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Determination of a thermally labile metabolite of a novel growth hormone secretagogue in human and dog plasma by liquid chromatography with ion spray tandem mass spectrometric detection

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Abstract

A sensitive and selective assay for the determination of *N*-{1(*R*)-[(1,2-dihydro-1-methylsulfonylspiro[3H-indole-3,4′-piperidin]-1′-yl)carbonyl]-2-(phenylmethoxy)-ethyl}-2-hydroxyamino-2-methylpropanamide (**I**), a hydroxyl amine metabolite of a novel growth hormone secretagouge (**II**) has been developed utilizing high-performance liquid chromatography with ion spray tandem mass spectrometric detection (HPLC–MS–MS). The analyte and an internal standard (**III**) were isolated from the basified biological matrix using a liquid–liquid extraction with methyl *tert*.-butyl ether (MTBE). The organic extract was evaporated to dryness at room temperature. The residue was reconstituted in the mobile phase and injected into the HPLC–MS–MS system. Multiple reaction monitoring using the precursor→product ion combinations of m/z 545→267 and 543 →267 was used to quantify **I** and **III**, respectively, after chromatographic separation under isocratic conditions. The assay was validated in the concentration range of 0.5 to 500 ng/0.1 ml in both human and dog plasma. The precision of the assay, expressed as relative standard deviation, was less than 10% over the entire concentration range with the exception of the low concentration of 0.5 ng/0.1 ml which was 14.0% for human plasma. The HPLC–MS–MS method provided sufficient sensitivity to completely map the pharmacokinetic time course of **I** following a single 5 mg dose of **II** to human subjects and a 0.5 mg/kg dose to beagle dogs.

Keywords: Growth hormone secretagogue

1. Introduction

Compound **I**, *N*-{1(*R*)-[(1,2-dihydro-1-methylsulfonylspiro[3H-indole-3,4′-piperidin]-1′-yl)carbonyl]-2-(phenylmethoxy)-ethyl}-2-hydroxyamino-2-methylpropanamide (Fig. 1), has been identified as a major metabolite of a novel growth hormone (GH) secretagouge (**II**) that was discovered and evaluated in our laboratories [1,2]. The GH secretagogues may offer a physiological advantage over recombinant GH therapy by providing a more sustained release of GH [3]. Clinical effectiveness of a GH-releasing hexapeptide has been demonstrated [4], but limited oral bioavailability inhibited the feasibility of its use as a therapeutic agent [5].

Compound **II** is a non-peptidyl GH secretagogue that has demonstrated growth hormone responses following oral administration in rats, dogs, swine,

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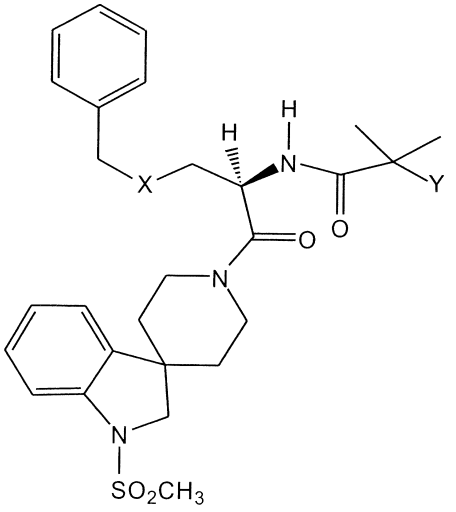

I; X=O Y=NHOH
II; X=O Y=NH_2
III; X=CH_2 Y=NHOH
IV; X=CH_2 Y=NH_2

Fig. 1. Chemical structures of **I**–**IV**.

and man. It represents an orally active GH secretagogue with potential therapeutic use. In order to support human pharmacokinetic (PK) studies, an assay for the determination of **II** in human plasma based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection was previously developed [6]. The method utilized an atmospheric pressure chemical ionization (APCI) interface and was applied successfully to support human PK studies. During the course of preclinical and clinical studies, compound **I** was identified as a major metabolite of **II** and an assay for determination of **I**, in addition to **II**, was required. Initially, the determination of **I** was attempted using HPLC–MS–MS with the APCI interface, a method similar to that used for the parent compound **II**. The use of the APCI interface allows a larger volume of the sample extract to be introduced directly to the MS system compared to that of the ionspray (ISP) interface, without the need for splitting of the HPLC effluent before entering the ionization region of the mass spectrometer. This feature of the HPLC–MS–MS system with APCI interface may increase the sensitivity of detection if the ionization efficiencies of the analyte between APCI and ISP are comparable. However, all attempts at validating the simultaneous assay for **I** and **II** using APCI interface and the same internal standard (I.S.; **IV**) as in the assay for **II** [6] were unsuccessful. The precision values for **I** were unacceptably high with relative standard deviations (RSDs) greater than 30% at all concentrations used for constructing the standard curve. High precision values (>20%) were also obtained after repeated injections of neat standards, indicating a possibility of **I** being thermally labile. In order to use APCI interface and maximize the low limit of quantification (LOQ), an analog (**III**) of **I** containing the same *N*-hydroxylamine moiety as in **I** was chosen as the internal standard instead of **IV**. It was assumed that any thermal instability of **I** in the APCI interface will be closely matched by an internal standard **III** containing the same thermally labile –NHOH moiety. Although the precision improved to under 20% RSD, the assay variation was still unacceptably high. In order to eliminate the exposure of **I** and **III** to heat after HPLC separation, the APCI interface was replaced with the ISP interface. Repeated injections of neat standards of **I** and **III** using ISP interface gave precision better than 3%. Based on these observations, a method for **I** utilizing ISP interface and **III** as the internal standard was developed and is the subject of this paper. This method exhibited adequate precision and accuracy and was used for supporting both preclinical and human PK studies.

In the past few years, HPLC–MS–MS has greatly improved the ability of bioanalytical chemists to develop methods for the determination of drug and metabolite(s) in biological matrices. A variety of these methods have been used to support large-scale clinical programs. Some of the recent examples from our laboratories are listed in Refs. [7,8]. In addition, multiple component analysis by HPLC–MS–MS for drug/metabolite screening in support of drug discovery has become quite common [9]. Although it is believed that the selectivity of these assays is ensured by using a specific multiple reaction monitoring (MRM) channel for each analyte simultaneously, this widely accepted belief may not be fully justified in view of the recent examples indicating a need for the careful assessment of assays selectivity

with regard to matrix effect and ion suppression or enhancement [10–13], interferences from metabolites [14–16], and a variety of ''cross-talk'' effects.

The example of the assay development for **I** presented in this paper provides a good illustration of how a seemingly small change in the physical and chemical properties of a molecule (metabolite) in comparison with the parent compound affect(s) the design of an analytical method, requiring development of a dramatically different assay than one used for the determination of the parent compound.

2. Experimental

2.1. Materials

I, **II**, and **III** were synthesized at the Merck Research Labs. (Rahway, NJ, USA). All solvents and reagents were of HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The drug-free human heparinized plasma originated from Biological Specialties (Lansdale, PA, USA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA, USA).

2.2. Instrumentation

A PE-Sciex (Thornhill, Canada) API III Plus tandem mass spectrometer equipped with APCI or with ISP interfaces, a Waters Associates (Waters–Millipore, Milford, MA, USA) WISP 715 auto-injector, and Perkin-Elmer biocompatible binary pump (Model 250) were used for all HPLC–MS–MS analyses. The data were processed using MacQuan software (PE-Sciex) on a MacIntosh Quadra 900 microcomputer. HPLC separation was performed using a Keystone Scientific C_{18} BDS 50×4.6 mm I.D., 3 μm analytical column coupled with a 2-μm in-line filter.

2.3. Chromatographic conditions

The aqueous portion of the mobile phase was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water and the addition of 820 μl formic acid. The mobile phase was a mixture of acetonitrile–water (62:38) containing 0.1% formic acid and 10 m*M* ammonium acetate, and was delivered at a flow-rate of 0.8 ml/min. The column eluent split ratio was 1:20. The retention times for **I** and **III** were 4.3 and 7.3 min, respectively.

2.4. MS–MS conditions

A PE-Sciex triple quadrupole mass spectrometer was interfaced via a Sciex APCI or ISP interface to HPLC system. The APCI interface was maintained at 500°C and the gas phase chemical ionization was effected by a corona discharge needle (+4 μA). The nebulizing gas (nitrogen) pressure and auxiliary flow were set at 550 kPa and 2.0 l/min, respectively. Curtain gas flow (nitrogen) was 0.9 l/min, and the sampling orifice potential was set at +55 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. The mass spectrometer was programmed to admit the protonated molecule $[M+H]^+$ at m/z 545 (**I**) and m/z 543 (**III**) via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, $280 \cdot 10^{13}$ atoms/cm^2), and to monitor the product ions via Q3 at m/z 267 for both analytes. The electron multiplier setting was −4.2 kV. Peak area ratios of **I**/**III** obtained from multiple reaction monitoring of analyte (m/z 545→267)/(m/z 543→267) were utilized for the construction of calibration curves, using weighted ($1/y^2$) linear least-square regression of the plasma concentrations and the measured area ratios. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.5. Standard solutions

A stock solution of **I** (1 mg/ml) was prepared in methanol. This solution was further diluted with methanol to give a series of working standards with the concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 μg/ml. The internal standard **III** was also prepared as a stock solution (1 mg/ml) in methanol by dissolving 10 mg of solid **III** in 10 ml of methanol. A working standard of 1.0 μg/ml was prepared by serial dilutions of stock standard with methanol, and was used for all analyses. All standards were prepared weekly and stored at 5°C.

A series of quality control (QC) samples at 2.5 and 400.0 ng/0.1 ml in plasma were prepared. Aliquots (0.25 ml) of these solutions were placed in 1.5-ml polypropylene tubes, stored at −20°C, and analyzed daily with clinical samples. The calculated concentrations of the QC samples were compared on a day-to-day basis to assess the inter-day assay reproducibility.

2.6. Sample preparation

A 0.1-ml aliquot of plasma was pipetted into a 15-ml centrifuge tube and 100 μl of the working standard of **III** (equivalent to 100 ng/0.1 ml of I.S.) was added followed by the addition of 1 ml of 0.2 *M* carbonate buffer (pH 9.8). After addition of 7 ml of methyl *tert*.-butyl ether and capping tubes with PTFE-lined caps, the mixture was mixed and rotated for 15 min. The tubes were then centrifuged and the organic layer was transferred to a clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at room temperature, the residue was reconstituted in 200 μl of the mobile phase and 75 μl aliquot was injected into the HPLC–MS–MS system.

2.7. Precision and accuracy

The precision of the method was determined by the replicate analyses ($n=5$) of human or dog plasma containing **I** at all concentrations utilized for constructing calibration curve. The linearity of each standard was confirmed by plotting the peak area ratio of the drug to I.S. versus drug concentration. The standard curve samples were prepared and assayed daily with quality control and unknown samples. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)]·100.

3. Results and discussion

3.1. The selection of the HPLC–MS–MS interface

Initially, an attempt was made to adapt the HPLC–APCI-MS–MS method developed for the determination of **II** [6] to the analysis of **I**. The validation attempts resulted in poor precision values (>30% RSD). Similarly, the replicate analysis of neat standards yielded an RSD greater than 20% clearly indicating some instrumental or chemical stability problems with **I**. In order to check for the former, a series of neat standards of **II** was injected to assess the precision of the instrument. The RSD for the replicate analysis of **II** (parent compound) was <3% indicating all components of the HPLC–MS–MS system were working properly. In order to assess the effect of temperature on the precision of determination, the APCI interface was replaced with ISP interface and replicate neat standards of **I** were injected. The precision values were less than 3% indicating the thermal instability of **I** was most likely responsible for the poor precision when APCI interface was utilized. The thermal and/or chemical instability of **I** was additionally confirmed by heating neat standard solutions of **I** in ACN–water (1:1, v/v) at 50°C and by analyzing aliquots at different time intervals by HPLC–ISP-MS–MS. The results of these experiments clearly indicated a significant decrease in the peak area of **I** after heating. Similar experiment performed for **I** in the same solvent at room temperature indicated that concentrations of **I** were unchanged. Clearly at elevated temperature compound **I** was labile that may have led to poor reproducibility when APCI interface at 500°C was utilized. Based on these observations, a method for **I** using ISP instead of an APCI interface needed to be developed.

Before a final decision was made to replace the APCI interface with the ISP interface for the determination of **I**, a new internal standard **III** containing a thermally labile *N*-hydroxylamino group, similar to **I,** was tested. It was hoped that any potential thermal instability of **I** will be closely matched and compensated for by **III**, improving the overall precision of the assay. The utilization of the APCI interface is usually beneficial from the assay sensitivity point of view since larger volume of plasma extracts could be injected for analysis with the assumption that similar efficiency of ionization between APCI and ISP interfaces is achieved. This improved assay sensitivity was especially important in our pediatric studies where volumes of plasma for analysis was limited to 0.1 ml. Using a structurally similar I.S. (**III**) and the APCI interface, the preci-

sion of determination of **I** improved to less than 20% RSD but was still insufficient to support human PK studies. Based on all these observations, a method for the determination of **I** using ISP interface and **III** as an I.S. was developed.

3.2. Assay validation

The mass spectra of the protonated molecules $[(M+H)^+]$ of **I** (m/z 545) and **III** (m/z 543) indicated the presence of intense product ions for both analytes at m/z 267 (Fig. 2).

By monitoring the precursor→product ion pairs at m/z 545→267 for **I** and m/z 543→267 for **III** in the MRM mode, a highly selective assay for **I** in plasma with the LOQ of 0.5 ng/0.1 ml was developed. Since the precursor ions at m/z 545 for **I** and m/z 543 for **III** were only 2 amu apart and the product ion at m/z 267 was the same for both **I** and **II**, there is a potential for the ''cross-talk'' between the analyte

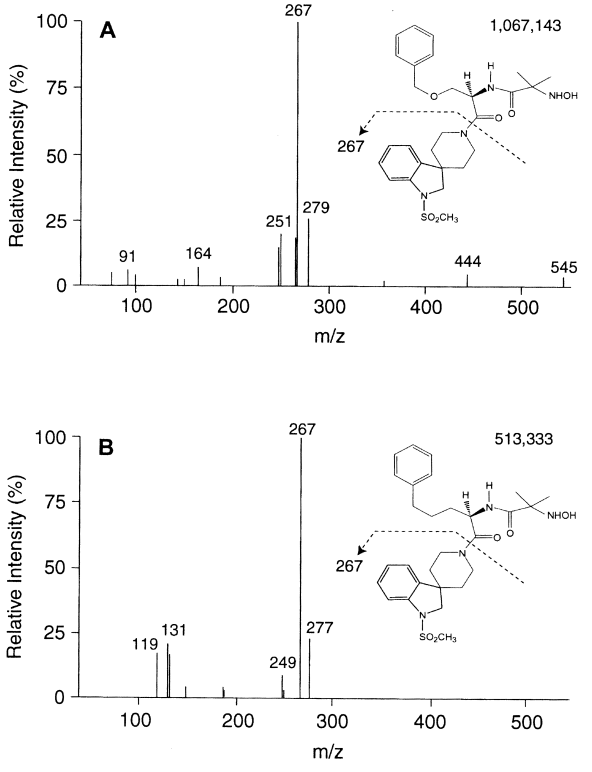

Fig. 2. Product ion mass spectra of the protonated molecules of (**I**) (m/z 545, A) and internal standard (**III**) (m/z 543, B).

and the I.S. MS–MS channels. This significant ''cross-talk'' is clearly illustrated in Fig. 3. Since compound **I** was chromatographically separated from **III,** the contribution of the **III** to **I** channel and vice versa had no affect on assay selectivity. The need for chromatographic separation of closely related analytes such as **I** and **III** to achieve selectivity of the HPLC–MS–MS assays was emphasized by us and others earlier [15,16]. Following the procedure described in the Experimental section, the assay was validated in the concentration range of 0.5 to 500 ng/0.1 ml. The intra-day precision, expressed as the RSD, was less than 10% at all concentrations within the standard curve range for human and dog plasma (Tables 1 and 2, respectively) with the exception of the precision of the low standard in human plasma that was 13.9% but well within our validation guidelines.

The isolation of the drug from biological fluids was based on a simple one step liquid–liquid extraction of **I** and **III** from basified plasma, evaporation of the extract to dryness, reconstitution of the residue in the mobile phase and injection onto the HPLC system. By minimizing the number of extraction steps high recoveries of **I** and **III** were achieved. The mean recovery of **I** from plasma was 97% and was practically the same at all concentrations within the standard curve range.

The limit of quantification (LOQ) of the assay was 0.5 ng/0.1 ml. The LOQ was defined here as the lowest concentration on the standard curve for which precision of the determination, expressed as RSD, was less than 15%, with an adequate assay accuracy (less than 10% deviation from nominal). Typical equations for the calibration curves were $y=0.011x-0.002$ (slope ± 0.0006; intercept ± 0.00007) and $y=0.0124x-0.003$ (slope ± 0.0009; intercept ± 0.00008) for human and dog plasma, respectively, with the correlation coefficient $r^2>0.99$. Representative chromatograms are shown in Fig. 3.

3.3. Assessment of matrix effect

The assessment of matrix effect and assay reliability is critical when analogs rather than stable isotope labeled parent compounds are selected for use as internal standards. The undetected but co-eluting endogenous impurities may affect the ioniza-

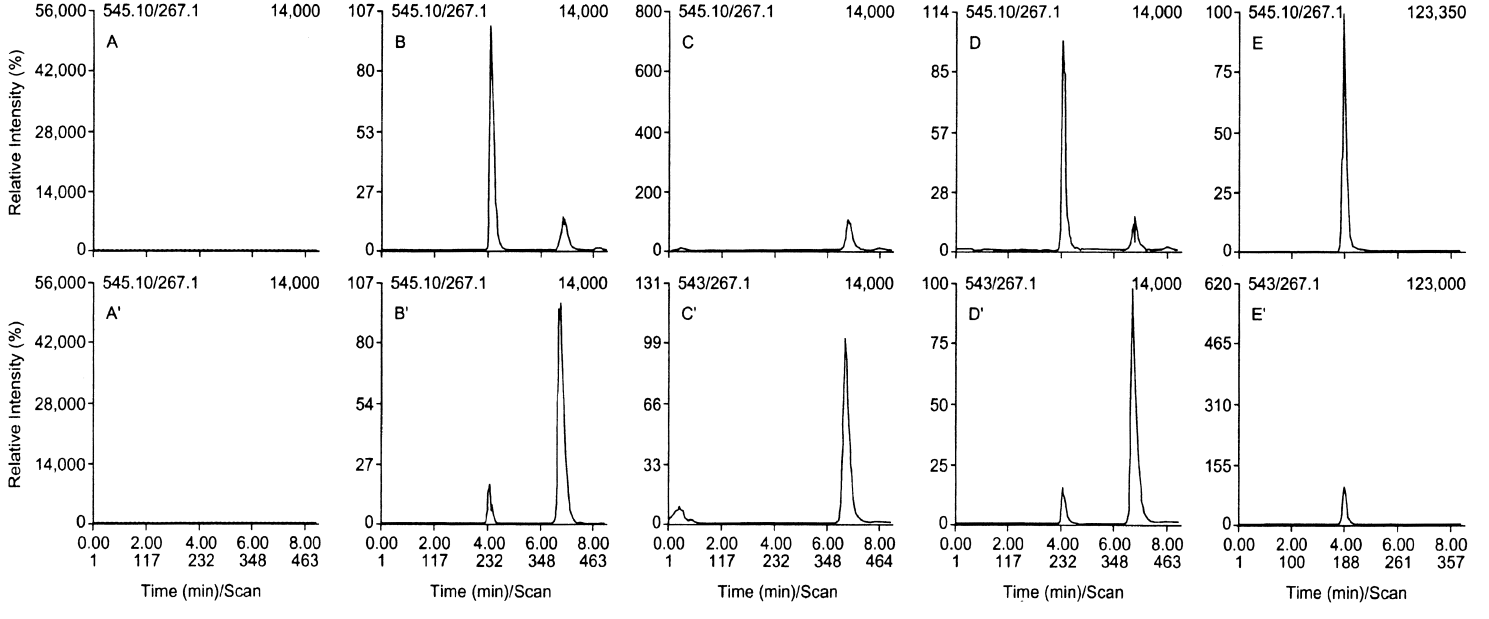

Fig. 3. Representative HPLC–MS–MS chromatograms of plasma (0.1 ml) extracts obtained by multiple reaction monitoring at m/z 545→267 (channel ''A'') for **I** and m/z 543→267 (channel ''B'') for internal standard (**III**); A, A′ – Blank control dog plasma monitored at channels ''A'' and ''B'', respectively; B, B′ – control dog plasma spiked with 50 ng of **I** and 100 ng of **III** monitored at channels ''A'' and ''B'', respectively; C, C′ control dog plasma spiked with 100 ng of **III** monitored at channels ''A'' and ''B'', respectively; D, D′ – dog plasma sample 0.5 h post-dose spiked with 100 ng **III** (calculated concentration 50.9 ng/0.1 ml) monitored at channels ''A'' and ''B'', respectively; E, E′ – control dog plasma spiked with 500 ng of **I** monitored at channels ''A'' and ''B'', respectively.

Table 1
Intra-day precision and accuracy of **I** and **III** spiked into five different lots of human control plasma

Nominal concentration (ng/0.1 ml)	Mean concentration[a] (ng/0.1 ml)	Precision[b] (RSD, %)	Accuracy[c] (%)	Mean area of **I**	Precision[d] (RSD, %)	Mean area of **III**	Precision[d] (RSD, %)
0.5	0.5	13.9	100	1027	15.9	206 695	24.3
1.0	1.0	5.1	105	2272	13.3	212 104	17.5
5.0	4.9	9.5	98	12 617	11.9	211 331	11.1
10.0	9.3	1.4	93	26 195	7.4	202 114	13.5
50.0	52.1	6.3	104	135 864	6.2	207 014	8.6
100.0	102.9	3.0	103	266 523	7.7	192 972	16.4
500.0	515.1	2.7	103	1 455 632	7.9	217 034	8.2

[a] $n=5$; mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as relative standard deviation (RSD) of peak area ratios of **I**/**III**.
[c] Expressed as [(mean observed concentrations)/(nominal concentration)·100] ($n=5$).
[d] $n=5$ at each concentration.

tion efficiencies of the analytes [10–13]. Therefore, in order to assess this effect, the ionization efficiency of both analytes, as measured by individual peak areas of **I** and **III** in different plasma matrices, was evaluated. Both **I** and **III** were spiked into five different sources of plasma, extracted, and their peak areas were determined. By comparing peak areas of the analyte in different lots of plasma, the differences in both recovery and ionization efficiency associated with a given plasma lot was assessed. As presented in Tables 1 and 2 the precision of the absolute peak areas of **I** and **III** were below 16 and 25%, respectively. The precision of peak area ratios (**I**/**III**) was under 15% at all concentrations spiked into five different sources of dog or human plasma. The beneficial and compensating effect of the presence of internal standard on the precision of determination of **I** was demonstrated by a significant increase in method precision when peak area ratios instead of absolute peak areas were calculated (Tables 1 and 2).

3.4. Assessment of assay selectivity

Assay selectivity was assessed by running blank control and patients' pre-dose plasma samples. No endogenous interferences were observed. In addition, internal standard (**III**) peak areas in plasma samples of subjects participating in a clinical study were compared for reproducibility at all time points after dosing with **II**. The mean values for **III** in post-dose

Table 2
Intra-day precision and accuracy of **I** and **III** spiked into five different lots of dog control plasma

Nominal concentration (ng/0.1 ml)	Mean concentration[a] (ng/0.1 ml)	Precision[b] (RSD, %)	Accuracy[c] (%)	Mean area of **I**	Precision[d] (RSD, %)	Mean area of **III**	Precision[d] (RSD, %)
0.5	0.5	5.9	100	1758	12.9	438 702	17.3
1.0	1.0	7.1	100	3563	13.6	419 892	12.1
5.0	5.0	5.9	100	18 763	8.7	409 974	12.5
10.0	9.7	1.4	100	34 324	12.8	371 006	15.9
50.0	51.2	2.0	102	202 201	14.7	302 496	18.6
100.0	102.2	3.1	102	423 977	12.2	473 256	16.0
500.0	500.5	4.2	100	2 493 134	9.6	501 845	11.9

[a] $n=5$; mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as relative standard deviation (RSD) of peak area ratios of **I**/**III**.
[c] Expressed as [(mean observed concentrations)/(nominal concentration)·100] ($n=5$).
[d] $n=5$ at each concentration.

Table 3
Precision and accuracy data of quality control dog and human plasma samples spiked with **I**

Nominal concentration (ng/0.1 ml)	No. of determinations	Dog plasma		Human plasma	
		Mean calculated concentration (ng/0.1 ml)	RSD (%)	Mean calculated concentration (ng/0.1 ml)	RSD (%)
2.5	4[a]	2.6	7.3	2.6	4.2
400.0	4[a]	403.0	4.6	426.0	12.8

[a] Over a period of 2 days.

samples were practically the same as in pre-dose samples spiked with **III** indicating that the metabolites of **II** did not interfere with the ionization and integration of **III** and that the recovery of **III** was practically the same from plasma samples originating from all subjects participating in the clinical study.

3.5. Analyses of samples from preclinical and clinical studies

The method was used to support preclinical and clinical pharmacokinetic studies both in human and dog with sufficient sensitivity (LOQ=0.5 ng/0.1 ml) for complete pharmacokinetic plasma concentration time course mapping. As an example, representative concentrations of **I** in plasma after oral administration of **II** to dogs participating in a single dose pharmacokinetic study are presented in Fig. 4. In addition, the analyses of QC standards indicated that **I** was stable in plasma when stored at −20°C (Table 3).

In conclusion, an assay for the determination of the thermally labile *N*-hydroxylamine metabolite (**I**) of a novel growth hormone secretagogue was developed, but required utilization of a different MS–MS interface than for the parent compound (**II**) and the use of an appropriate and different internal standard. In addition, the new assay required considerable changes in the extraction procedure and chromatographic conditions utilized. The development of an assay for **I** illustrates the difficulties associated with the transition from a method established for the parent compound to an assay for the determination of metabolite(s). The transition from the assay developed for determination of a single analyte to multiple component methods involving potentially labile analytes (metabolites) is generally not simple, and the difficulties associated with this transition are not always fully appreciated within pharmaceutical development community. This transition is considerably much more complex when assays for multiple metabolites (analytes) of different chemical and physical properties and at widely different concentration ranges are required.

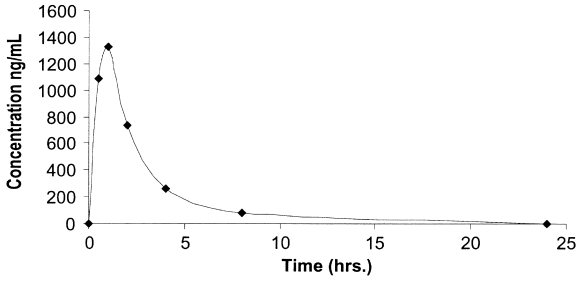

Fig. 4. Representative plasma concentration–time course for dogs receiving a 25 mg/kg dose of **II**.

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