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Column switching in high-performance liquid chromatography with tandem mass spectrometric detection for high-throughput preclinical pharmacokinetic studies

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Abstract

A high-throughput liquid chromatography–tandem mass spectrometry method is described for the determination of multiple compounds in dog and rat plasma. After acetonitrile precipitation of plasma proteins, the analytes are pre-concentrated and back-flushed on a reversed-phase column for separation using a switching valve. The analytes are ionized using TurboIon Spray in a positive mode, and detected by multiple reaction monitoring. Automatic tuning software is used for fast method development. The data processing is greatly speeded up by using a powerful quantitation software package. Chromatography of multiple compounds takes only 4 min. The linear calibration curve ranges from 0.5 to 1000 ng/ml. This method was successfully used in the analysis of multi-compounds for preclinical pharmacokinetic studies. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Column switching; Pharmaceutical analysis

1. Introduction

In the pharmaceutical industry, there is currently a great interest and demand in establishing an advanced technology for automated high-throughput bioanalysis of drug candidates for pharmacokinetic studies. Liquid chromatography–tandem mass spectrometry (LC–MS–MS) has proved to be a reliable, rapid, robust, sensitive and selective analytical method in support of preclinical and clinical studies [1–3].

Tularik's research drug candidates compound **I**, and compound **II** are lead compounds with antiviral activity, in particular against human cytomegalovirus. A preclinical study was designed to study pharmacokinetic behavior of the two compounds in different species under identical conditions. The dose of multiple compounds into a single animal (*N*-in-1 or cassette dosing) provides advantages in minimizing sample processing time, the number of animals required, and the animal variability [4,5]. In this study, both compounds were administered simultaneously to rats and dogs in a parallel dosing experimental protocol. To quantify multiple compounds in animal plasma, a selective, sensitive and high-throughput analytical method is needed. The simultaneous quantitation of multiple compounds in bio-matrices using LC–MS–MS has increased its popularity recently. The simultaneous determination of unlabeled and isotopically labeled analytes has been reported [6]. Other researchers have studied on simultaneous determination of mixtures of drug candidates by LC–MS–MS as an in vivo drug screening procedure [7]. To speed up

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process and reduce sample preparation efforts, fast assays have also been published for the quantitation of drugs and their metabolites in human plasma and urine using column switching LC–MS–MS [8,9]. Our approach is to establish flexible, sensitive and selective analytical methods which are based on simple sample preparation procedures, fast gradient chromatography and fully automated LC–MS–MS. The following method describes a rapid, high-throughput, highly sensitive and a selective column switching LC–MS–MS method for simultaneous quantitation of compounds **I** and **II** in rat and dog plasma.

2. Experimental

2.1. Materials and animals

HPLC-grade solvents were obtained from PE Applied Biosystems (Foster City, CA, USA) and analytical-grade reagents were purchased from Aldrich (Milwaukee, WI, USA) and Fisher (Pittsburgh, PA, USA). Compounds **I** (T900066) and **II** (T216349) and the internal standard were synthesized by Tularik (South San Francisco, USA). Male and female Sprague–Dawley rats, (200–250 g), were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA), and housed at Tularik at least 1 week prior to the study. Mongrel dogs (8–12 kg), were also obtained from Harlan Sprague–Dawley, and housed at the Laboratory for Toxicology Research of the University of Illinois at Chicago at least 1 week before test compound administration.

2.2. Dose formulation

For intravenous administrations, compound **I** was dissolved in 30% PEG-200 in water for injection, and compound **II** was dissolved in 10% dimethylsulfoxide (DMSO) in 5% dextrose. Solutions were injected sequentially intravenously (i.v.) into animals via a lateral tail vein and a cephalic vein in rats and dogs, respectively. The i.v. dose was 0.5 mg/kg for each compound in a volume of 1 ml/kg. For oral administration, compound **I** was dissolved in ethanol and mixed thoroughly with an excess of dry methylcellulose. The ethanol was evaporated to formulation dryness. The residue containing compound **I** was mixed with compound **II**. The two compounds were mixed to a total quantity of 2.5 mg of each compound for every 50 mg of formulation. This formulation was mixed with water until completely suspended, and dosed orally to rats via gastric gavage. The dose was at 2.5 mg/kg for each compound in a total volume of 5 ml/kg. For the dog studies, gelatin capsules, size 000, containing 50 mg/kg total formulation corresponding to 2.5 mg/kg each for both compounds, were administered orally followed by a flush of 20 ml water.

2.3. Plasma sample collection

Rats were randomly assigned to groups of four animals each. All animals received the test compounds, either intravenously or orally, and blood samples were taken under brief isoflurane anesthesia from the ventral tail artery at different intervals after dosing. A maximum of three 1.0-ml blood samples was taken from each rat. The animals were sacrificed following the final blood sample. Groups of rats were staggered in such a way that four samples per time-point were available. Blood samples were collected at 0, 2.5, 5, 10, 15, 30, 60 min and 2, 4, 8, 12 and 24 h following intravenous dosing and 0, 5, 15, 30, 60 min and 2, 4, 8, 12 and 24 h following oral dosing. Dogs were randomly assigned to two groups of three animals each, for intravenous and oral administration of test compounds. Blood samples (2 ml) were taken from a cephalic vein sequentially at 0, 2, 5, 10, 15, 30, 45, 60 min and at 2, 4, 8, 12, 24, 48 and 72 h after intravenous dosing; and 0, 15, 30, 60 min and 2, 4, 8, 12, 24, 48, and 72 h following oral dosing. All blood samples were collected into Vacutainer tubes containing EDTA as anticoagulant and placed on ice until centrifugation at 3000 *g* for 10 min. Plasma was collected and frozen at −70°C until analysis.

2.4. Sample preparation

Aliquots (0.100 ml) of animal plasma samples were diluted two-fold with acetonitrile. The acetonitrile solution contains the internal standard at 100 ng/ml. The sample was then vortexed to mix and centrifuged at approximately 8000 *g* for 2 min. The

supernatant was transferred to a glass autosampler vial for analysis.

2.5. Calibration standards and quality control samples

Blank blood samples were collected from test rats and dogs. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −70°C until needed. Stock standard solutions were added to blank animal plasma to make concentrations of compounds **I** and **II** at 1000 ng/ml each. Then the solution was diluted with the blank animal plasma to make a series calibration standard over the range 0.1–1000 ng/ml. Quality control (QC) samples were made using the pooled rat plasma at 16.0, 125 and 500 ng/ml. The spiked samples were then extracted following the procedure described above.

2.6. LC conditions

An aliquot (20 μl) of the plasma extract was injected. The analytical column used was a C_8 cartridge (50×2.1 mm, 3 μm) (YMC, Wilmington, NC, USA). The LC systems consisted of a Shimadzu (Columbia, MD, USA) LC-10AD pump system and a PE Series 200 autosampler (Perkin-Elmer, Norfolk, CT, USA). The gradient program was:

Time (min)	Acetonitrile (mobile phase A) (%)	0.1% Formic acid (mobile phase B) (%)
0.0	30	70
0.6	30	70
2.0	70	30
2.1	90	10
3.0	90	10
3.1	30	70
4.0	30	70

The flow-rate was 0.3 ml/min. The time between injections was 4 min.

2.7. Column switching operation

The column switching system consisted of the switching valve (LabPro 6 or 10 port, Rheodyne, Cotati, CA, USA) and a pre-concentration cartridge (PE Applied Biosystems, Newguard, C_{18}, 10×2.1 mm, 5 μm). The loading solution was 10% acetonitrile in 0.1% formic acid. The switching valve was controlled by PE Sciex software Sample Control. Following injection under the load position (Fig. 1), pump 1 which delivers the weak organic solution, loads the sample on the pre-concentration cartridge. The solvent front from the cartridge is directed to the waste. Another pump (pump 2) delivers the mobile phase at initial gradient composition of 30% acetonitrile through the analytical column to the mass spectrometer. The solvent front containing most of the endogenous components of the sample is eluted from the cartridge at 0.6 min, monitored using an UV detector. At that time, the valve is switched to the inject position and the analytes are back-flushed to the analytical column for separation and detection by the mass spectrometer. At 3.0 min, the valve is switched back to the load position for equilibration of the pre-concentration cartridge and preparation for next sample.

2.8. Mass spectrometric conditions

Mass spectrometric detection was performed using a Perkin-Elmer Sciex (Toronto, Canada) API 365 triple quadrupole mass spectrometer, equipped with a Sciex TurboIon Spray ionization source operated at 350°C and 7 l/min of nitrogen gas. The ion source was operated in the positive ionization mode. Multiple reaction monitoring (MRM) was used for quantitation with the dwell time of 350 ms for each compound. The collision energy was set at 36 eV. Sciex Autotune software was used for the automatic tuning MS parameters for each compound. Data were acquired and quantitated using Sciex Sample Control and TurboQuan software, respectively.

2.9. Pharmacokinetic analysis

Non-compartmental analysis was performed using SAAM II software (SAAM Institute, University of Washington, Seattle, WA, USA). Clearance, volume of distribution at steady state, elimination half-life and oral bioavailability of each compound were determined using this software.

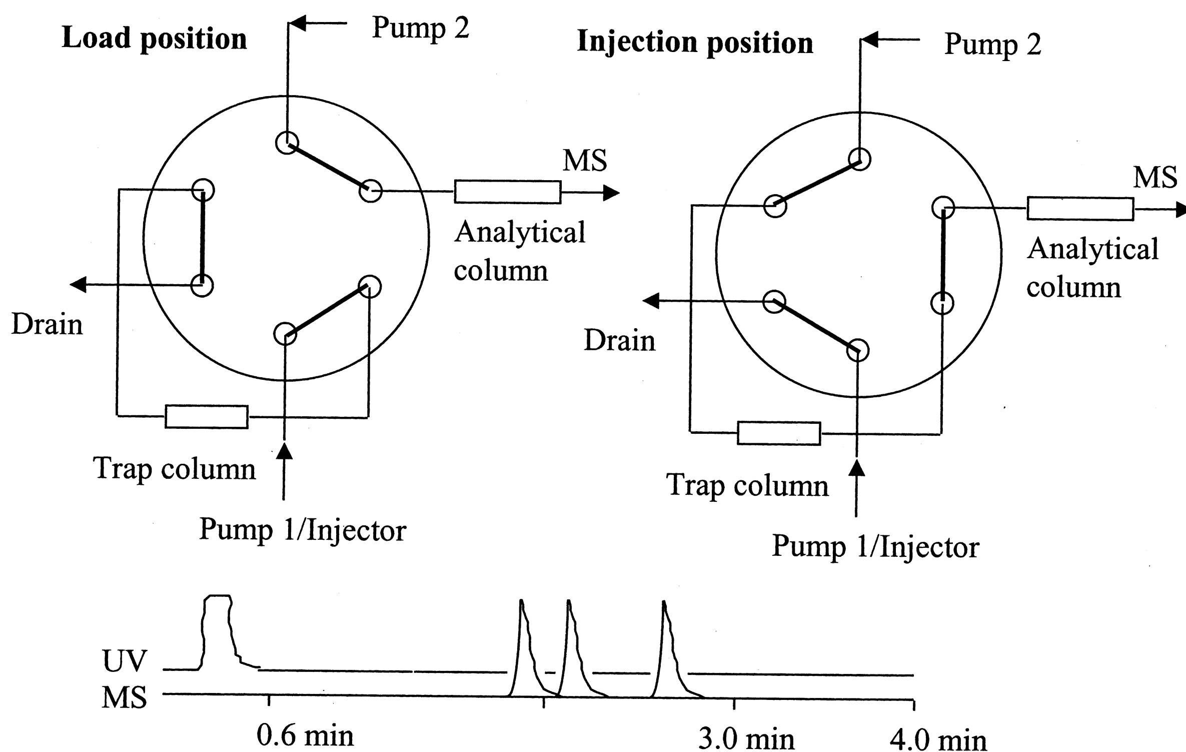

Fig. 1. Column switching diagram and corresponding UV and MRM profiles.

3. Results and discussion

3.1. LC–MS–MS method development

LC–MS–MS method development usually takes considerable amount of time even for an experienced chemist. To speed up this process, we used Sciex software to automatically tune MS and MS–MS parameters for target compounds. As with manual tuning, the automatic tuning also requires infusion of an analyte solution (e.g., at 1 μg/ml) at 5–10 μl/min using either a regular IonSpray or TurboIon Spray source. After desired information was entered in the Autotune window, the precursor ion intensity was maximized by ramping parameters of each MS lens up to the collision cell. The process continues to find eight largest product ions, maximize their sensitivities, and select the most intense fragment ion. Finally, the MS–MS experimental and state files are automatically built. The optimization report can be printed out. The automatic tuning process takes about 5 min for one compound. This software is user friendly and it greatly increases productivity.

Since the compounds selected for *N*-in-1 dosing are usually similar in structure, the optimized state file for one particular compound may be used simply as a general state file for all compounds. In a case where different compounds may require different MS parameters for an optimized sensitivity, e.g., different ion energies, these parameters for each compound can be entered into the experimental file. The mass spectrometer will scan these parameters during the course of an analytical run and the highest sensitivity for each compound can be achieved.

To speed up the LC method development process, we used a short and narrow bore column and a fast gradient. A short column requires a shorter re-equilibrium time, thus, a shorter analytical time. Generally, a gradient saves the time of not having to change mobile phase compositions as for an isocratic method. A narrow bore column facilitates narrower peak width, lower solvent consumption and slower

flow-rate, at which the LC eluent can be directly introduced into the TurboIon Spray source without splitting. YMC C_8 and C_{18} cartridges (50×2.1 mm, 3 μm) were tested. The C_8 cartridge was chosen for better retention and peak shapes of tested compounds. To develop a gradient method, we start with a steep gradient program to get all compounds out of the column, then revise the method to achieve the shortest run with satisfactory separation (capacity factor $k'>1$ for the first peak). It usually takes two separate runs to finalize a gradient LC method. Typical extracted ion chromatograms (list individual ion chromatogram for each MS–MS transition) of compounds **I** and **II** spiked in rat are demonstrated in Fig. 2. Compounds **I**, **II** and the internal standard were eluted within 3 min with symmetric peak shape and narrow band.

3.2. Calibration curve

The calibration standards of the mixed compounds **I** and **II** were prepared from 0.5 to 1000 ng/ml in rat and dog plasma and analyzed following the procedures described above. The linear dynamic ranges were 3.5 orders of magnitude for both compounds. The calibration curves were constructed using observed concentrations versus peak area ratios. A linear fit with weightings of either $1/X$ or $1/X^2$ was tested. The use of $1/X^2$ weighting gave better precision over the standard curve range; therefore, it

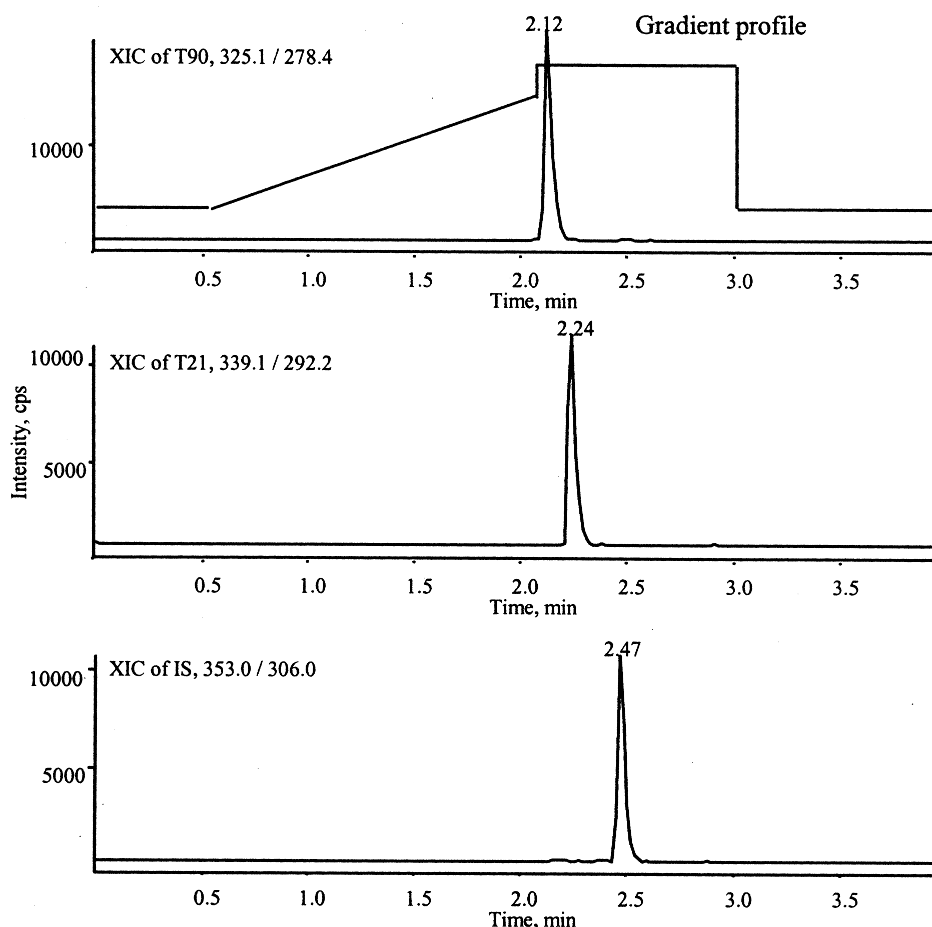

Fig. 2. Typical extracted ion chromatograms of a mixture of analytical standards. See Section 2.6 Section 2.7 Section 2.8 for LC–MS–MS conditions.

was used to best describe the data. The line equations were $y=0.028x+0.015$ ($R=0.996$) and $y=0.057x+0.019$ ($R=0.997$) for compounds **I** and **II**, respectively.

3.3. Assay sensitivity, accuracy and recovery

The lower limit of quantitation (LLOQ) was 0.5 ng/ml for both compounds **I** and **II** in rat and dog plasma. The upper quantifiable limit was 1000 ng/ml. As demonstrated in Fig. 3, the signal to noise ratio (S/N) was at least 5:1 at 0.5 ng/ml in plasma. Samples with concentrations in excess of this limit can be diluted with blank plasma. Since this method was mainly developed for the fast and high-throughput pre-clinical studies, little effort was made for repeated assays for statistical purpose. Since only limited assays were performed, the calculation provided qualitative numbers for process accuracy and recovery. For QC samples at 16.0, 125 and 500 ng/ml of compounds **I** and **II** in rat plasma, the overall accuracy was estimated as 95.4–109% for compound **I** and 97.2–109% for compound **II**, respectively. The qualitative extraction recovery was determined greater than 97% for compounds **I** and **II**.

3.4. Rat and dog plasma sample analysis

Sample preparation and the LC–MS–MS analysis (an overnight run) of multiple compounds from more than 200 samples were finished within 1 day. This multiple component analysis minimized sample processing time and the number of animals required. The mixed standards and the QC samples from this study were not much more difficult to prepare than a single compound dosing approach. A gain in assay speed is evident for the cassette dosing. The representative extracted ion chromatograms and plasma concentration profiles for i.v. and oral doses are demonstrated in Figs. 4 and 5, respectively. The extracted ion chromatograms illustrated narrow and symmetric peaks, free of any interferences.

The use of the column-switching valve provided advantages in pre-concentration and on-line clean up of matrices. The composition of loading mobile phase and injection volume can be optimized to achieve the speed and sensitivity. The inexpensive trap cartridges can be replaced every few hundred injections to maintain efficiency and eliminate carry-over. Main drawbacks are an additional pump and mobile phase.

A high-throughput quantitation approach usually generates lots of raw data. Fast data processing has become as important as sample analysis. We used a powerful quantitation package, TurboQuan (PE Sciex), to process the data generated from this study. In addition to many built-in features, such as statistics, sort and query; the high speed for peak integration and quantitation is quite impressive. It took less than 5 s to integrate and calculate 630 peaks from the study samples.

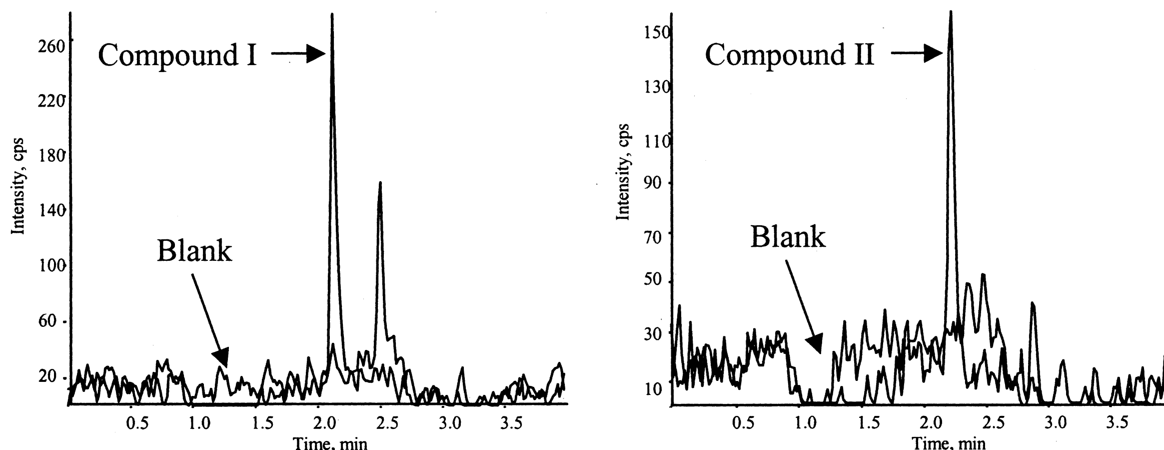

Fig. 3. Lower limit of quantitation for compounds **I** and **II**. Injections of 10 pg on column for both compounds.

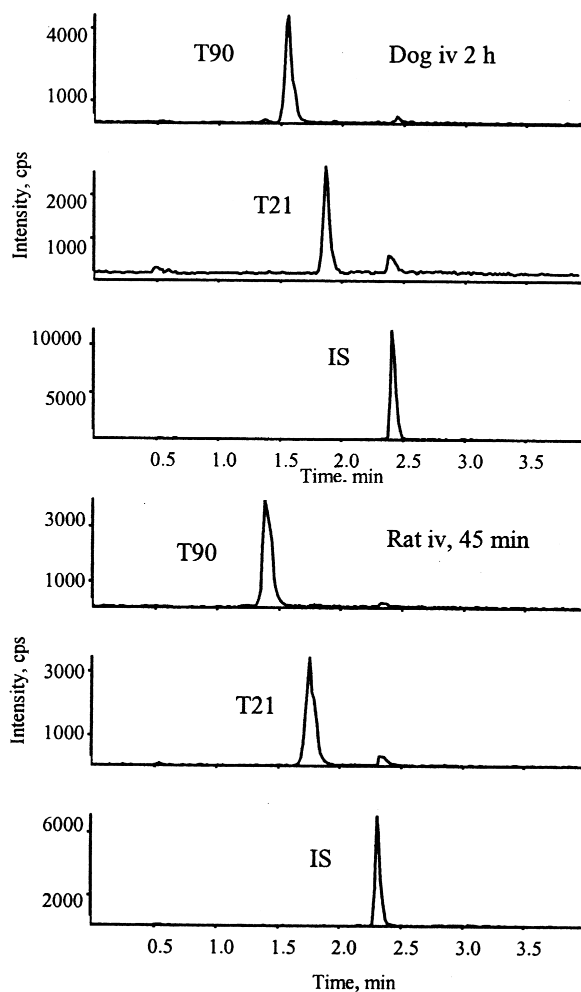

Fig. 4. Extracted ion chromatograms and plasma concentrations following i.v. administration of mixed compounds to animals. Co-administration of compounds **I** and **II** at a single i.v. dose of 0.5 mg/kg.

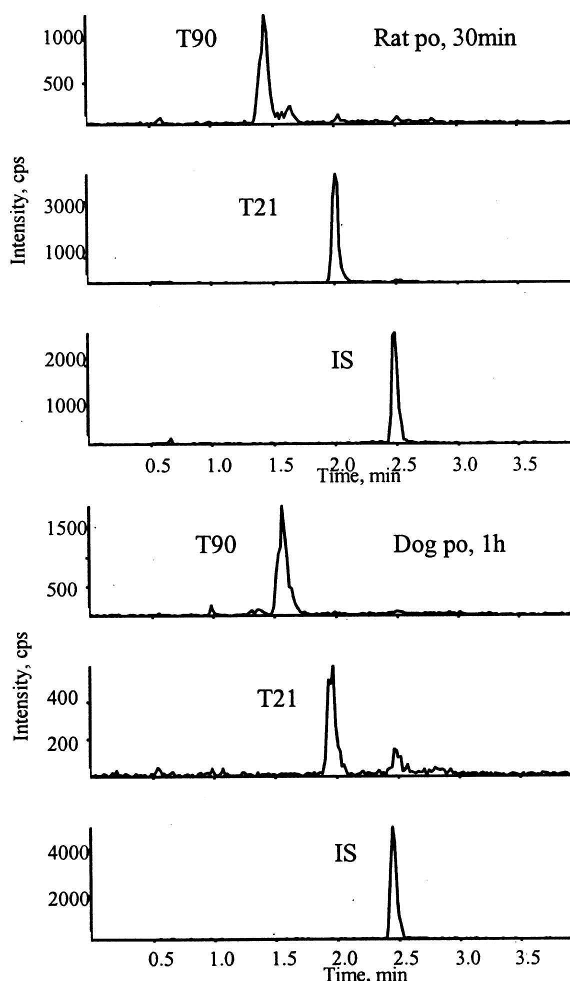

Fig. 5. Extracted ion chromatograms and plasma concentrations following oral (p.o.) administration of mixed compounds to animals. Co-administration of compounds **I** and **II** at a single oral dose of 2.5 mg/kg.

3.5. Pharmacokinetic analysis

The plasma concentration profiles obtained from the average data points showed a similar trend between the two species following either i.v. or oral administrations of the two compounds, indicating similar pharmacokinetic behavior of the two compounds. The pharmacokinetic parameters are illustrated in Table 1. Plasma concentrations after i.v. and oral administration of compound **II** in rats were highly variable, due to high inter-animal and inter-group variability, and deemed insufficient to allow a meaningful assessment of pharmacokinetic parameters. Overall, the plasma concentrations of the two compounds in dogs and rats after i.v. administration show that the compounds are relatively rapidly cleared. The clearance is around 2 l kg^{-1} h^{-1}, which indicated the compounds were metabolically cleared rather than renally excreted unchanged. Since the volume of distribution is moderate at 1.3–2.3 l/kg, the elimination half-lives are short (27–50 min). The low plasma concentrations after oral administration indicates that the oral bioavailability is very low (%F=3–6). It cannot be deduced from the present

Table 1
Pharmacokinetic parameters

Compound/species	Bioavailability (%)	Clearane, CL (l kg^{-1} h^{-1})	Volume of distributions, V_{dss} (l/kg)	Half-live ($t_{1/2}$)
T900060/rat	6.4	2.6	1.7	27.7
T900060/dog	11	2.1	1.3	24.9
T216349/rat	N/C[a]	N/C	N/C	N/C
T216349/dog	2.8	1.9	2.3	49.7

[a]Non-calculable due to high inter-animal and inter-group variability.

data whether the low oral bioavailability is due to limited absorption or first-pass metabolism.

This initial study with the two lead compounds indicates that improvements in the i.v. and oral pharmacokinetic profiles are required for this class of compounds to produce realistic drug candidates. Since the high clearance indicates metabolism as a major elimination route, the in vitro metabolic fate of these leads will be investigated to identify metabolite structures and routes of biotransformation. This may lead to the design of metabolically more stable compounds and hence to drug candidates with a more acceptable pharmacokinetic profile.

4. Conclusions

This study demonstrated a fast and reliable LC–MS–MS analytical approach for high-throughput bioanalysis. This approach was successfully used for the determination of lead compounds **I** and **II** in rat and dog plasma in support of a pre-clinical study. The results indicated that the method was fast, precise, accurate and rugged, and is suitable for the routine bioanalysis of drugs and metabolites. The advent of analytical methodologies for *N*-in-1 dosing pharmacokinetic studies allows rapid evaluation of modified compounds based on the initial leads and allows a more rapid data turnaround for structure–activity relationship development than was possible with the traditional, labor-intensive discrete pharmacokinetic studies.

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