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## **Determination of the active metabolite of molsidomine in human plasma by reversed-phase high-performance liquid chromatography**

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### SUMMARY

A reversed-phase high-performance liquid chromatographic method, with ultraviolet detection, is proposed for the plasma determination of SIN-1, the active metabolite of molsidomine, which involves propoxycarbonyl derivatization. The internal standard is the ethoxycarbonyl derivative of SIN-1 (i.e. molsidomine). Derivatization and extraction are each performed in one step (2 min) with 70% yield. The nature of a by-product is discussed. The method provides rapid elution (less than 15 min), linearity over the range 0.4–200 ng/ml, day-to-day precision between 2.5 and 11.3% and a limit of determination of 0.5 ng/ml. This method is also suitable for the simultaneous

determination of molsidomine and SIN-1. In this case the internal standard is an ethoxycarbonyl derivative of a piperazino-3-sydnonimine, a SIN-1 analogue

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## INTRODUCTION

Linsidomine hydrochloride, or 3-morpholinosydnonimine hydrochloride or SIN-1, is an active metabolite of the prodrug molsidomine or Corvasal<sup>®</sup>. Intravenous infusion (Corvasal Intracoraire<sup>®</sup>) shows SIN-1 to be a potent coronary vasodilator, probably owing to its rapid transformation into SIN-1A (N-nitroso-N-morpholinoaminoacetonitrile) by opening of the sydnone ring [1-3]. SIN-1A can yield the pharmacologically active NO<sup>•</sup> radical and is transformed non-enzymically into SIN-1C (N-cyanomethylaminomorpholine) (Fig. 1).

In order to study the possible variations of the pharmacokinetic parameters in humans in different physiological and pathological situations, we developed an analytical method for the quantitative determination of SIN-1 in plasma. The measurement of molsidomine previously described by Dell and Chamberlain [4] was not sufficiently sensitive for low concentrations. Bevan and Modha [5] proposed, for the plasma determination of molsidomine and SIN-1, derivatization with butyl chloroformate to form the SIN-1 butoxycarbonyl derivative. Koyama et al. [6] proposed a high-performance liquid chromatographic (HPLC) method with UV detection for the simultaneous determination of molsidomine and its non-active metabolite SIN-1C in plasma, as the active metabolites SIN-1 and SIN-1A were thought to be readily degraded in neutral aqueous solution [1,7-10].

We have re-examined the data for the plasma determination of SIN-1 after its parenteral administration, and propose a new method based on the formation of a propoxycarbonyl SIN-1 derivative with the ethoxycarbonyl derivative of SIN-1 (molsidomine) used as an internal standard (I.S.). This method also allows the simultaneous determination of molsidomine and SIN-1 after oral administration of molsidomine in clinical studies. Therefore the analytical procedure for the simultaneous determination of molsidomine and SIN-1 is based on the formation of a propoxycarbonyl derivative for the SIN-1 and the use of the ethoxycarbonyl derivative of a SIN-1 analogous compound as an internal standard. This method only requires one extraction step and one chromatographic injection for the two compounds, and is consequently more simple, rapid and sensitive than the previous methods.

## EXPERIMENTAL

### *Chemicals*

SIN-1, molsidomine and 3-(N-methylsulphonylpiperazino)sydnonimine hydrochloride (C78-0757) (used as I.S.) were a gift of the Drug Development Department of Hoechst Pharmaceutical Labs. (Paris, France).

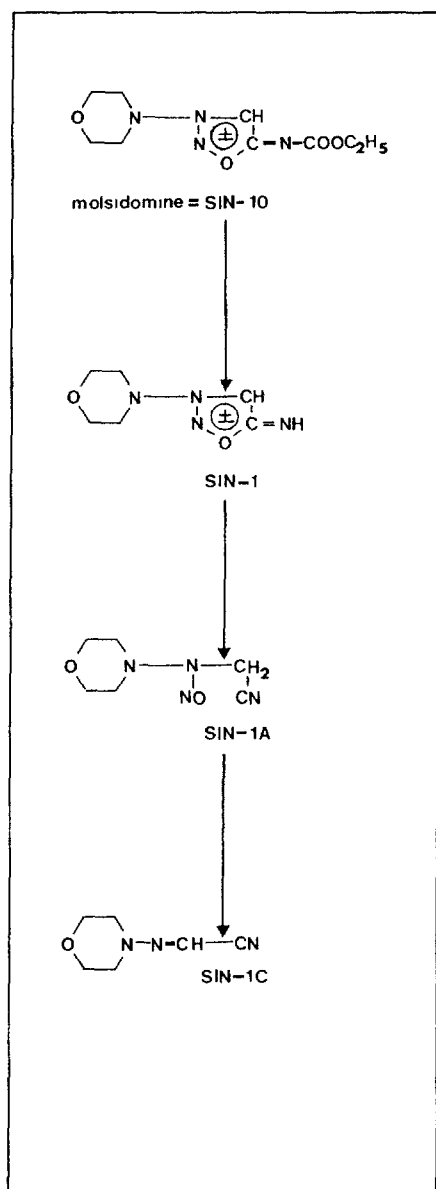


Fig. 1. Metabolism of molsidomine.

1,2-Dichloroethane (Uvasol), acetonitrile (LiChrosolv) and potassium dihydrogenphosphate (ISO) were purchased from Merck (Darmstadt, F.R.G.) and propyl (98%), ethyl (97%) and butyl (98%) chloroformates from Aldrich

(Strasbourg, France). All other chemicals were purchased from Prolabo (Paris, France) in the RP Normapur quality.

All aqueous solutions were made up in distilled water. Citrate buffer was prepared from 1.5 M citric acid and adjusted to pH 2.1 with 1.0 M potassium dihydrogenphosphate. Tris buffer was prepared from tris(hydroxymethyl)-aminomethane (Tris) and adjusted to pH 8.5 with hydrochloric acid. Acidic methanol solution was a solution of hydrochloric acid ( $d=1.19$ ) in methanol (1:1000, v/v).

### *Reference solutions*

Working solutions of SIN-1 (0.5–100 ng/ml) and of molsidomine (0.5–100 ng/ml) were prepared by dilution with the acidic methanol solution of stock standards of SIN-1 and molsidomine at 0.5 mg/ml in the acidic methanol.

Molsidomine was used as I.S. for determination of SIN-1 after SIN-1 administration, and the ethoxycarbonyl derivative of 3-(N-methylsulphonylpiperazino)sydnominine hydrochloride was used as I.S. for determination of SIN-1 and molsidomine after molsidomine administration.

### *Derivatization of 3-(N-methylsulphonylpiperazino)sydnominine hydrochloride*

Undiluted ethyl chloroformate (100  $\mu$ l), 10 ml of 1,2-dichloroethane and 2.0 ml of Tris buffer were added to 500  $\mu$ l of a stock solution of 3-(N-methylsulphonylpiperazino)sydnominine hydrochloride (1 g/l in acidic methanol). The tube was vigorously shaken on a vortex mixer for 2.0 min and centrifuged for 5.0 min, and the upper phase was withdrawn. The lower organic phase was evaporated under a stream of nitrogen at 45°C. The residue was dissolved in 5.0 ml of acidic methanol. This working solution was stored at -30°C.

### *Apparatus and chromatographic conditions*

The HPLC system consisted of a Varian 5020 pump with a manual injector (Valco) equipped with a 50- $\mu$ l loop (Varian, Les Ulis, France) and coupled with a Kratos Spectroflow 773 spectrophotometer (Cunow, Cergy, France) and a Shimadzu recorder (5 mm/min). Chromatography was carried out on a column (150 mm  $\times$  4.6 mm I.D.) packed with Beckman Ultrasphere IP ( $C_{18}$ , particle size 5  $\mu$ m) (Beckman, Gagny, France). The device was completed with a precolumn ( $C_{18}$ , 20 mm  $\times$  4.6 mm I.D., particle size 10  $\mu$ m). The mobile phase was acetonitrile-aqueous 0.01 M potassium dihydrogenphosphate (18:82, v/v) (buffer pH 4.5), and used at the flow-rate of 1.0 ml/min. The column effluent was monitored at a wavelength of 312 nm. The mass spectra were obtained with a Nermag (France) quadrupole R10.10C instrument, coupled with a Girdel 32 gas chromatograph equipped with a ross injector, cross-linked methylsilicone capillary column (25 m  $\times$  0.32 mm I.D.) (column temperature, 180°C; helium flow-rate, 1 ml/min; source temperature, 140°C).

## Methods

**Sample collection.** Blood samples (5 ml) were collected in tubes containing 100  $\mu$ l of citrate buffer (pH 2.1); the final pH was 5.4. Collected blood samples were immediately put into ice-water and centrifuged at 4°C for 5 min at 3000 g. If determinations were not to be made immediately, the separated plasma was stored immediately in plastic tubes at -80°C until analysis. As sydnonimines are sensitive to light, the tubes must be protected.

**Derivatization and extraction for determination of SIN-1 in absence of molsidomine.** A 2.0-ml volume of plasma, 100  $\mu$ l of acidic methanol, 25  $\mu$ l of molsidomine solution (2  $\mu$ g/ml) as I.S., 100  $\mu$ l of undiluted propyl chloroformate, 15 ml of 1,2-dichloroethane and 2.0 ml of Tris buffer solution (pH 8.5) (the Tris or buffer is added just before mixing) were introduced into a glass centrifuge tube. The tube was vigorously vortexed for 2.0 min and immediately centrifuged for 10.0 min. The upper phase was withdrawn and the lower organic phase was transferred to another glass tube and evaporated under a stream of nitrogen at 45°C. The residue was dissolved in a mixture of 3.0 ml of diethyl ether and 200  $\mu$ l of 0.01 M hydrochloric acid, gently vortex-mixed for 1.0 min and centrifuged for 2.0 min. The diethyl ether phase was withdrawn. The lower aqueous phase was evaporated under a stream of nitrogen at 45°C. The residue was dissolved in 150  $\mu$ l (or 75  $\mu$ l for concentrations below 1.0 ng/ml) of mobile phase and injected into the chromatograph.

**Derivatization and extraction for simultaneous determination of SIN-1 and molsidomine.** The procedure was the same as previously described, but the ethoxycarbonyl derivative of 3-(N-methylsulphonylpiperazino)sydnonimine hydrochloride was used as I.S. instead of molsidomine.

**Calibration standards.** These were prepared by adding 100  $\mu$ l of a standard solution of SIN-1 and/or molsidomine to acidified plasma [prepared using 200 ml of drug-free plasma added to 2.5 ml of citrate buffer (pH 2.1)]. They were subjected to the same treatment as the samples.

## RESULTS AND DISCUSSION

### Collection conditions

Sydnonimines are sensitive to light in the solid state and in aqueous solution. They are stable in weak acidic media but react in alkaline media [1,8-10]. We verified that aqueous solutions of SIN-1 at pH 4.5 remained stable for up to 4 h at room temperature and up to 3 h at 30°C. The instability of SIN-1 in plasma at physiological pH shows the need to adjust the pH to 5.4-5.5 in blood (5 ml) with a citric phosphate-buffered solution (pH 2.1, 100  $\mu$ l), and to cool the samples to 4-8°C. These conditions completely stabilize SIN-1 in plasma for up to 1 h; the stability drops to 97.5% 3 h after collection. If the same buffer (pH 2.1) is used but the samples are kept at room temperature, the stability is 81.5% after 1 h and 72.5% after 3 h.

### *Derivatization reaction*

The separation of SIN-1 on reversed-phase HPLC was achieved by the formation of alkoxy carbonyl linsidomine derivatives via reaction with alkyl chloroformates in alkaline media (pH 7.5–9.5) and extraction of the derivatives in a slightly polar organic solvent [11]. Reactions with ethyl, propyl, and butyl chloroformate were developed at the optimal pH of 8.5, fixed by a Tris-buffered solution.

For the plasma determination of SIN-1, the formation of the propoxycarbonyl linsidomine derivative in 0.5 M Tris buffer (pH 8.5) was chosen. This derivative has a shorter retention time (11.1 min) than the butoxycarbonyl SIN-1 derivative (28.6 min) and allows simultaneous determination of SIN-1 and molsidomine (which was not transesterified by propyl chloroformate).

The analytical procedure for derivatization must be carefully performed, and especially there must be no exposure to neutral or alkaline media. The propyl chloroformate, the extraction solvent and the Tris buffer must be added successively without delay and mixed vigorously for 2 min. The optimum conditions for the reaction of derivatization and extraction of the derivative are then achieved. The overall yield was assumed to be 70%, from the results obtained for the ethoxy derivative of SIN-1, which is molsidomine (derivatization yield 82% and extraction recovery 88%).

### *Chromatographic separation*

Representative chromatograms show the selective determination of SIN-1 in plasma (Fig. 2A). The chromatograms obtained for the simultaneous determination of molsidomine and SIN-1 are shown in Fig. 2B. Molsidomine and SIN-1 were separated within 15 min. The retention times were 5.6 and 11.1 min, respectively, and that of the I.S. (ethoxycarbonyl derivative of 3-(N-methylsulphonylpiperazino)sydnominine hydrochloride) was 7.4 min.

A compound I (peak 1, retention time 4.6 min) was present in all chromatograms and could be identified as N-chloroacetyl-3-morpholinosydnominine, formed as a by-product during the derivatization procedure.

The limits of determination for SIN-1 and molsidomine in spiked plasma were 0.5 and 0.4 ng/ml, respectively.

The linearity was verified over the range 0.4–200 ng/ml. The linear regression equation was  $y = 27.39x + 0.033$ , where  $y$  is the HPLC signal (peak height) and  $x$  is the spiked SIN-1 concentration. The correlation coefficient ( $r$ ) was 0.9999. The diethyl ether clean-up procedure improved the quality of the chromatograms and the detection limits.

The precision of the SIN-1 determination in spiked plasma samples was determined for different added concentrations (1, 25 and 100 ng/ml) and varied from 3.2% (for 100 ng/ml) to 7.5% (for 1 ng/ml) for within-day and between 2.5% (for 100 ng/ml) and 11.3% (for 1 ng/ml) for day-to-day studies. The measured concentrations were in a good agreement with the spiked con-

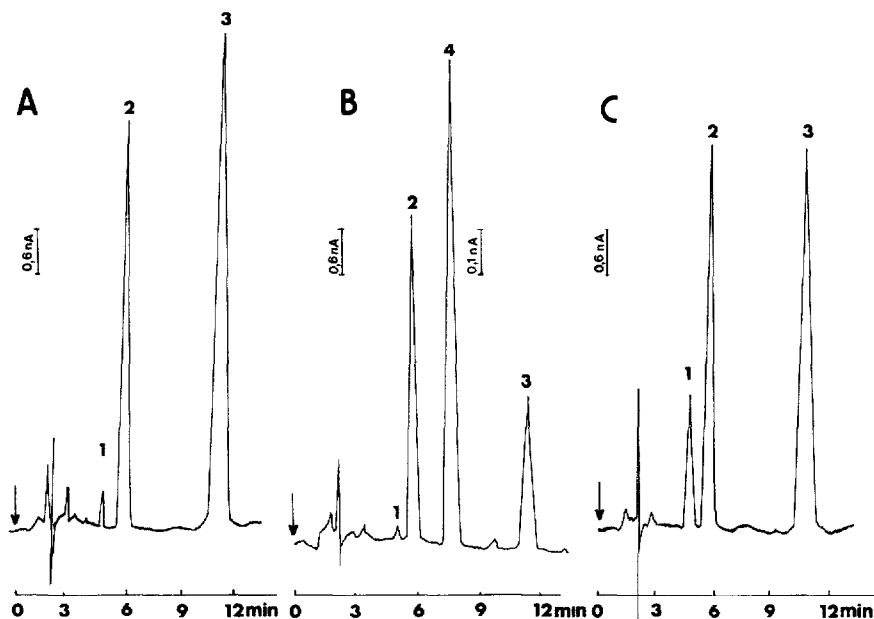


Fig. 2. Chromatograms of sample extracts. (A) HPLC of SIN-1: 1-ml plasma samples spiked with 100 ng/ml SIN-1. Peaks: 1 = compound I; 2 = molsidomine (I.S.); 3 = SIN-1 propoxycarbonyl derivative. Detector sensitivity, 0.005 a.u.f.s.; retention times, 4.6, 5.6 and 11.1 min, respectively, for the three peaks. (B) Simultaneous determination of molsidomine and SIN-1: 2-ml plasma samples spiked with 25 ng/ml molsidomine and 5 ng/ml SIN-1. Peaks: 1 = compound I; 2 = molsidomine; 4 = 3-(N-methylsulphonylpiperazino)syndonimine hydrochloride ethoxycarbonyl derivative (I.S.); 3 = propoxycarbonyl derivative of SIN-1. Detector sensitivity 0.012 a.u.f.s. for the three first peaks and set at 0.002 a.u.f.s. before the elution of SIN-1 derivative. The retention time of the I.S. is 7.4 min. (C) HPLC of products formed by derivatization of SIN-1 with commercial propyl chloroformate in aqueous solution (100 ng/ml). Peaks: 1 = compound I; 2 = molsidomine (I.S.); 3 = propoxycarbonyl-SIN-1.

centrations. The relative error ranged from  $-3.0\%$  to  $+10\%$  (for the 1.0 ng/ml concentration closest to the limit of determination). Quality control of spiked SIN-1 plasma samples indicated that SIN-1 is stable ( $18.3 \pm 1.5$  ng/ml) in the frozen state ( $-20^\circ\text{C}$ ) for at least twelve months.

With this HPLC method the retention times of the HPLC peaks of the ethoxy; propoxy- and butoxycarbonyl linsidomine derivatives were 5.6, 11.1 and 28.6 min, respectively. Independent of the reagent used, the minor peak of compound I was observed at a retention time of 4.6 min.

After derivatization of SIN-1 in aqueous solutions (pH 5.4) at 40 and 100 ng/ml (Fig. 2C) and in a spiked 100 ng/ml plasma sample (pH 5.4) (Fig. 2A), the formation of compound I (peak 1) in plasma is lessened and that of the propoxycarbonyl SIN-1 derivative (peak 3) is increased. This result suggests that compound I may depend on the degree of SIN-1 uptake. Therefore it is

necessary to use a stable I.S. derivatized prior to its addition to plasma. The ratio of the peak areas of I and propoxycarbonyl SIN-1 is 0.2 for aqueous solutions up to 1000 ng/ml and 0.05 for spiked plasma up to 1000 ng/ml.

SIN-1 plasma concentrations ranged between 10 and 100 ng/ml after a single intravenous administration of 1 mg of SIN-1, and from 1 to 10 ng/ml after a 2-mg single oral dose of molsidomine. Determination of SIN-1 as its propoxycarbonyl derivative is reliable, as the linearity of the calibration curve verified (1–100  $\mu\text{g/ml}$ ). Compound I was formed in negligible amounts in the plasma of patients and did not impair the determinations of SIN-1 in the proposed analytical procedure.

#### *Identification of compound I by gas chromatography–mass spectrometry (GC–MS)*

In order to study the characteristics of the observed peaks, the mass spectra of molsidomine, propoxycarbonyl SIN-1 and compound I were analysed by MS and GC–MS (electron impact). The molecular masses were confirmed by chemical ionization. Molsidomine and propoxycarbonyl SIN-1 are differentiated only by the molecular peak,  $m/z$  242 and 256, respectively, in low abundance (Fig. 3). For both molecules the observed fragmentations can result from the carbonyl or the morpholinic nitrogen ionization. Ionization of the carbonyl group induces the ejection of the ethoxy or propoxy radical to generate the  $m/z$  197 ion or, after rearrangement (McLafferty), the formation of the  $m/z$  168 radical ion, which produces the  $m/z$  140 ion (the most abundant ion) and the  $m/z$  70 radical ion (Fig. 4a). Cleavage of the N–morpholino bond (Fig. 4c) yields the morpholine ion  $m/z$  86 (abundance 35–45%). The M–30 ion (loss of NO) is produced capture of the hydrogen of the oxadiazole ring, and then successive fragments  $m/z$  113 and 87 or 101 may result from the ionization of the N-3 nitrogen of the oxadiazole cycle.

The  $m/z$  57 and 42 ions (Fig. 3) may result from fragmentation of the morpholine ring or of the  $m/z$  168 ion.

Compound I has a mass spectrum similar to those of the other two compounds (Fig. 3), with a molecular ion of 246 (197+49), which indicates the acylated morpholinonydnonimine structure (RCO with R=49) (Fig. 4). The existence of four pairs of fragments at  $m/z$  246 and 248 [M, (M+2)], 216 and 218 [M–30, (M+2)–30], 77 and 79 [49+18, (49+2)+18] and 49 and 51 [M–197, (M+2)–197] indicates the presence of a chlorine atom. Thus we can postulate a formula for I with R=CH<sub>2</sub>Cl (Fig. 3). The MS peaks for compound I correspond to the following events: (1) rupture of the bond alpha to the carbonyl group, with  $m/z$  197 and then  $m/z$  77 and 79 ions being formed (Fig. 4a); (2) chlorine ionization (Fig. 4b) with formation of the CH<sub>2</sub>Cl<sup>+</sup> ( $m/z$  49 and 51) ions (abundance 30%); (3) rearrangement of the  $m/z$  125 radical ion, with the formula proposed in Fig. 4b.

The proposed formula for compound I is drawn in Fig. 3c. It is chloroacetyl-



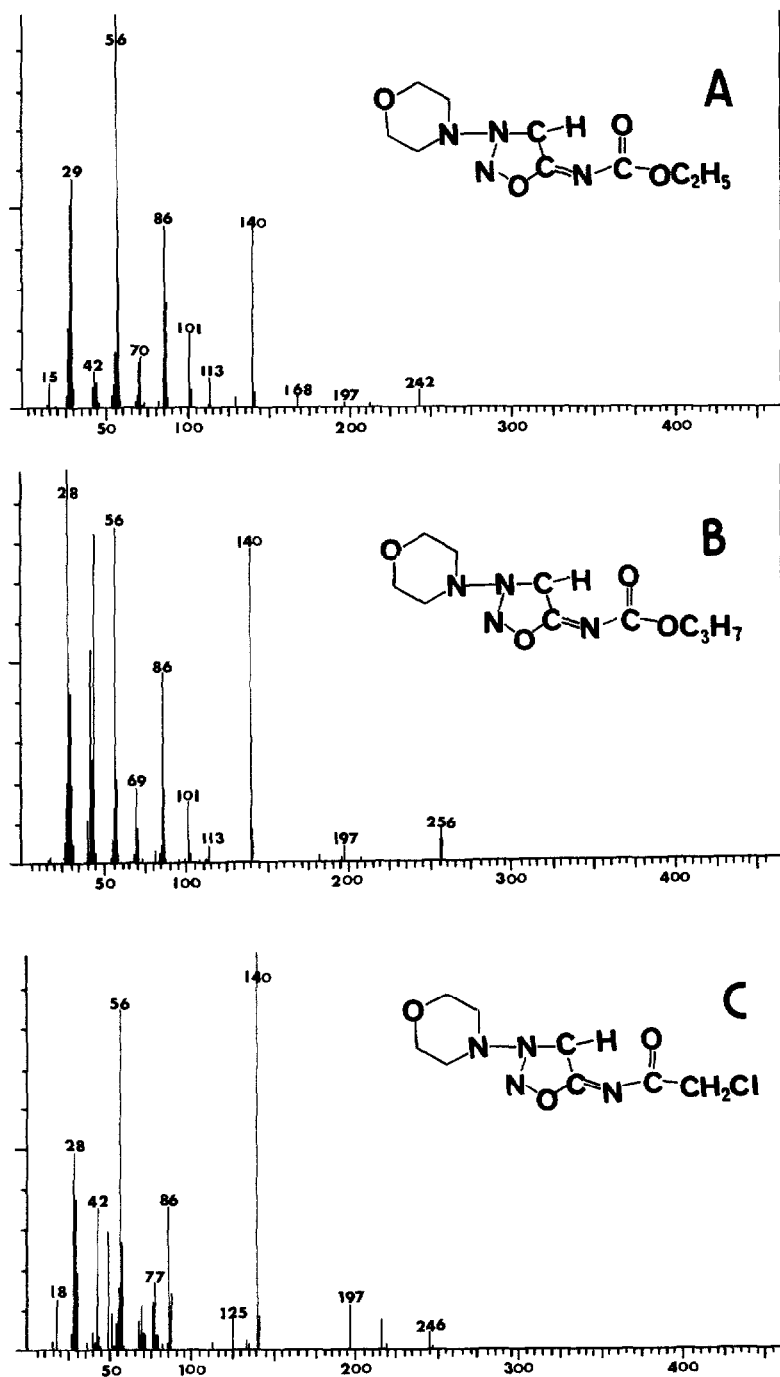


Fig. 3. Electron-impact mass spectra and structures of (A) molisdimine, (B) propoxycarbonyl linsidimine (P-SIN-1) and (C) chloroacetyl linsidimine (I) (by-product in the derivatization of SIN-1 with propyl chloroformate).



linsidomine, a secondary product found during SIN-1 derivatization with propyl chloroformate, by reaction of SIN-1 with a derivative of monochloroacetic acid ( $\text{ClCH}_2\text{COX}$ ) present as an impurity (less than 2%) in the alkyl chloroformate reagent used.

These findings were confirmed by an additional assay showing that the reaction of SIN-1 with monochloroacetyl chloride (Aldrich) formed, under the proposed analytical conditions for the SIN-1 determination, a product with the same chromatographic characteristics as compound I.

### Applications

A pharmacokinetic pilot study was performed in two coronary patients (48 and 56 years old) each receiving a 1-mg intravenous single dose of SIN-1 (Corvasal Intracronaire). The time-related decrease of the SIN-1 plasma concentrations (ng/ml) is shown in Fig. 5A. The half-life of SIN-1 is  $29.5 \pm 1.7$  min.

A second pharmacokinetic study was performed in six healthy young volunteers ( $25.5 \pm 3.1$  years old) after a 2-mg single oral dose of molsidomine (Corvasal). The time-courses of the plasma concentration were determined by the described analytical method. The results for molsidomine and SIN-1 are shown in Fig. 5B:  $C_{\text{max}}$  is  $17.9 \pm 1.7 \mu\text{g l}^{-1}$  for molsidomine and  $5.3 \pm 0.5 \mu\text{g l}^{-1}$  for SIN-1. The molsidomine half-life is  $1.2 \pm 0.1$  h and the SIN-1 apparent half-

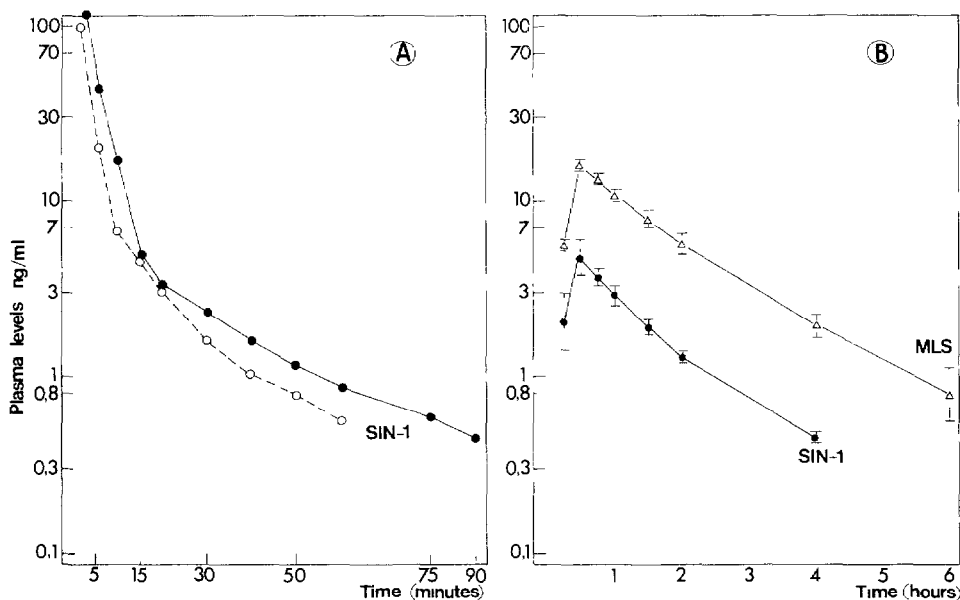


Fig. 5. Plasma concentration-time curves of (A) SIN-1 after a single 1-mg intravenous dose in two coronary patients and (B) molsidomine (MLS) and SIN-1 after a single 2-mg oral dose of molsidomine in six young healthy volunteers.

life is  $1.0 \pm 0.08$  h. These data are in good agreement with those previously reported [6,12-14].

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