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Journal of Chromatography B, 805 (2004) 21–25
JOURNAL OF CHROMATOGRAPHY B
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Determination of a cyclooxygenase II inhibitor in human plasma by capillary gas chromatography with mass spectrometric detection

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Received 28 October 2003; received in revised form 27 January 2004; accepted 2 February 2004

Abstract

Sensitive methods based on capillary gas chromatography (GC) with mass spectrometric (MS) detection in a selected-ion monitoring mode (SIM) for the determination of a cyclooxygenase II (COX-II) inhibitor (3-isopropoxy-4-(4-methanesulfonylphenyl)-5,5′-dimethyl-5H-furan-2-one, **I**) in human plasma, in two concentration ranges of 0.1–20 and 5–1000 ng/ml, are described. Following liquid–liquid extraction, the residue, after evaporation of the organic phase to dryness, was reconstituted in acetonitrile (20 μl) and part of the extract (1 μl) was analyzed by GC/MS/SIM. The drug (**I**) and internal standard (**II**) were separated on a 25 m × 0.2 mm capillary column with HP Ultra 1 (100% dimethylpolysiloxane, 0.33 μm) phase and analyzed by MS/SIM monitoring ions at *m/z* 237 and 282 for **I** and **II**, respectively. The standard curve was linear within the lower concentration range of 0.1–20 ng/ml and the lower limit of quantification (LLOQ) in plasma was 0.1 ng/ml. Intraday coefficients of variation (CV, $n = 5$) were 8.9, 4.2, 5.7, 3.1, 1.9, 1.9, and 4.4% at 0.1, 0.2, 0.5, 1.0, 5.0, 10, and 20 ng/ml, respectively. The standard curve was also linear within the higher concentration range of 5–1000 ng/ml and the LLOQ in plasma was 5 ng/ml. Intraday coefficients of variation (CV, $n = 5$) were all below 9% at all concentrations within the standard curve range. The accuracy for **I** in human plasma was 91–112% and the recovery of **I** and **II** was greater than 70% at all concentrations within both standard curve ranges. The details of the assay methodology are presented.

Keywords: Cyclooxygenase II inhibitor

1. Introduction

Compound **I** [3-isopropoxy-4-(4-methanesulfonylphenyl)-5,5′-dimethyl-5H-furan-2-one, Fig. 1] is a potent, orally active and selective cyclooxygenase II (COX-II) inhibitor [1–5]. It belongs to the class of compounds that was shown to relieve inflammation and pain. In order to support early clinical studies with this compound, analytical methods for the determination of **I** in human plasma were required. Contrary to two other structurally similar COX-II inhibitors studied in our laboratories that are currently on the market in the US (Rofecoxib or VIOXX™) and other international markets (ARCOXIA™), the molecule of **I** does not contain the "*cis*-stilbene-like" moiety and does not form highly fluorescent photocyclization products after irradiation [6–8]. Therefore, a need existed for evaluating and applying means of detection of **I** other than post-column photochemistry/fluorescence detection.

Although HPLC methods with tandem mass spectrometric (MS/MS) detection for both VIOXX™ and ARCOXIA™ were developed [9,10] they required, especially in the case of VIOXX™, long-term studies to design unique MS ionization conditions for sensitive detection of these difficult to ionize molecules [9]. Compound **I** was also shown to ionize with difficulty using HPLC/MS/MS detection under atmospheric pressure chemical ionization conditions. Therefore, gas chromatography (GC) with MS detection under electron impact (EI) ionization conditions and/or the relatively weak native fluorescence of the molecule were utilized for the detection of **I**. An analytical method based on HPLC with fluorescence detection for the determination of **I** in human plasma in the concentration range of 5–400 ng/ml was originally developed [11]. Concurrently, the GC/MS method with the much lower limit of quantification (LLOQ) of 0.1 ng/ml was designed to support studies with the anticipated lower doses of **I**. The development and validation of the GC/MS method for **I** with the improved (50×) LLOQ of 0.1 ng/ml is the subject of this communication. The mechanism of fragmentation of **I** under the GC/EI ionization conditions is

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doi:10.1016/j.jchromb.2004.02.003

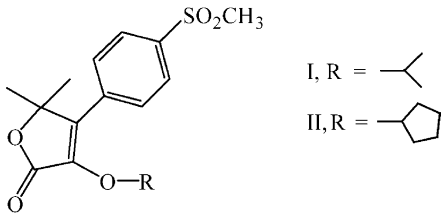

Fig. 1. Chemical structures of **I** and **II**.

proposed and the stability of **I** in human plasma at these low concentrations under freeze–thaw conditions is examined.

2. Experimental

2.1. Materials

Drug **I** and internal standard **II** were obtained from the Medicinal Chemistry Department of Merck Frosst Canada (Montreal, Canada). HPLC and GC grade solvent came from Baxter (McGaw Park, IL, USA). The GC column used was a fused−silica capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness) with methylsilicone stationary phase (HP-1, Hewlett-Packard, Wilmington, DE, USA). A multi-tube vortexer (Baxter, Scientific Products, McGaw Park, IL, USA) and a TurboVap LV evaporator ZW 700 (Zymark Co., Hopkinton, MA, USA) were used during preparation of plasma samples for analyses.

2.2. Instrumentation

A Hewlett-Packard model 5890 series II gas chromatograph equipped with a 7673 autosampler and an HP 5971A mass selective detector (MSD) was used. The GC operating conditions were: injection port temperature, 300 °C; GC/MSD interface temperature, 300 °C; column temperature programming, 100 °C for 1 min, then rising to 300 °C at 30 °C/min and remaining at that temperature for 3 min. After cooling to 100 °C, an equilibration time of at least 30 s was allowed. Helium was used as the carrier gas, with a flow rate of 0.998 ml/min. A mass selective detector in the electron-ionization mode was operated at an ion source pressure of 1.5×10^{-5} Torr. The electron multiplier (EM) voltage was 200–600 V above calibration voltage. Data was processed using HP-Chemstation version B.00 in selected-ion monitoring mode (SIM).

2.3. Standard solutions

Stock standard solutions of **I** and **II** at the concentration of 1.0 mg/ml were prepared in acetonitrile and stored at −20 °C. Under these conditions, the compounds were stable for at least 6 months. These solutions were further diluted to give a series of working standards with concentrations of 10000, 5000, 2000, 1000, 500, 200, 100, 50, 10, 5, 2 and 1 ng/ml. The standard curve in the assay was constructed by analyzing control human plasma samples (1 ml) spiked with standard solutions of **I** (100 μl) and **II** (100 μl). The concentrations of the analytes used in the assay of **I** were 0.1, 0.2, 0.5, 1.0, 5.0, 10, and 20 ng/ml and 5, 10, 50, 100, 200, 500, and 1000 ng/ml for standard curves in the low and high concentration range, respectively. Quality-control samples were prepared from standard solutions different from those used for the construction of standard curves. Appropriate aliquots of the diluted solution were added to 100 ml of control human plasma to yield the concentrations indicated in Table 2. Aliquots (1.25 ml) of the well-mixed plasma aliquots were stored at −20 °C until taken for assay with a set of test samples.

2.4. Sample preparation

To 1 ml of plasma in a 15 ml centrifuge tube, 10 ng of internal standard (100 μl of 0.1 μg/ml working standard solution of **II** in acetonitrile, and 100 μl acetonitrile to mimic standard solution addition during preparation of standard curve samples) was added. After vortex mixing for 10 s, the sample was extracted with 20 ml of toluene for 20 min on a multi-tube vortexer. After centrifugation for 10 min at 3000 × *g*, the upper organic layer was transferred to a clean centrifuge tube and evaporated to dryness at 35 °C. The residue was dissolved in 20 μl of acetonitrile. After vortex mixing for 10 s, the extract was transferred to a glass HPLC-insert and 1 μl of the solution was injected into GC/MSD system.

2.5. Precision, accuracy, recovery, and selectivity

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma containing **I** at all concentrations utilized for constructing calibration curves. The linearity of each standard line was confirmed by plotting the peak area ratios of the drug to internal standard versus concentration. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted ($1/y^2$) linear regression of the standard line. The accuracy of the assay was expressed by [(mean observed concentration)/(spiked concentration)] × 100.

Assay selectivity was assessed by running blank control plasma samples. No endogenous interferences were observed. The recovery was determined by comparing the peak area of **I** extracted from human plasma to that of standards injected directly.

3. Results and discussion

3.1. Mass spectra and SIM chromatograms

The electron ionization mass spectra revealed the presence of four major fragment ions at *m*/*z* 130, 209, 237, and 282 for both **I** and **II** (Fig. 2). In the mass spectra of **I** and

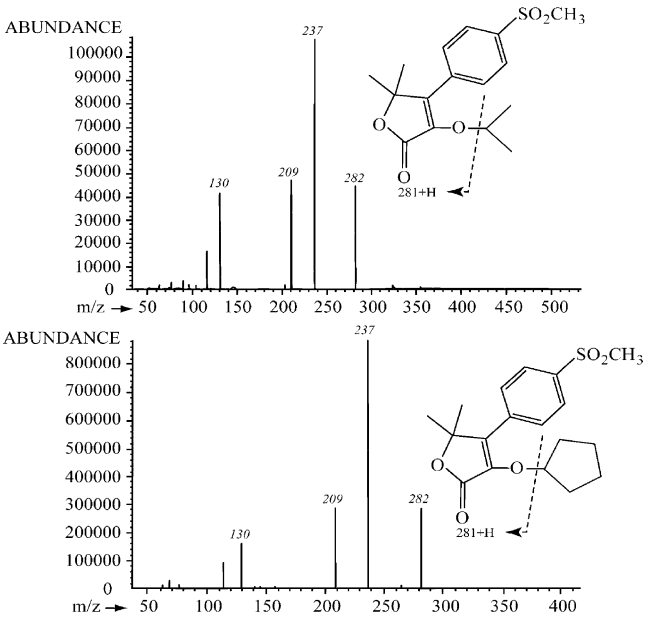

Fig. 2. Electron ionization mass spectra of **I** and **II**.

II, the *m*/*z* 282 fragment was formed by elimination of the isopropyl and cyclopentyl moieties (elimination of propene and cyclopentane from **I** and **II**, respectively), whereas the base peak at *m*/*z* 237 most likely corresponded to the further elimination of a carboxyl group from the fragment at *m*/*z* 282. The proposed fragmentation mechanism of **I** and **II** is shown in Fig. 3. The most intense fragment ion peak in the MS spectrum for both **I** and **II** was at *m*/*z* 237. However, endogenous interference was observed when monitoring the response in this channel for the internal standard. Therefore, the response at *m*/*z* 282 for **II** was monitored and the interference in this MS channel for **II** was not observed. The sensitivity of detection at *m*/*z* 282 for both **I** and **II** was sufficient for reliable quantification of **I**, and the same fragment ion of **I** and **II** at *m*/*z* 282 was used for selected ion monitoring of these compounds. Representative chromatograms of **I** and **II** are presented in Fig. 4.

3.2. Assay validation

The assay for **I** was validated in human plasma in the concentration ranges of 0.1–20 and 5–1000 ng/ml. Typical equations for the calibration line of **I** were $y = 0.071x - 0.000322$ and $y = 0.109x - 0.168$, respectively, with correlation coefficients of >0.990/0.992. The intraday assay precision, expressed as the coefficient of variation (CV %), was less than 9% at all concentrations used for constructing both standard lines. The accuracy of **I** in human plasma at all concentrations within the standard curve ranges were within 91–112%. The recovery of **I** from plasma was greater than 70% at all concentrations within standard curve ranges studied. Intraday precision data at all concentration within the standard curve ranges and for the quality-control samples are presented in Tables 1 and 2.

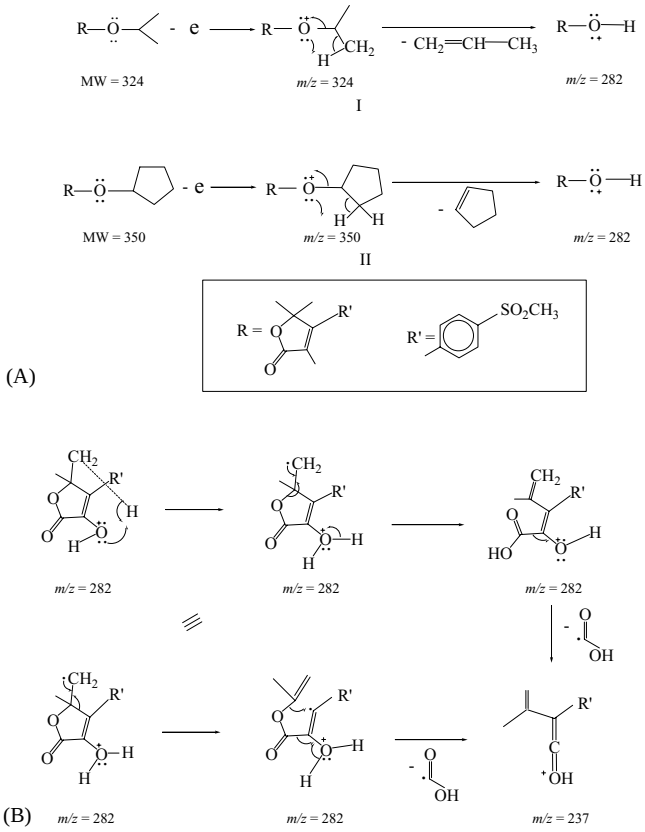

Fig. 3. Proposed fragmentation mechanism of **I** and **II** leading to formation of major fragments at *m*/*z* 282 (A) and *m*/*z* 237 (B).

Table 1
Intraday precision and accuracy data for the determination of **I** in human plasma as assessed by the replicate ($n = 5$) analysis of standards

Nominal standard concentration (ng/ml)	Mean ($n = 5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	Precision[b] (%)
(A) Standard curve range: 0.1–20 ng/ml			
0.1	0.10	104	8.9
0.2	0.20	101	4.2
0.5	0.47	95	5.7
1.0	0.91	91	3.1
5.0	5.10	101	1.9
10.0	10.60	106	1.9
20.0	22.50	112	4.4
(B) Standard curve range: 5–1000 ng/ml			
5	5.2	103	3.0
10	9.4	94	2.2
50	46.5	93	3.8
100	98.8	99	3.7
200	208.9	104	3.4
500	559.3	112	3.5
1000	1024.6	102	6.4

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

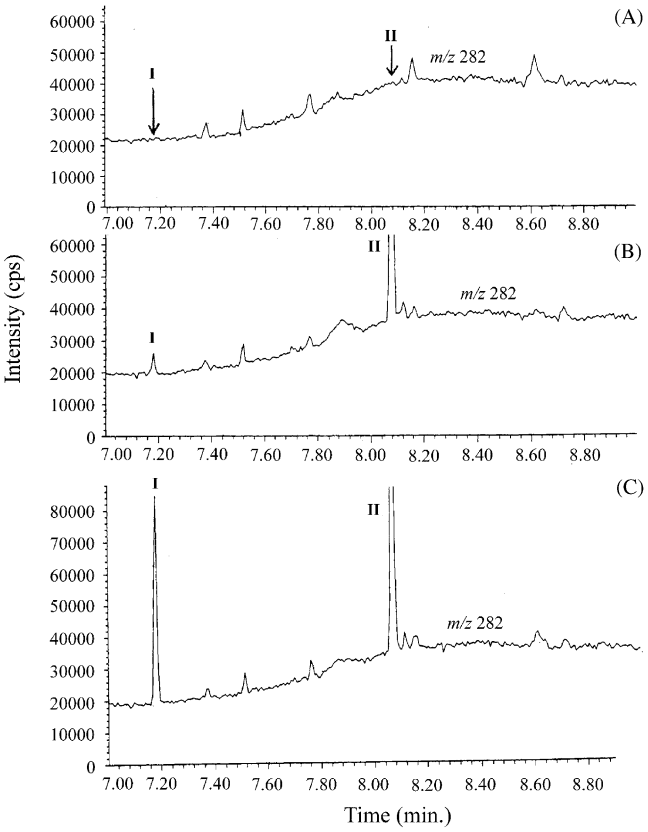

Fig. 4. Representative chromatograms of **I** and **II** in human plasma. (A) Blank control plasma; (B) control plasma spiked with 0.5 ng/ml of **I** and 10 ng/ml of **II**; and (C) control plasma spiked with 5.9 ng/ml of **I** and 10 ng/ml of **II**.

In addition, a cross validation was performed against a method for **I** based on HPLC with fluorescence detection that was previously reported [11]. The reference method utilized SPE for sample preparation in a 96-well format followed by HPLC with fluorescence detection. The plasma concentrations of **I** determined with the GC/MSD/SIM method versus the concentrations determined with the reference method, using the standard curve in the concentration range of 5–1000 ng/ml, are presented in Fig. 5. The results indicated a good correlation ($R^2 = 0.993$) between the two methods. A plasma concentration of **I** versus time-course is presented in Fig. 6.

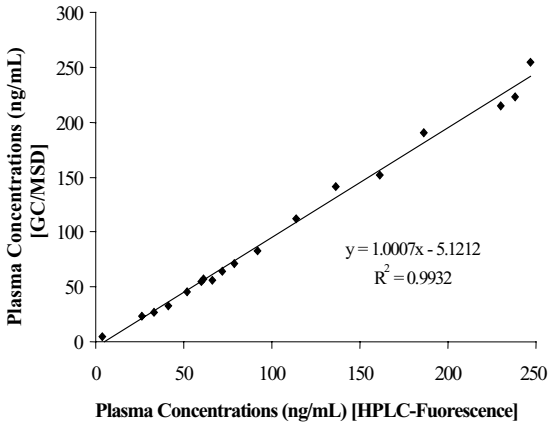

Fig. 5. Cross validation of methods for the determination of **I**; plot of plasma concentrations of **I** in selected human clinical samples assayed with the GC/MSD vs. the method based on HPLC with fluorescence detection [11].

Table 2
Stability data for the determination of **I** in human plasma as assessed by the replicate analysis of quality-control (QC) samples

Nominal standard concentration (ng/ml)	Mean ($n = 5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	Precision[b] (%)
(A) Standard curve range: 0.1–20 ng/ml			
0.3 (low QC)	0.31[c] ($n = 5$)	103	3.9
	0.31[d] ($n = 2$)	99	
	0.28[e] ($n = 2$)	91	
15 (high QC)	16.2[c] ($n = 5$)	108	1.9
	16.8[d] ($n = 2$)	103[f]	
	16.3[e] ($n = 2$)	100[f]	
(B) Standard curve range: 5–1000 ng/ml			
15 (low QC)	16.1[c] ($n = 5$)	107	1.7
	16.2[d] ($n = 2$)	101[f]	
	16.2[e] ($n = 2$)	101[f]	
315 (high QC)	331[c] ($n = 5$)	105	0.8
	334[d] ($n = 2$)	101[f]	
	330[e] ($n = 2$)	100[f]	

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.
[c] Initial mean concentration of quality-control samples following one freeze–thaw cycle.
[d] Mean concentration of quality-control samples following two freeze–thaw cycles.
[e] Mean concentration of quality-control samples following three freeze–thaw cycles.
[f] Expressed as [(mean measured concentration)/(mean ($n = 5$) of the initial measured concentration)] × 100.

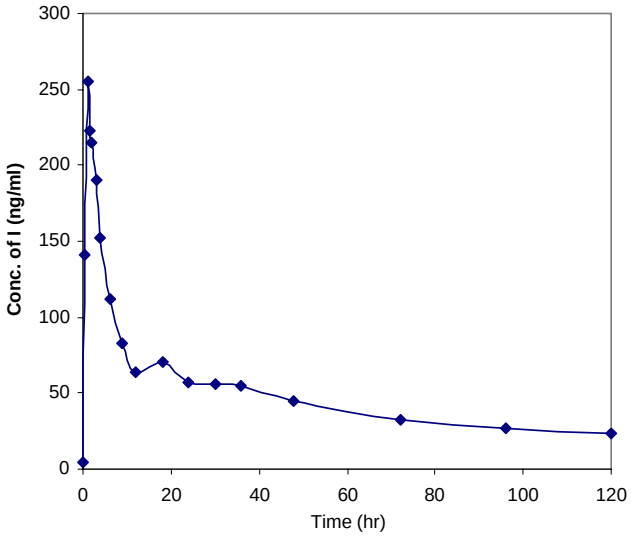

Fig. 6. Mean pharmacokinetic time-course of subjects ($n = 6$) receiving a 10 mg oral dose of **I**.

3.3. Freeze–thaw stability

Freeze–thaw stability was examined by exposing quality-control (QC) samples to three freeze–thaw cycles (freezer nominal temperature of −20 °C.). By comparing the initial mean values at three different concentrations of QC standards after one freeze–thaw cycle to the similar mean values after subsequent freeze–thaw cycles, the effect of freeze–thawing on the stability of **I** in plasma was determined. As can be seen from Table 2, there were no significant differences (<4%) in the assay concentrations following multiple freeze–thaw cycles, thus indicating analyte/sample stability.

4. Conclusions

The method for **I** based on GC/MS detection described here was shown to be much more sensitive (50×) than the previously described HPLC assay with fluorescence detection that was based on a weak, "native" fluorescence of the analyte [11]. Although the relative ease of operation of the HPLC system with fluorescence detection in comparison with GC/MS favors the use of the former methodology, the much improved sensitivity of detection, when needed, and the relative difficulty with ionization of **I** under a variety of HPLC/MS/MS conditions, makes the GC/MS approach for assaying this relatively small (MW = 324) and neutral molecule quite attractive at low oral doses of **I**. The GC/MS method would allow for the complete mapping of the pharmacokinetic time-course following administration of low doses of **I**.

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