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Short Communication

Determination of the calcium antagonist SIM6080 in plasma by high-resolution gas chromatography

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Abstract

A sensitive and selective gas chromatographic method for the determination of the calcium antagonist SIM6080 in plasma has been developed and validated. A three-step extraction procedure is employed followed by capillary gas chromatographic analysis using nitrogen-selective detection and the programmed temperature vaporizer injection technique. The defluorinated analogue was used as the internal standard. The analysis of spiked plasma demonstrated the good accuracy and precision of the method with limit of detection of 1 ng/ml. The method has been used for pharmacokinetic studies in laboratory animals.

1. Introduction

SIM6080 (I) (Fig. 1) is a new calcium antagonist combining transmembrane and intracellular calcium antagonistic properties [1–3]. The haemodynamic profile showing peripheral and coronary vasodilator activity without myocardial depression parallels the in vitro separation of vascular, inotropic and chronotropic effects and suggests that I is a potentially useful agent for the treatment of coronary heart disease. Anti-atherosclerotic effects were observed and could provide an additional dimension to the therapeutic potential of the compound [4,5].

In order to study the pharmacokinetic profile of I in laboratory animals a sensitive analytical technique capable to measure concentrations for up to 24 h after administration was required. Other calcium antagonists which share some common substructure units such as the

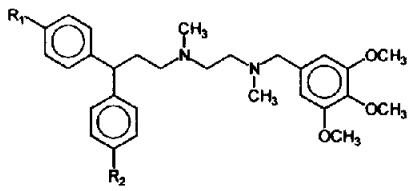

Fig. 1. Structures of I (SIM6080) and internal standard (I.S.).

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alkylarylamines verapamil and gallopamil or the piperazine derivative flunarizine were analyzed by gas chromatography with selective detection [6,7] or by high-performance liquid chromatography, mainly with the high-sensitive fluorimetric detection [8,9].

This paper reports an assay for the quantification of I in plasma. The drug was isolated by a three-step extraction procedure and analyzed by high-resolution gas chromatography (HRGC) with a nitrogen-selective detector (NPD) and the programmed temperature vaporizer (PTV) injection technique. These features give the method the sensitivity and the specificity required to determine plasma concentration in laboratory animals following single or multiple oral doses in the range 3–10 mg/kg. Due to the availability of an automated sample injector, up to 48 samples can be assayed daily by a single operator.

2. Experimental

2.1. Materials

N_1,N_2 - Dimethyl - N_1 - [3,3 - di - (4 - fluorophenyl)propyl] - N_2 - (3,4,5 - trimethoxybenzyl)-ethylenediamine dihydrochloride, (SIM6080, I) and the internal standard E974/1 (I.S.), the defluorinated analogue (Fig. 1) were synthesized by the Medicinal Chemistry Department of Zambon Research. Heptane (Resy-analyzed reagent) was purchased from Baker (Deventer, Netherlands). Analytical grade organic solvents and chemicals were purchased from Carlo Erba (Milan, Italy).

Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The disposable Pyrex glass culture tubes with PTFE-lined screw-cap (16 × 100 mm) and pipettes (5 ml) were from Corning Inc. (Corning, NY, USA). Automatic micropipettes were from Brand (Wertheim-Main, Germany).

2.2. Equipment and chromatographic conditions

The HRGC system was a DANI 3800 HR-PTV chromatograph equipped with a NPD 68/20 detector, a PTV 382/88 injector and an ALS 3940 autosampler (all from DANI, Monza, Italy). A fused-silica capillary column (10 m × 0.32 mm I.D.) coated with CP Sil-5 chemically bonded phase at a film thickness of 0.12 μm (Chrompack, Middelburg, Netherlands) was used. The programmed temperature vaporizer injection technique was selected with an initial temperature of 80°C and a final temperature of 270°C. A 4-μl sample was injected. The carrier gas was helium at a flow-rate of 1.2 ml/min (measured at 200°C). The combustion gases hydrogen and air were supplied at a flow-rate of 5 ml/min and 130 ml/min respectively. The make-up gas was helium at a flow-rate of 15 ml/min. The initial oven temperature was 200°C which was maintained for 1 min and was then increased by 5°C/min to 285°C. The NPD detector temperature was 300°C and the rubidium pearl temperature was generally set at 460°C at the installation and then gradually increased to compensate for aging. The chromatograms were acquired with an SP-4270 integrator linked to a Chromstation/2 system, all from Spectra-Physics (San Jose, CA, USA).

2.3. Preparation of stock and working solutions

Stock solutions of I and I.S. (1 mg/ml) were prepared in methanol and were stable for at least one month when stored at − 20°C.

Working solutions of I (1 μg/ml) and I.S. (2 μg/ml) were prepared daily by dilution of the corresponding stock solutions with acetonitrile–0.01 *M* HCl (25:75, v/v).

2.4. Extraction

The frozen plasma (− 20°C) was thawed at room temperature and a 0.1–1.0 ml aliquot was pipetted into a disposable glass culture tube. If less than 1 ml was used, water was added to obtain a final volume of 1 ml. Internal standard (100 ng = 100 μl of the working solution), 4 *M* sodium hydroxide (125 μl) and 5 ml of the organic extracting solvent (heptane–*n*-pentanol, 95:5, v/v) were added to each tube.

The plasma sample was extracted for 20 min on a horizontal mechanical shaker and then

centrifuged for 1 min at 1200 *g*. The organic phase was transferred to another disposable tube and extracted into 2 ml of 0.1 *M* hydrochloric acid for 5 min. After centrifugation (1 min, 1200 *g*) the organic phase was aspirated off, the sample was made alkaline with 175 μl of 4 *M* sodium hydroxide and then extracted with 5 ml of the organic extracting solvent (heptane–*n*-pentanol, 95:5, v/v) for 20 min. The organic phase was transferred to another disposable tube and evaporated to dryness under nitrogen at 40°C. The residue was dissolved in 100 μl of *n*-hexane, containing 1% triethylamine and 1% *n*-pentanol, which were transferred into the autosampler vial.

2.5. Calibration

Standard curves were prepared using 1 ml of drug-free heparinized plasma obtained from the appropriate animal species. The standards spanned one order of magnitude, *i.e.* 25, 50, 75, 100, 150 and 200 ng/ml of I, and were prepared by adding appropriate volumes of its working solution. The calibration curve was obtained from the least squares linear regression of the peak-area ratio of I to the internal standard against the concentration of I.

3. Results and discussion

3.1. Selectivity and sensitivity

Chromatograms of drug-free plasma, plasma spiked with known concentration of I, and plasma from a dog receiving a single oral dose of I 10 mg/kg taken 10 h after administration are shown in Fig. 2. The chromatograms show both the sensitivity of the assay, which gives a quantitation limit of 5 ng/ml and a detection limit (at a signal-to-noise ratio $S/N = 3$) of 1 ng/ml, and the selectivity, as no interfering peaks are seen at the retention time of I. While the selectivity is due to the extensive clean-up of the three-step extraction procedure, to the specificity of the NPD detector and to the efficiency of the capillary column the main contribution to the sensitivity is given by the PTV injector [10] which allows the introduction of a large amount of sample (up to 5 μl). Additional advantages offered by the PTV, compared with the on-column injector, are the absence of contamination of the analytical column since non-volatile matter is trapped in the injector precolumn, and the rapid analysis time because only the injector and not the whole column has to be cooled to near ambient temperature which can be done in only a few seconds.

3.2. Absorption to glassware

Absorption turned out to be one of the major problems in the development of the assay. If the same test tubes that were employed for the analysis of samples containing I were next used to analyze blank plasma samples after our usual glassware cleaning procedure, an unacceptable residual signal was generally found due to contamination from previous samples. Washing the tubes with sodium hydroxide and rinsing with water and methanol as well as silylation decreased but not eliminated the problem. The use of disposable glassware eliminated both the contamination and a tedious cleaning procedure.

Even using disposable glassware and autosampler vials a peak at the retention time of I was seen when pure hexane was used to prepare the solution for the analysis. The intensity of this peak was in the range 1–10 ng/ml and could interfere with the determination of the low levels occurring in the low-concentration range of the pharmacokinetic curve. This interference was due to binding of I to active centres of the chromatographic system. This could be avoided by using hexane containing small amounts of triethylamine and *n*-pentanol.

3.3. Validation

The within-day accuracy and precision of the method were assessed by extracting and analyzing plasma samples spiked with I at five different concentrations four times on one day. Accuracy was within 5% of the theoretical value at each concentration of I. The coefficient of variation (C.V.) was $\leq 6\%$. The results are summarized in Table 1.

The day-to-day accuracy and precision were

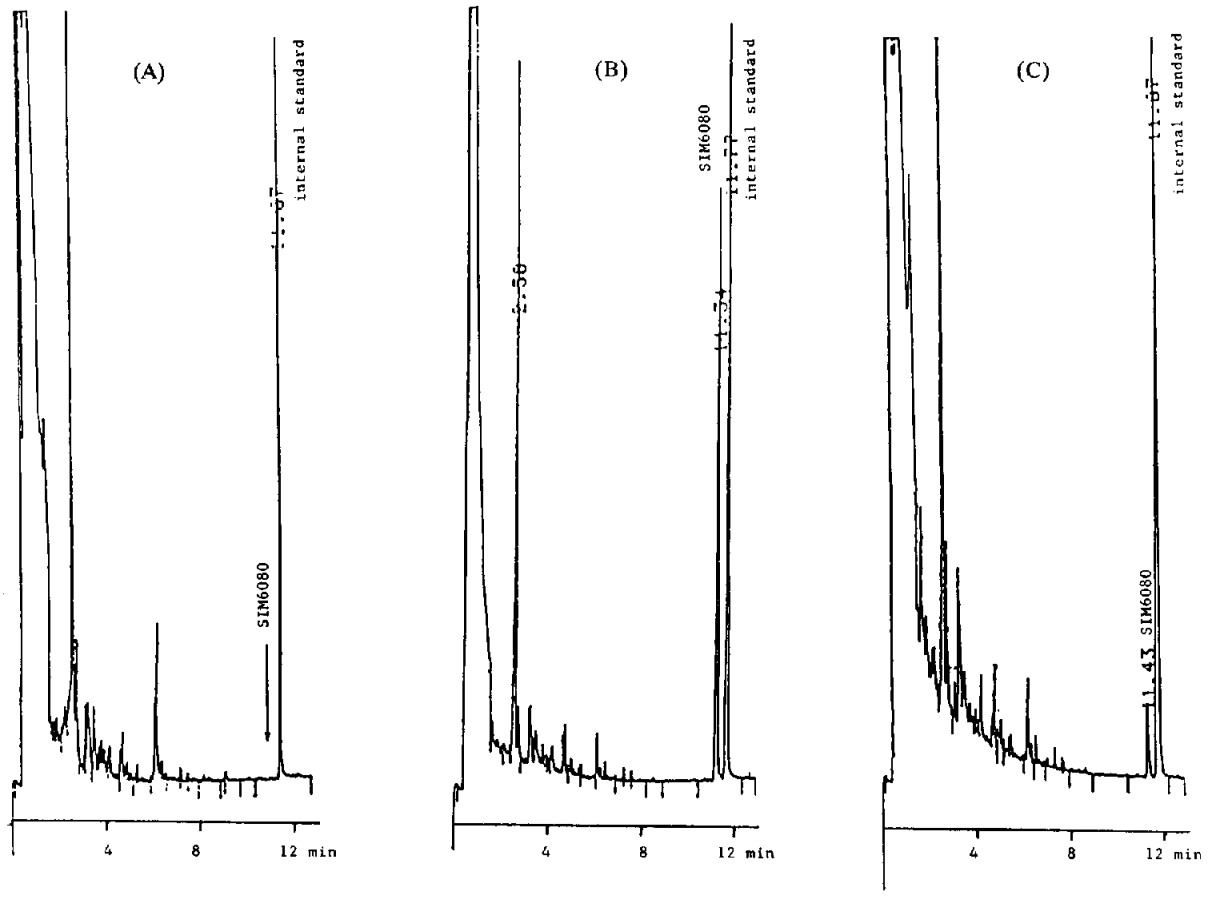

Fig. 2. Chromatograms of extracts from (A) drug-free plasma sample spiked with internal standard (200 ng/ml), (B) plasma sample spiked with I (SIM6080) (150 ng/ml) and internal standard (200 ng/ml), and (C) plasma sample from a dog 10 h after a single oral dose of I 10 mg/kg. Drug concentration is 18 ng/ml.

determined by extracting and analyzing plasma samples spiked with I at five different concentration on five consecutive days. All samples were evaluated using the calibration curve obtained on day 1. The results are listed in Table 2 and show the very good accuracy and precision of the method in the concentration range tested.

The precision of the HRGC analysis was

Table 1
Within-day accuracy and precision of the determination of I in spiked plasma by HRGC-NPD analysis

Concentration added (ng/ml)	*n* (mean ± S.D.) (ng/ml)	Concentration found (%)	C.V. (%)	Accuracy (%)
25	4	25.1 + 1.2	4.8	+ 0.4
50	4	51.9 + 3.1	6.0	+ 3.2
75	4	77.0 + 3.5	4.6	+ 2.7
100	4	104.6 + 4.7	4.4	+ 4.6
200	4	196.0 + 3.1	1.6	− 2.0

Table 2
Day-to-day accuracy and precision of the determination of I in spiked plasma by HRGC-NPD analysis

Concentration added (ng/ml)	Concentration found (ng/ml)					Mean ± S.D.	C.V. (%)	Accuracy (%)
	day 1	day 2	day 3	day 4	day 5			
25	25.1	22.6	23.8	27.7	24.0	24.3 ± 2.2	9.1	−0.7
50	51.9	47.3	49.1	51.9	47.5	49.5 ± 2.3	4.6	−0.5
75	77.0	79.4	73.1	79.1	76.7	75.1 ± 5.3	7.1	+0.1
100	104.6	99.0	103.1	100.7	105.1	103.8 ± 4.8	4.6	+3.8
200	196.0	196.5	199.4	192.6	195.5	195.9 ± 2.4	1.2	−2.1

evaluated from six injections of a plasma sample spiked with 100 ng/ml of I. The low C.V. (1.2%) allowed a single injection for each sample.

The stability of the drug in plasma was assessed by preparing plasma samples spiked with 100 ng/ml of I.

The spiked samples were immediately frozen, kept at −20°C and analyzed over a five-month period. The results showed that storage of the frozen plasma sample does not affect the recovery of the drug.

4. References

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