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Short Communication

Determination of the platelet activating factor antagonist 6-(2-chlorophenyl)-9-[(4-methoxyphenyl)thiocarbamoyl]-1-methyl-7,8,9,10-tetrahydro-4H-pyrido[4′,3′-4,5]-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4] diazepine in human plasma by liquid chromatography–thermospray mass spectrometry☆

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Abstract

A sensitive and specific method for the determination of the platelet activating factor (PAF) antagonist 6-(2-chlorophenyl)-9-[(4-methoxyphenyl)-thiocarbamoyl]- 1-methyl-7,8,9,10-tetrahydro-4H-pyrido[4′, 3′-4, 5]thieno-[3, 2-*f*][1, 2, 4]triazolo[4, 3-*a*][1, 4] diazepine (I) in human plasma is described. The target molecule was analyzed by high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) after extraction by ion-exchange chromatography. HPLC was carried out using a C_{18} column and the coupling to the MS was done by a thermospray (TSP) interface working in the direct ion-evaporation ionization mode in presence of 0.1 *M* ammonium acetate. Selected-ion monitoring (SIM) was carried out for the ion *m/z* 370 and its $[M + 2]^+$ isotopic peak. Evaluation of the intensity matching of such ions has been used in the validation results. The method gives good accuracy and precision over the concentration range 1–200 ng/ml in human plasma.

1. Introduction.

6 - (2 - Chlorophenyl) - 9 - [(4 - methoxyphenyl)-thiocarbamoyl] - 1 - methyl - 7,8,9,10 - tetrahydro-4H pyrido[4′,3′ - 4,5]thieno[3,2 - *f*][1, 2, 4]triazolo[4, 3 - *a*][1 , 4] diazepine (I) is one of the most representative molecules of a series of synthetic dual antagonists of the platelet activating factor (PAF) with a captodative center allowing free radical scavenging activity, the chemical structure of which is shown in Fig. 1. These compounds are of potential therapeutic value in several pathologies, particularly those concerning the bronchopulmonary system, inflammatory processes and allergic responses [1]. Since I is under clinic development, precise analytical methods are needed to measure its levels in biological fluids.

In a previous paper [2], we have reported the

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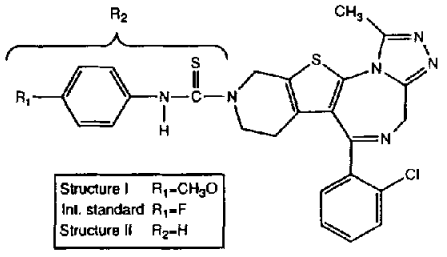

Fig. 1. Structures of the compounds used in this study.

mass spectrometric properties of several constituents of this series and we concluded that LC–MS using a thermospray interface, where strong peaks were present in the spectra, could be a good technique to quantify these compounds.

In the present paper we describe a method to measure the level of I in human plasma by HPLC–TSP-MS after extraction by ion-exchange chromatography. This method allows the measurement of concentrations of I over the range 1–200 ng/ml of human plasma with good accuracy and precision.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile and methanol were purchased form SDS (Peypin, France), HPLC grade. Water was purified by a Milli Q system from Millipore (Milford, MA, USA). Ammonium acetate was from Aldrich (Steinheim, Germany), ACS reagent. Ammonia solution was from Merck (Darmstadt, Germany), p.a. 25%. Polyethylene glycol was from Janssen Chimica (Beerse, Belgium), average molecular mass 400. Compound I (BN50730) and the internal standard (BN50765) were from the Institute Henri Beaufour (Paris, France), compound I being under phase II of clinical development.

2.2. Preparation of calibration curves

A standard stock solution (500 μg/ml) was prepared by dissolving 5.0 mg of I in 10 ml of methanol. A serial dilutions scheme in the same solvent was applied to get the suitable range of calibration standard solutions of 4000, 2000, 800, 400, 200, 100, 50 and 20 ng/ml. Internal standard stock solution (500 μg/ml) was prepared by dissolving 5.0 mg of this compound in 10 ml of methanol and the working internal standard solution (400 ng/ml) was prepared by two consecutive 1:25 and 1:50 dilutions with methanol of the former solution.

Calibration curves were prepared as follows: 8 aliquots of 1.0 ml of untreated human plasma were transferred into 10-ml glass centrifuge tubes fitted with PTFE lined screw caps and spiked with 50 μl of internal standard solution (effective concentration 20 ng/ml) and 50 μl of each standard solution. Effective concentrations of I in plasma samples were 200, 100, 40, 20, 10, 5, 2,5 and 1 ng/ml. One plasma blank with only 100 μl of methanol and another with 50 μl of internal standard and 50 μl methanol were also prepared for each calibration curve.

2.3. Sample preparation

The plasma samples with the suitable standard solutions added, were vortex-mixed for a short time and extracted with Bond Elut CBA 1-ml columns that were previously conditioned with 1 ml of methanol and 1 ml of water. After a cleaning step with 2 ml of water, elution was carried out with 1 ml of methanol. After evaporation of the solvent under a stream of nitrogen, the samples were resuspended in 200 μl of the mobile phase.

2.4. HPLC–TSP-MS

The HPLC system consisted of two Applied Biosystems (San Jose, USA) SF-400 pumps and one Applied Biosystems 1000S diode-array UV detector fitted with a high-pressure flow-cell to permit direct in-line UV detection. A Spark Holland (Aj Emmen, Netherlands) Must HP 6 multiport stream-switch allowed to pass the flow through the column in the analyzing mode or to bypass it in the calibration step. The column used was packed with Kromasil C_{18} from Eka Nobel (Bohus, Sweden), 5 μm particle size,

250 × 4.6 mm I.D. A 2-μm pore screen filter from Supelco (Bellefonte, CA, USA) was placed between the Must HP 6 multiport stream-switch and an Applied Biosystems 419 dynamic mixer/injector. Elution was isocratic with 0.1 *M* ammonium acetate buffer (pH 8.0)–and acetonitrile (55:45, v/v) during 16 min. A washing step with 100% acetonitrile was also carried out after each injection. The flow-rate was 1.0 ml/min and the injection volume was 100 μl.

A Kratos (Manchester, UK) Profile HV-3 double focussing mass spectrometer fitted with a thermospray interface and a liquid nitrogen trap was used for this study. Data were acquired and analyzed by a Kratos Mach 3 networked data processing workstation. Thermospray source temperature was 160°C. The average inlet and tip temperatures were 122°C and 195°C, respectively. Positive thermospray spectra were obtained at full acceleration voltage (4 kV) with a scan speed of 3 s per decade in the nominal data collection mode. SIM experiments were carried out at a cycle time of 0.4 s and the apparatus was focused on the ions *m/z* 370 and 372. Calibration was achieved with a solution of polyethylene glycol, average molecular mass 400 (0.3 mg/ml) in 0.1 *M* ammonium acetate buffer pH 8.0, injected in the flow injection mode. Mass chromatograms were smoothed prior to integration.

3. Results and discussion

Mass spectra of both I and the internal standard (Fig. 2) show a cluster peak at *m/z* 370 that corresponds to the $[M+H]^+$ ion of 6-(2-chlorophenyl)-1-methyl-7, 8, 9, 10-tetrahydro-4H-pyrido[4′, 3′-4, 5]thieno[3, 2-*f*][1, 2, 4]triazolo[4, 3-*a*][1,4] diazepine (II) that is produced by thermal degradation of compound I in the TSP interface [2]. The weak peaks at lower masses correspond to the ion $[M+NH_4]^+$, M being a isothiocyanate that also results from degradation of the parent compound. A peak isotopic coefficient (I_p) was defined as the quotient between the intensities of the peaks of *m/z* 370 and 372. The theoretical value $I_p = 2.54$, obtained from the relative isotopic intensities calculated with the Atoms computer program in the Mach 3 software package, agreed with the experimental value of the coefficient: $I_p = 2.40 \pm 0.17$ ($n = 9$, C.V. = 6.9%, −5.6% of difference between experimental and theoretical values).

A blank plasma chromatogram showed no interfering peaks at the retention times of the compounds studied. The mean retention time for 12 runs was 10.5 min (C.V. = 0.4%) for I and 13.1 min (C.V. = 0.4%) for the internal standard. The measured response ratio of I was calculated as the quotient between its area response and the area response of the internal

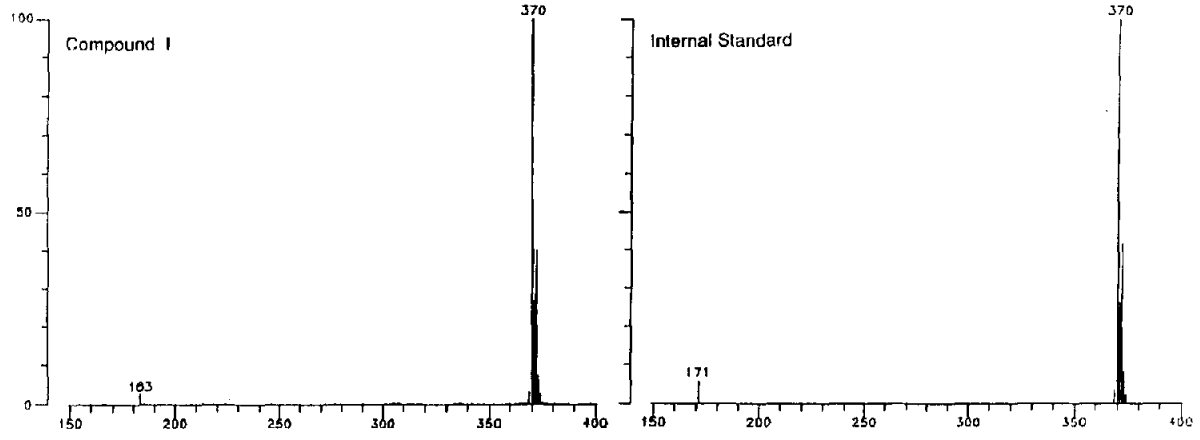

Fig. 2. Thermospray mass spectra of compound I and the internal standard.

standard. Weighted least squares (weighting factor = concentration^{-2}) fitting was performed of the effective concentration *vs.* response ratio to equation: C(concentration) = slope × response ratio ± intercept. Quantitation was found to be linear over the concentration range 1–200 ng/ml. In all cases the correlation coefficient was between 0.989 and 0.997.

The limit of quantitation was established as 1 ng/ml of plasma with an accuracy and a precision of 3% for five spiked samples. Fig. 3 shows the mass chromatograms of a human plasma sample with 5 ng of I/ml of plasma.

Accuracy of the analysis was estimated as the percent difference (error) between the mean values and the true or known concentrations. Precision was estimated as the relative standard deviation (coefficient of variation) of the measured concentrations of replicate samples. Two series of four calibration curves were prepared with an interval of two month. During each period, calibration curves were prepared on four consecutive days, one each day. Intra-period accuracy and precision values were calculated for the two series of replicates and inter-period data were obtained from these two series considered as a unique series of eight replicates. Table 1 shows the accuracy and precision data.

Even though blank plasma chromatograms showed that no interfering peaks appeared at the retention times of the compounds studied, we have performed an evaluation of the chromatographic isotopic coefficient I_c defined as the quotient between the peak areas of the mass chromatograms at m/z 370 and 372. Good agreement of the value of this coefficient with the theoretical value of the previously defined I_p indicates that no interference substances are coeluting with both I and the internal standard [3].

Table 2 shows the mean values of I_c at the different concentrations of I used in the calibration curves. Although the relative standard deviation (C.V.) is relatively high for the lowest concentrations due to the difficulty to measure the small $[M+2]^+$ peak, the accuracy expressed as the percent differences between the mean I_c values and the theoretical values are not higher than 10%. The overall I_c for compound I is 2.49 ± 0.35 (n = 53, C.V. = 10%, −2.1% of difference) and for the internal standard is 2.48 ± 0.34 (n = 54, C.V. = 14%, −2.4% of difference)

4. Conclusions

The coupling of high-performance liquid chromatography with mass spectrometry using a thermospray interface allows the measurement of compound I over the concentration range 1–200 ng/ml in a human plasma sample with a

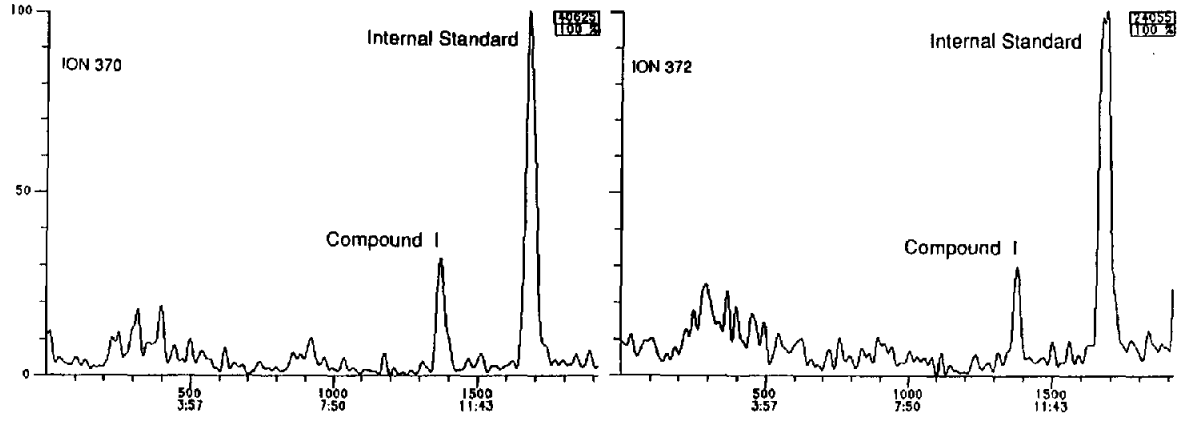

Fig. 3. Representative mass chromatograms of ions m/z 370 and 372 of a plasma sample with 20 ng/ml of internal standard and 5 ng/ml of compound I.

Table 1
Accuracy and precision data for the LC–MS analysis of compound I

Period	Parameter	Concentration (ng/ml)							
		1	2.5	5	10	20	40	100	200
1	mean	1.03	2.35	5.03	9.88	20.2	41.4	92.7	205
	Error (%)	3.2	−6.0	0.56	−1.2	0.78	3.4	−7.3	2.4
	C.V. (%)	3.3	7.9	8.2	9.9	8.9	5.6	7.9	4.0
	n	3	4	4	4	3	4	4	4
2	mean	1.01	2.49	5.05	8.89	21.4	41.3	92.8	194
	Error (%)	1.2	−0.53	0.90	−11	6.8	3.3	−7.2	−2.9
	C.V. (%)	5.7	10	5.3	8.2	6.5	6.9	11	1.8
	n	2	4	4	3	4	4	3	4
Overall	mean	1.02	2.42	5.04	9.45	20.9	41.3	92.7	200
	Error (%)	2.4	−3.3	0.73	−5.5	4.2	3.3	−7.3	−0.22
	C.V. (%)	4.5	9.5	6.9	11	8.1	6.3	9.5	4.1
	n	5	8	8	7	7	8	7	8

Table 2
Values of I_c for the LC–MS analysis of compound I

Concentration (ng/ml)	Mean I_c	C.V. (%)	*n*	Difference (%)[a]
1	2.60	22	5	2.4
2.5	2.28	16	6	−10
5	2.65	9.5	7	4.3
10	2.60	17	7	2.4
20	2.33	9.2	7	−8.2
40	2.46	7.6	7	−3.0
100	2.57	9.9	7	1.0
200	2.40	10	7	−5.4

[a] Difference between mean I_c and theoretical I_p.

volume of 1 ml with a good accuracy and precision. The limit of quantitation of this method is 1 ng/ml with an accuracy and precision of 3% for five spiked samples. Evaluation of the isotopic coefficient of the peak areas used in the selected-ion monitoring helped to corroborate the high specificity of this analytical method.

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