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Rapid quantitative determination of a collagenase inhibitor and its major metabolite by on-line liquid chromatography with ionspray tandem mass spectrometric detection

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Abstract

A rapid, sensitive and specific analytical method has been developed and validated to quantify the collagenase inhibitor N2-(2(5)-[(hydroxycarbamoyl)methyl)-4-methylvaleryl]-N1,3-dimethyl-L-valin-amide (I) and its major metabolite (II) from plasma and urine. This method employs an automated solid-phase extraction procedure to isolate the analytes and the internal standard from the biological matrix. Reconstituted extracts were analyzed by HPLC–ionspray MS–MS. Chromatography was performed on a 4.6 mm I.D. reversed-phase guard column. The retention times of the analytes and the internal standard were approximately 1.3 min. The assay has a limit of quantification of 5 ng/ml plasma and a limit of detection of 1 ng/ml, based on 100-μl plasma aliquots. No sample-drying step was required. The standard curves were linear from 5 to 5000 ng/ml using weighted linear regression analysis ($1/y^2$). The intra- and inter-assay precision were better than ± 10% with intra- and inter-assay accuracies between 95 and 105%. This new HPLC–MS–MS assay procedure for I and II in plasma and urine has proven to be specific, sensitive, accurate and robust, and is being used routinely for the analysis of I in pre-clinical and clinical trial samples. Up to 200 unknowns may be analyzed each 24 h per analyst.

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1. Introduction

N2-(2(5)-[(Hydroxycarbamoyl)methyl)-4-methylvaleryl]-N1,3-dimethyl-L-valin-amide (I, Fig. 1) belongs to a new class of hydroxamic acids designed as so-called "disease modifying anti-rheumatic drugs" for the treatment of rheumatoid arthritis, which is considered the most common form of inflammatory polyarthritis affecting approximately 1% of the adult population [1]. Compound I has been shown in vitro to be a potent inhibitor of human fibroblast collagenase and gelatinase A, which are known as the key enzymes that catalyse the cartilage degradation in rheumatoid arthritis [2]. In vivo studies in novel animal models revealed that the compound is orally active, and clearly demonstrated the ability of the compound to protect articular cartilage from damage associated with arthritic disease. In rats, I is excreted predominantly in the urine, the major metabolic pathway being a reduction of the hydroxamic acid moiety to a corresponding amide (II) [3]. It was thought the amide metabolite might be responsible for

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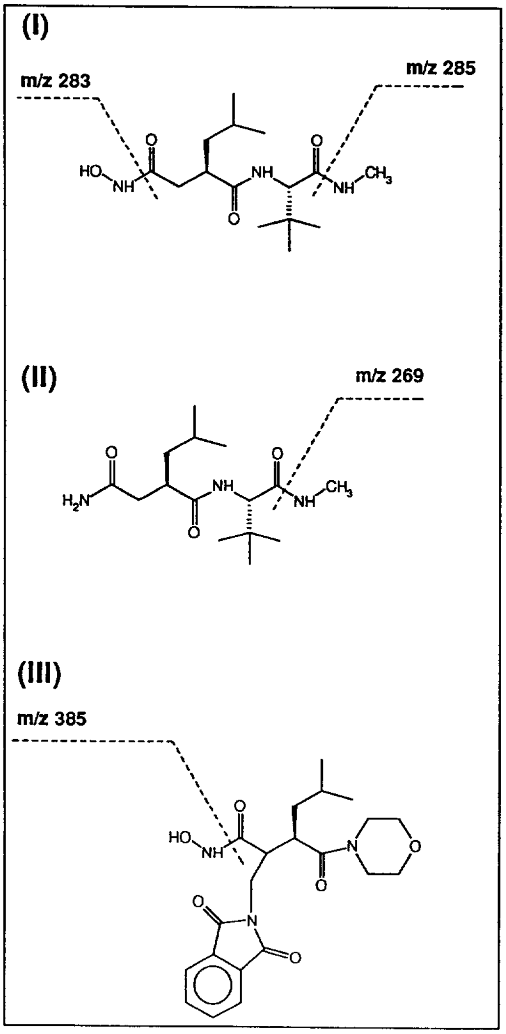

Fig. 1. Structures of the collagenase inhibitor (I), the amide metabolite (II) and the internal standard (III). The loss of 31 (33) u to yield the major fragments, is indicated.

unwanted side effects of the drug. Therefore, an assay had to be developed and validated which allowed the simultaneous determination of the parent drug and its major metabolite.

The development of a method for the quantitative determination of the collagenase inhibitor (I) and its major metabolite (II) in plasma and urine has challenged the advantages afforded by modern HPLC–thermospray MS techniques. The elevated temperature conditions common to thermospray interfaces can cause problems with the quantification and characterization of trace level amounts of thermally labile compounds [4]. Although reversed-phase HPLC provides a good separation technique, the compound's lack of a suitable UV chromophore excludes conventional detection at the nanogram level required for pharmacokinetic and toxicokinetic studies. Therefore, on-line HPLC with tandem mass spectrometry, using the mild ionization conditions of pneumatically assisted electrospray (ionspray) interfaced to tandem mass spectrometry should provide the required fast analytical capability for trace level quantification of thermally labile compounds [5,6].

Our aim was to develop a fast mass spectrometric method using pneumatically assisted electrospray (ionspray) with on-line HPLC–MS–MS to be able to quantify the thermally labile compounds I and II in human plasma and urine samples in the nanogram per millilitre range during an ongoing pharmacokinetics study in healthy volunteers. In order to enable us to report plasma concentrations to the clinical pharmacology unit as fast as possible, we envisaged having an efficient, automated sample workup procedure without time-consuming sample concentration/redissolving and sample transfer steps.

2. Experimental

2.1. Materials

N2 - (2(5) - [(Hydroxycarbamoyl)methyl) - 4 - methylvaleryl] - N1,3 - dimethyl - L - valinamide (I), N2 - [2(*R*)(carbamoylmethyl) - 4 - methylvaleryl] - N1,3 - dimethyl - L - valinamide (II) and 4 - [2(*R*) - [1(R,*S*) - 4 - methylvaleryl]morpholine (diastereoisomer) (III) were obtained from Roche Products (Research and Development, Welwyn Garden City, UK). Methanol was from Rathburn (Walkerburn, Scotland) and was of HPLC grade. All other reagents and chemicals were of HPLC-grade and were used without any further purification.

2.2. Method

Sample preparation

Blood samples were collected into heparinised or EDTA-pretreated tubes, cooled in ice and centrifuged for plasma (3000 *g*) as soon as possible (< 1 h). Urine and plasma samples were frozen at −20°C. Frozen samples were thawed at room temperature. To 100 μl of plasma (or urine), 100 ng internal standard in 100 μl 25% aqueous methanol and 500 μl of ammonium formate (0.1 *M*, pH 3.0) were added. In order to give a final volume of 1 ml, ammonium formate (0.1 *M*, pH 3.0) was added to each vial, followed by brief vortex-mixing. The solid-phase extraction (SPE) was carried out by an automatic sample processor (Aspec XL, Gilson, Villier-le-Bel, France) running overnight using 1 ml C_8 Bond Elut-SPE cartridges (Varian, Harbor City, CA, USA). Conditioning of the SPE cartridges with 1 ml each of methanol and 0.1 *M* aqueous ammonium formate (pH 3.0) was followed by loading of 0.95 ml of plasma dilution (i.e. 100 μl plasma + 800 μl 0.1 *M* ammonium formate (pH 3.0) + 100 μl internal standard (100 ng)), washing with 0.4 ml of 0.1 *M* aqueous ammonium formate (pH 3.0) and 0.4 ml of methanol–0.1 *M* aqueous ammonium formate (1:9, v/v) and elution with 0.4 ml of methanol–0.0025 *M* aqueous ammonium formate (pH 3.0) (7:3, v/v) (mobile phase). An automatic sample processor (Aspec XL, Gilson) or a robotic sample processor (Tecan 5052, Hombrechtikon, Switzerland) was used to transfer the extracted samples to the appropriate HPLC autosampler vials.

HPLC–API-MS–MS

HPLC was performed on a Phenomenex Ultracarb 5 μm ODS (30) Guard column (30 mm × 4.6 mm) (Torrance, CA, USA) using a Pharmacia LKB-HPLC Pump 2248 series (Uppsala, Sweden) and a Waters 717plus Autosampler (Milford, MA, USA). The injection volume was 50 μl onto the column. The mobile phase was water–methanol (70:30, v/v) containing 0.0025 *M* ammonium formate (pH 3.0) and the flow-rate was 0.5 ml/min.

Mass spectrometric detection was carried out using a PE Sciex API IIIplus triple-quadrupole instrument (Toronto, Ontario, Canada) operating in the ionspray interface mode using positive-ion pneumatic assisted electrospray (ionspray). In order to get the maximum response for I from a flow-rate of 0.5 ml/min, a T-piece splitter was installed after the column, employing a flow-rate of approximately 36 μl/min (split ratio 1/14) towards the orifice of the mass spectrometer. Multiple reaction monitoring (MRM) was employed using argon as collision gas (no other gases or gas mixtures were tested). The mass spectrometer's parameter "collision gas thickness" (CGT) was set at density of $150 \cdot 10^{13}$ molecules per cm^2 (instrument readout) with the collision energy at 20 eV to provide the most abundant product ions for I (*m/z* 285) and II (*m/z* 269). Precursor to product ion transitions were monitored for *m/z* 316 to *m/z* 285 for I, for *m/z* 300 to *m/z* 269 for the amide (II), and for *m/z* 418 to *m/z* 385 for the internal standard (III). Dwell-time for each transition was 188 ms.

Data were acquired by the PE Sciex API data system (RAD, version 2.5b5) and the peak areas measured using the Macintosh software program MacQuan (version 1.3.b2). Owing to the fact that during operation of Sciex API III mass spectrometers nitrogen is condensed inside on a cryoshield, it is necessary that the instrument is turned into the recycle mode every 24 h to enable the evaporation of the condensate. For that purpose the mass spectrometer was connected with an automatic shutdown and recycle mode inducing device (MasterGate and MasterLink, MGT-Systems, Milton Keynes, Bucks, UK).

Preparation of calibration curve

Calibration samples and quality control (QC) samples were prepared with each batch of unknown test samples to cover the range of 5.0 to 5000 ng ml^{-1}. To 100 μl of blank human plasma (urine) were added 0.5, 1, 2, 5, 10, 30, 100, 300 and 500 ng of each analyte (I and II) in combined standard dilutions of 25% methanol–water in volumes of 50 μl. The internal standard (100 ng) was added to each tube in volumes of 100 μl. After brief vortex-mixing, the samples were centrifuged and submitted to automated solid-phase extraction as described before.

Preparation of quality control samples

QC samples were prepared by an independent analyst at two concentrations towards the top and bottom of the calibration curve (QC1: 25 ng/ml; QC2: 1000 ng/ml). Two of each QC sample were stored and analyzed with the unknown test samples in every batch.

Quantification

Calibration curves were constructed by plotting peak-area ratios of each of the analytes and the internal standard against the analytes' concentrations. The results of the raw data were transferred to an "inhouse"-developed laboratory information management system (LIMS) using Excel and Apple exchange software. This LIMS system was used to calculate the weighted linear regression fit of the peak areas of the standards of I and II relative to the internal standard. The weighted ($1/y^2$) linear regression line was fitted over the 1000-fold concentration range. Drug concentrations in the unknown and quality control samples were calculated from this line.

3. Results and discussion

3.1. Mass spectrometry

The PE Sciex API III triple-quadrupole mass spectrometer with the atmospheric pressure ionization (API) source interfaced to the ionspray for on-line HPLC–MS has not been used for quantitative determinations as much as the heated nebulizer interface [7]. Pneumatically assisted electrospray (ionspray) is more often used for qualitative analysis such as peptide sequencing, mass determinations of high-molecular-mass proteins, other biomacromolecules [8]. A further area of application of ionspray HPLC–MS in the pharmaceutical industry is the metabolite identification and/or metabolite profiling of smaller molecules (molecular mass <1000 u), especially for labile compounds of biological origin, because of its extremely mild ionisation [9].

The lack of a chromophore excluded conventional HPLC with ultraviolet or fluorescence detection. Initial experiments on mass spectrometric characterization of the collagenase inhibitor (I), demonstrating its the thermal instability, were done on FAB-MS (Finnigan MAT 8400) and thermospray (TSP) single MS instruments (Finnigan 4500). Additionally, a previously developed HPLC–thermospray MS assay, with solid-phase extraction clean-up and chromatographic separation on a 100 mm × 8 mm C_{18} reversed-phase column for the quantification of I in plasma on a Finnigan 4500 mass spectrometer had shown matrix interferences and a inferior limit of quantification (50 ng/ml). The deficiency in sufficient sensitivity and selectivity in single MS instruments (Finnigan 4500 with TSP interface) made it necessary to use a triple-quadrupole mass spectrometric approach. Therefore, it was required to monitor the analytical product ions of I, II and III simultaneously by means of collisionally activated dissociation (CAD) to achieve acceptable performance for high sample throughput analysis (Tables 1 and 2).

The major product ion fragment of I is formed by an α-cleavage (Fig. 1) with a loss of 31 u (methylamine), yielding the product ion used for quantification at m/z 285. Another, competing, fragment of I is formed by the loss of 33 u (hydroxylamine) from the "hydroxamic acid part" of the molecule, exhibiting a minor fragment ion at m/z 283. Owing to the relatively high mass resolution in the third mass analyzer (mass resolution at half peak height was 1 u) the isotopic contribution of the minor fragment at m/z 283 to the analytical product ion at m/z 285 is less than 0.1% and hence neglected. The mass spectrometer's parameters (state file) were set such as to optimize the abundance of the major fragment ion (m/z 285), exhibiting the loss of methylamine. Fig. 2a shows the product ion mass spectrum of I using the protonated molecule (m/z 316) as the precursor ion. The major fragment of II ("amide metabolite") after collisionally activated dissociation is also formed by

Table 1
Assay performance of I in plasma

Sample code	True value (ng/ml)	Number	Calculated value (ng/ml)	Error (mean) (%)	C.V. (%)
A. Inter-assay					
Standards					
S01	5.000	3	4.787	−4.253	7.626
S02	10.00	4	10.60	5.955	7.003
S03	20.00	4	20.24	1.187	10.42
S04	50.00	4	49.85	−0.3100	7.872
S05	100.0	4	99.84	−0.1600	6.035
S06	300.0	4	295.6	−1.475	5.520
S07	1000.0	4	993.7	−0.6350	7.526
S08	3000.0	4	2950.0	−1.683	3.453
S09	5000.0	4	5011.0	0.2250	7.109
			Mean	−0.1277	6.951
QCs					
Q01	22.93	8	23.34	1.772	7.309
Q02	947.3	8	955.2	0.8287	6.826
			Mean	1.300	7.067
B. Intra-assay					
Standards —pre-study validation					
S01	5.000	1	5.077	1.540	0.0000
S02	10.00	1	9.893	−1.070	0.0000
S03	20.00	1	18.35	−8.250	0.0000
S04	50.00	1	43.88	−12.24	0.0000
S05	100.0	1	99.87	−0.1300	0.0000
S06	300.0	1	312.7	4.233	0.0000
S07	1000.0	1	1031.0	3.100	0.0000
S08	3000.0	1	3226.0	7.533	0.0000
S09	5000.0	1	5296.0	5.920	0.0000
			Mean	0.0707	0.0000
QCs —pre-study validation					
Q01	25.08	4	22.93	−8.583	4.717
Q02	1003.0	4	947.3	−5.551	1.427
			Mean	−7.067	3.072

the α-cleavage of methylamine, as shown in Fig. 1. No competing fragment ion similar to I was observed in the product ion mass spectrum of II (Fig. 2b). Therefore, the major (i.e., most abundant) fragment ions were used as analytical traces for the quantification. The major product ion fragment of the internal standard (III) used for the quantification is represented by the loss of hydroxylamine (33 u) from the hydroxamic acid side of the molecule (Figs. 1 and 2c). Our approach in optimizing the analytical product ions for I was to tune the mass spectrometer's parameters for relatively lower mass resolution (Δm) in the first mass analyzer Q1 (Δm_{Q1} of the protonated molecular ion at half peak height was 2 u), and for a higher mass resolution in the second mass analyzer Q3 (Δm_{Q3} of the product ion at half peak height was 1 u) in order to be able to discern between the above mentioned competing product ions at m/z 285 and 283.

Table 2
Assay performance of II in urine

Sample code	True value (ng/ml)	Number	Calculated value (ng/ml)	Error (mean) (%)	C.V. (%)
A. Inter-assay					
Standards					
S01	5.000	4	4.859	−2.820	3.191
S02	10.00	4	10.15	1.452	8.189
S03	20.00	4	20.61	3.025	3.364
S04	50.00	4	52.95	5.890	10.09
S05	100.0	4	101.9	1.873	6.055
S06	300.0	4	299.3	−0.2333	5.846
S07	1000.0	4	992.5	−0.7525	5.802
S08	3000.0	4	2850.0	−5.008	5.468
S09	5000.0	4	4825.0	−3.495	8.540
			Mean	−0.0077	6.283
QCs					
Q01	25.96	8	25.32	−2.485	7.315
Q02	878.4	8	886.3	0.9036	3.871
			Mean	−0.7905	5.593
B. Intra-assay					
Standards —pre-study validation					
S01	5.000	1	5.013	0.2600	0.0000
S02	10.00	1	10.13	1.300	0.0000
S03	20.00	1	19.39	−3.050	0.0000
S04	50.00	1	48.78	−2.440	0.0000
S05	100.0	1	104.7	4.700	0.0000
S06	300.0	1	320.1	6.700	0.0000
S07	1000.0	1	1015.0	1.500	0.0000
S08	3000.0	1	2992.0	−0.2667	0.0000
S09	5000.0	1	4570.0	−8.600	0.0000
			Mean	0.0115	0.0000
QCs —pre-study validation					
Q01	25.45	4	25.96	2.014	2.017
Q02	1018.0	4	878.4	−13.71	1.772
			Mean	−5.850	1.894

3.2. Chromatography

In order to develop an assay with high sample throughput and to obtain maximum response for the analytes, we required as short a retention time as possible. The great advantage of having analytes with different molecular masses and similar retention times in combination with tandem mass spectrometric detection, enabled us to aim at a separation that removes salts and major matrix components that can suppress or interfere with the analysis from the target components, while maintaining good sample throughput. Previous experiments have shown that it is essential to implement such a crude chromatographic separation of I and II for adequate sensitivity and to prevent blockages of the ionspray capillary and the orifice at the front end of the mass spectrometer. Another reason why the sample should be separated from contaminants is that the reactant ions may be depleted when sample and a large excess of other components coelute.

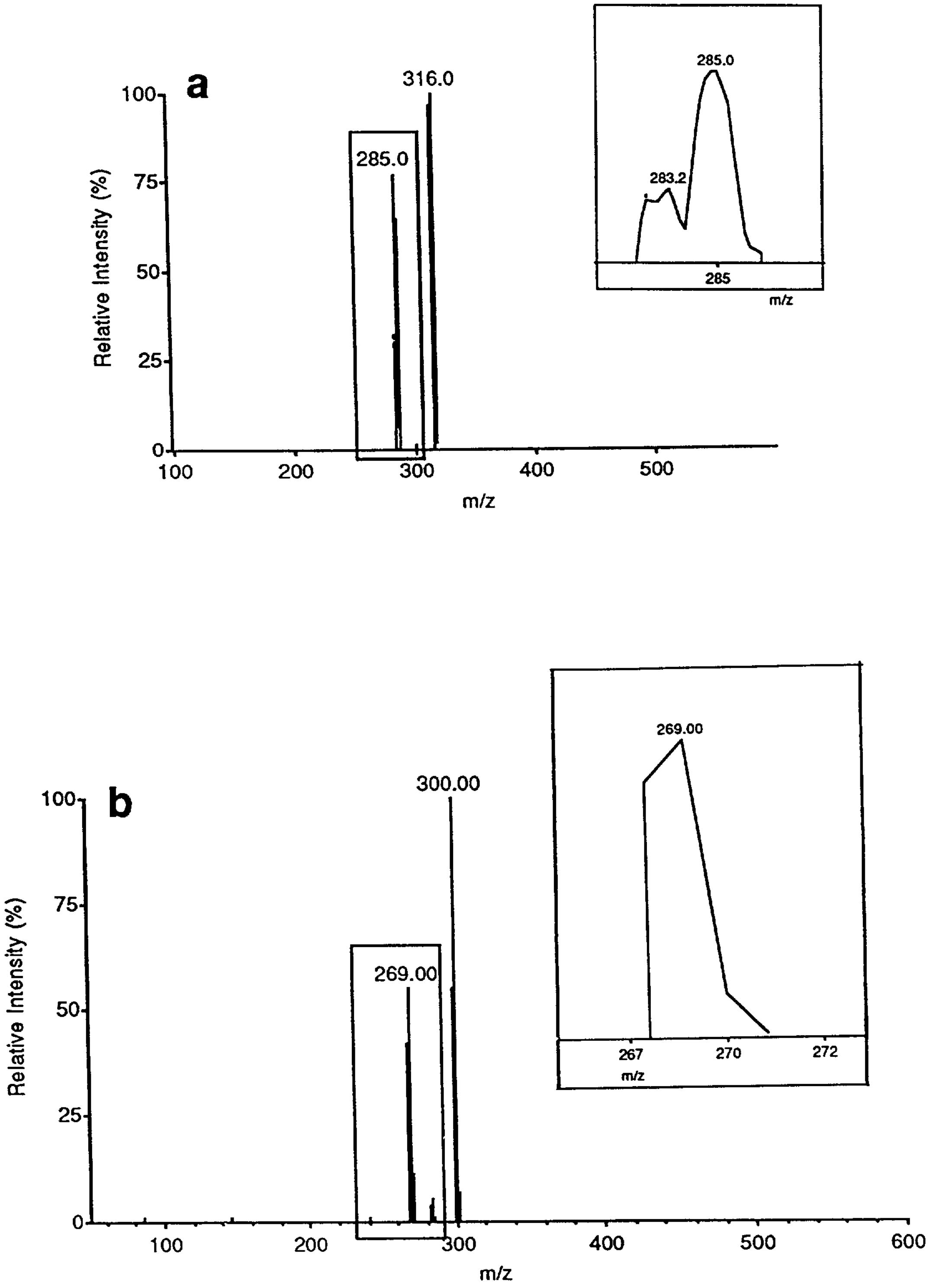

Fig. 2 (*Continued on p. 220*)

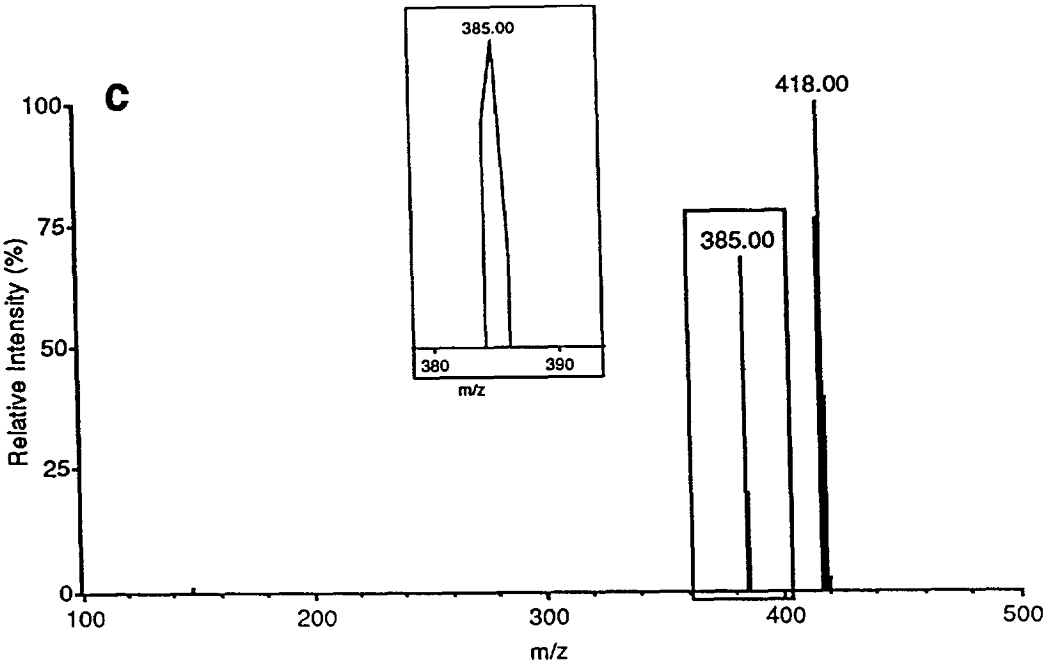

Fig. 2. (a) Positive product ion mass spectrum of the protonated molecule ion of I; 50 pg/μl presented to the source by API (ionspray)-HPLC–MS–MS mode. (b) Positive product ion mass spectrum of the protonated molecule ion of II; 50 pg/μl presented to the source by API (ionspray)-HPLC–MS–MS. (c) Positive product ion mass spectrum of the protonated molecule ion of III; 50 pg/μl presented to the source by API (ionspray)-HPLC–MS–MS mode.

As a consequence proper formation of protonated sample molecules could no longer take place and quantification would be impossible [10]. After testing several short columns of the immense variety available on the market, we obtained the best results with the Phenomenex Ultracarb 5 μm ODS (30) 30 mm × 4.6 7 mm (I.D.) guard column. This column provided us with the best compromise in terms of chromatographic reproducibility, flow-rate, high sample throughput and, last but not least, cost effectiveness. One column was used for approximately 200 samples without deterioration, such as peak tailing and decreasing signals, which we observed after the injection of approximately 250 plasma extracts.

The lack of a suitable stable labelled form of I to be used as internal standard, forced us to implement another, structurally related hydroxamic acid. The reason for choosing III as the internal standard was its similarity to I in terms of the lipophilicity (log $p = 0.40$ at pH 4.2) and recovery from biological matrices after solid-phase extraction from C_8 solid-phase extraction cartridges (recovery of I, II and III $\geq 96\%$).

MRM chromatograms of plasma extracts from a blood sample taken 6 h after oral administration of 5 mg of I to a healthy volunteer, are shown in Fig. 3. Both analytes and the internal standard nearly coelute with a retention time of 1.3–1.4 min. The cycle time per sample was 3 min. The detector response was linear over the range of 5.0–5000 ng/ml for I and II. Typical calibration curves for I and II comprised a mean slope of 0.001 ($n = 4$; R.S.D. = 0.0005) with a mean intercept of 0.005 (R.S.D. = 0.0002) and a slope of 0.003 ($n = 4$; R.S.D. = 0.0005) with a mean intercept of 0.097 (R.S.D. = 0.004), respectively. In order to evaluate the inter-assay performance of the analytical method we prepared and analyzed standard curves on four subsequent days with four QC samples with each standard

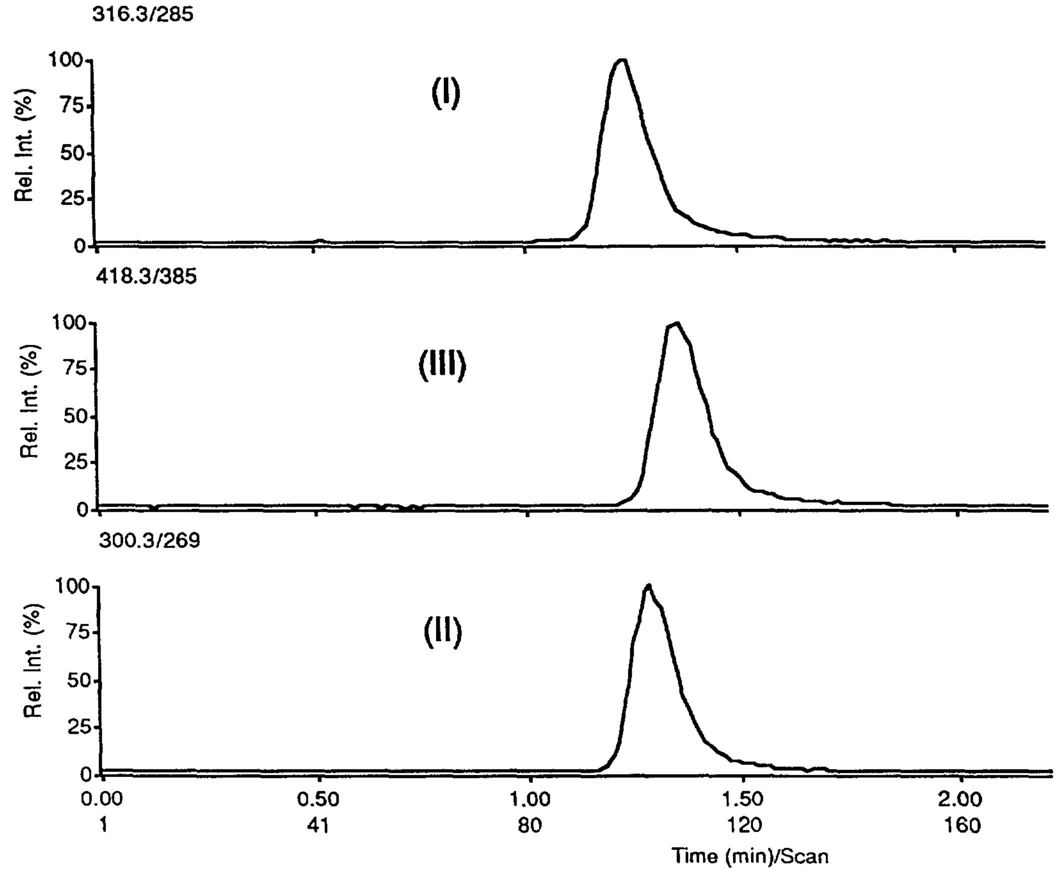

Fig. 3. Typical chromatograms obtained by multiple reaction monitoring (MRM) during the determination of I (back-calculated concentration 7.2 ng/ml), the amide (II) (back-calculated concentration 5.0 ng/ml), and the internal standard (III) (1 μg/ml) in plasma extracts of a blood sample taken from a healthy volunteer 6 h after oral administration of 5 mg of I.

curve (duplicate QC samples towards the lower and duplicate QC samples towards the upper quartile of the calibration range). Furthermore, a "pre-study validation" standard curve was analyzed with eight QC samples (four QC samples towards the lower and four QC samples towards the upper quartile of the calibration range) prior to the start of the analysis of samples with unknown concentrations of I and II. Thus, the intra-assay performance was assessed to ensure that the results were acceptable, and could be used for pharmacokinetic analysis.

The mean inter-assay precision (C.V.) for the standards of I in plasma was 6.9% with a mean error of −0.1%, and for the QC samples 7.1% with a mean error of +1.3%. The mean intra-assay precision for the QC samples was 3.1% with a mean error of −7.1% (Table 1) (assay performance of I in urine was similar to that in plasma, data not given).

The mean inter-assay precision (C.V.) for the standards of II in urine was 6.3% with a mean error of −0.01%, and for the QC samples 5.6% with a mean error of −0.8%. The mean intra-assay precision for the QC samples in urine was 1.9% with a mean error of −5.9% (Table 2) (assay performance of II in plasma was similar to that in urine; data not given). Therefore, the

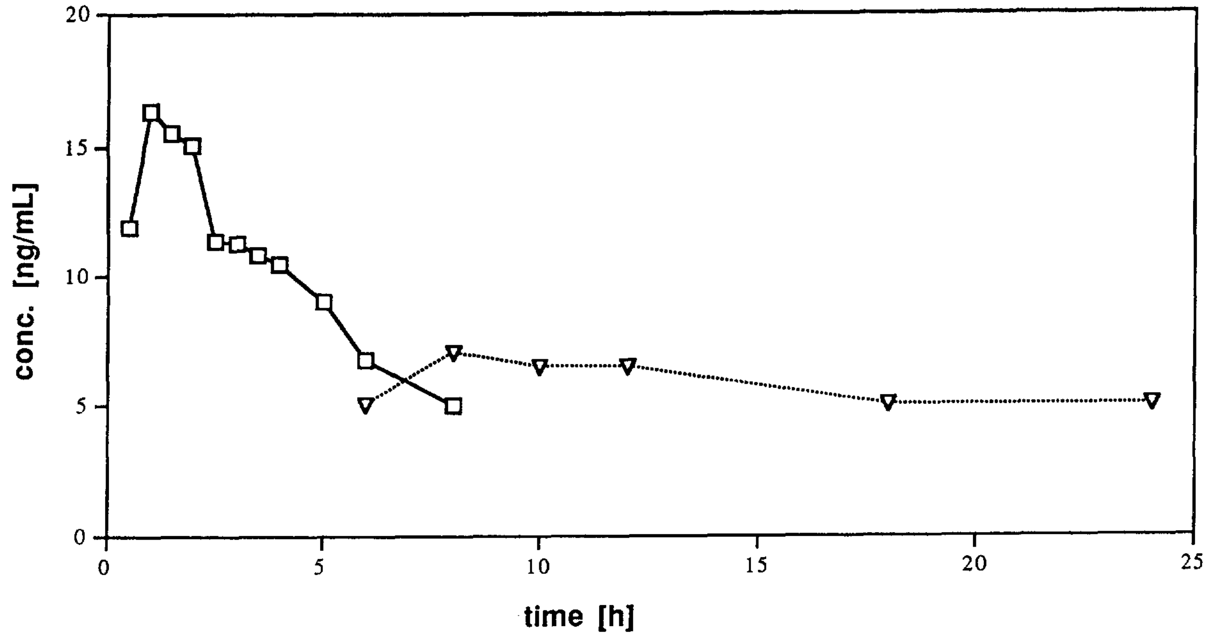

Fig. 4. Plasma concentration–time profile after oral administration of a single dose of 5 mg of I to a healthy volunteer. □ = I; ▽ = II.

inter- and intra-assay performances for the standards and QC samples were satisfactory for routine on-line HPLC–MS analysis.

4. Conclusion

In conclusion, a very fast, sensitive, selective and robust assay with tandem mass spectrometric detection has been developed. This method has been employed successfully for the determination of the pharmacokinetics of the collagenase inhibitor (I) and its amide metabolite (II) in a clinical pharmacokinetic study in which volunteers received a single dose of 5 or 3 mg of I. An example of a concentration versus time profile is outlined in Fig. 4, in which drug concentrations could be measured up to 8 h for I and 24 h for II post-dose, respectively. Thus, HPLC–MS–MS with an ionspray interface has demonstrated itself to be an excellent tool for fast analysis of non-volatile, thermally labile compounds in pharmacokinetic studies.

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