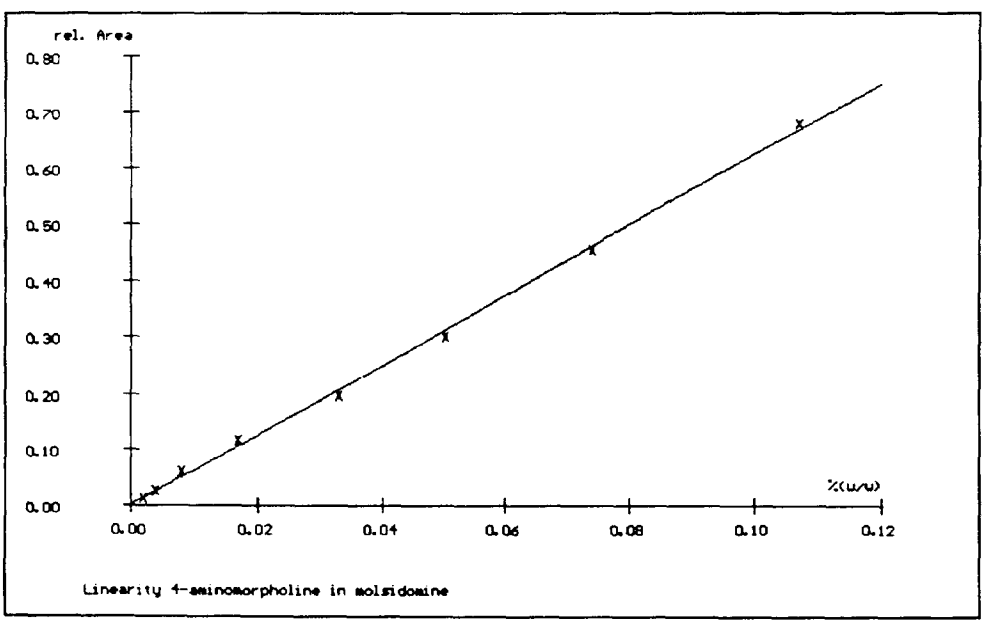


Fig. 5. Linearity shown for both methods, i.e., precolumn derivatization and electrochemical detection.

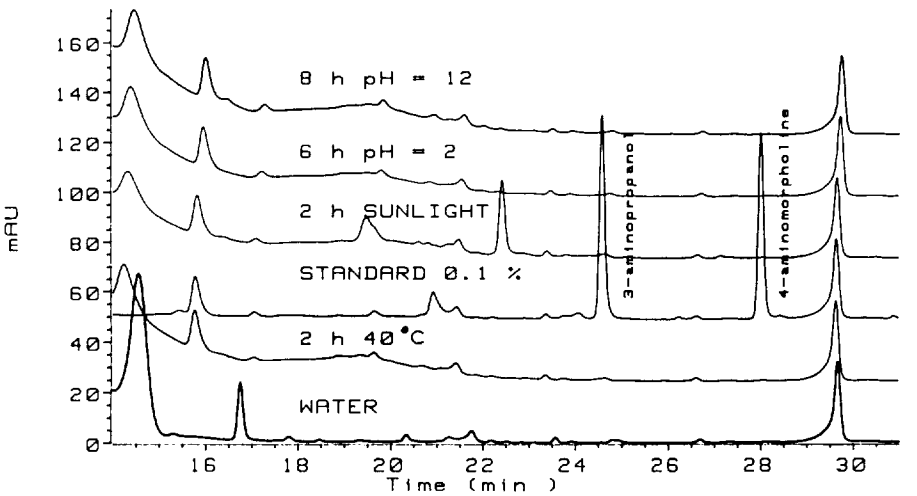


Fig. 6. Stability of molsidomine under different storage conditions. No 4-aminomorpholine was formed in the test solutions.

variation of the parameters for electrochemical detection.

3.2. Electrochemical detection

Oxidation of the strongly reducing hydrazines takes place under relatively mild conditions. A potential as low as 350 mV was found to be most suitable with respect to high sensitivity and maximum selectivity (Fig. 7). By-products were fully separated, as was shown by UV detection, and a potential as high as 1200 mV was needed to obtain a signal for other electrochemically active compounds (Fig. 8). The PCX-100 column turned out to be most convenient for chromatography as strong non-ionic interactions of the

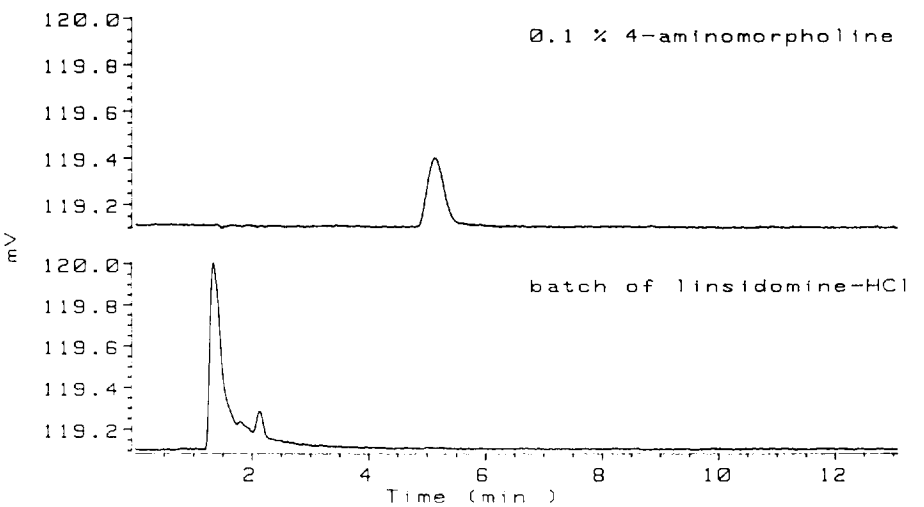


Fig. 7. Chromatograms of synthesis intermediate linsidomine hydrochloride and a standard solution of 4-aminomorpholine [0.1% relative to molsidomine (1 g l^{-1})].

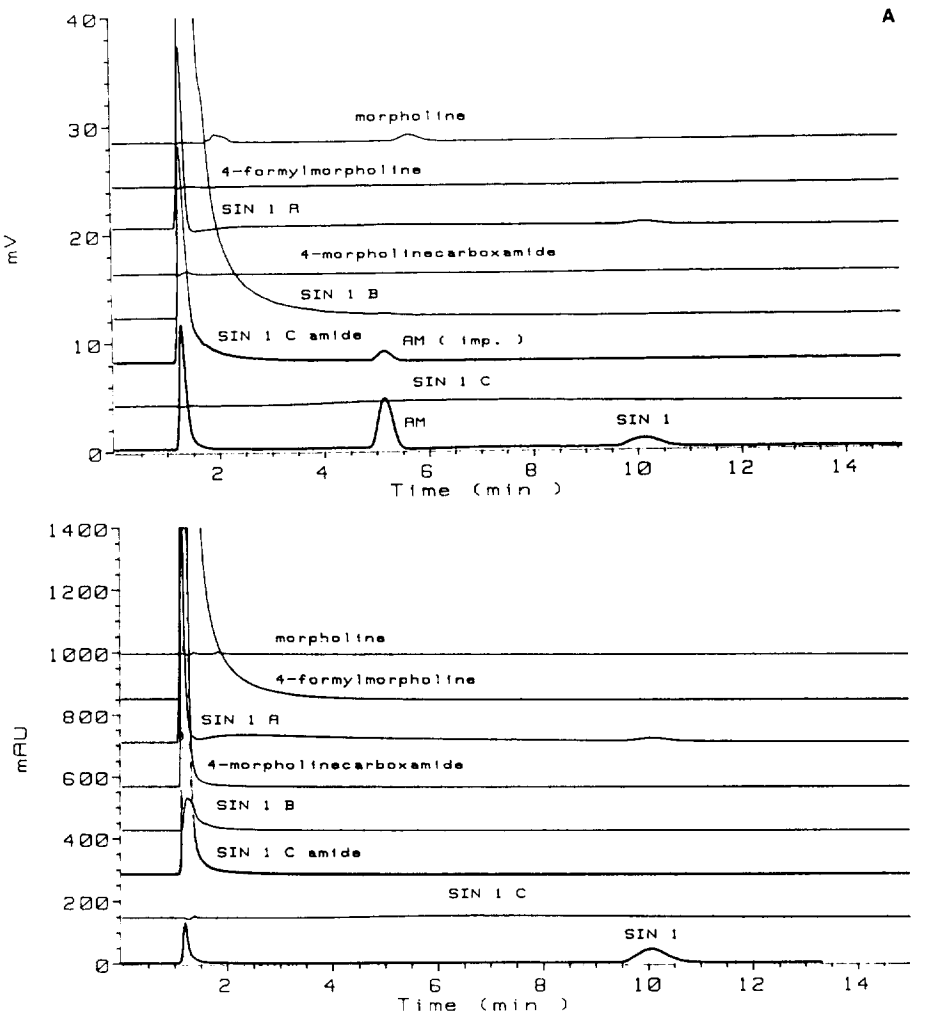


Fig. 8. Chromatograms demonstrating selectivity for the determination of 4-aminomorpholine in linsidomine. (A) Obtained by setting the potential at 1200 mV; (B) obtained by measuring at 210 nm. All by-products are well separated and are either only electrochemically active at the high potential of 1200 mV or inactive.

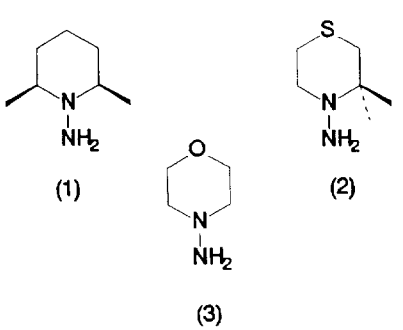


Fig. 9. Structures of the hydrazines investigated: 1 = *cis*-2,6-dimethylpiperidine; 2 = 3,3-dimethyl-4-aminothiomorpholine; 3 = 4-aminomorpholine.

ionized substances were observed on most cation-exchange stationary phases.

PCX-100 is a polymer-based stationary phase, resistant to organic solvents. Variation of the acetonitrile content in the mobile phase may easily be adjusted to influence the selectivity and to reduce non-ionic contributions to retention [6]. In addition, the pH of the mobile phase was adjusted to 4.0 so that degradation of linsidomine hydrochloride could be significantly reduced during analysis. Degradation of linsidomine hydrochloride and molsidomine was observed in alkaline solutions [5].

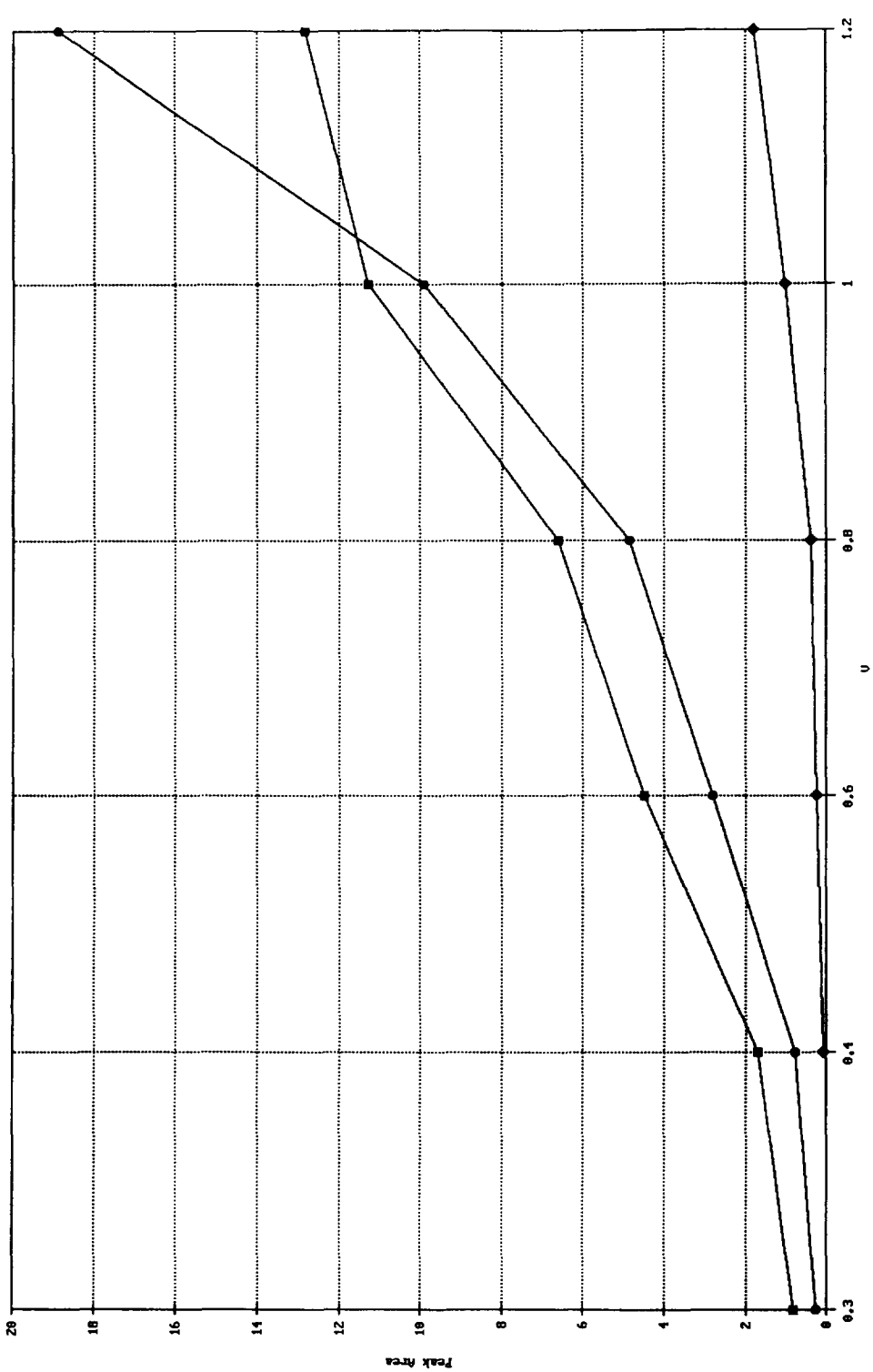


Fig. 10. Hydrodynamic voltammograms obtained for (◆) *cis*-2,6-dimethylpiperidine, (■) 3,3-dimethyl-4-aminothiomorpholine and (●) 4-aminomorpholine

The precision of the method at the limit of quantification (50 ppm) was sufficient (relative standard deviation = 7%) and no contamination of the working electrode surface could be observed, as was demonstrated by repetitive injections of the same solution without electrode pretreatment.

This mode of detection was applicable to the structurally similar 4-amino-*cis*-2,6-dimethylpiperidine and to 3,3-dimethyl-4-aminothiomorpholine (Fig. 9).

Hydrodynamic voltammograms for the three hydrazines investigated are shown in Fig. 10.

Low responses were observed for 4-amino-*cis*-2,6-dimethylpiperidine whereas similar responses were obtained for 4-aminomorpholine and 3,3-dimethyl-4-aminothiomorpholine. This may be explained by a greater steric hindrance due to the methyl groups for 4-amino-*cis*-2,6-dimethylpiperidine than for the others. We also found that the SIN 1 C analogous compound of 3,3-dimethyl-4-aminothiomorpholine yielded a good response, whereas its sulfoxide was electrochemically inactive, so oxidation obviously occurs at the sulfur atom.

4. Conclusions

Two methods suitable for routine pharmaceutical quality control for the determination of trace amounts of 4-aminomorpholine in molsidomine and linsidomine were presented, based on precolumn derivatization and ion chromatography–electrochemical detection.

The reactivity of some other hydrazines investigated was shown to be very dependent on their pattern of substitution.

Procolumn derivatization may be run on standard HPLC equipment with an appropriate autosampler. High sensitivity can be obtained using standard UV detection.

If high selectivity is required, electrochemical detection should be preferred but again the pattern of substitution has to be kept in mind when choosing suitable analytical conditions with respect to the pH value, the working potential and the stationary phase.

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