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Column-switching High-performance Liquid Chromatography Combined with Ionspray Tandem Mass Spectrometry for the Simultaneous Determination of the Platelet Inhibitor Ro 44-3888 and its Pro-drug and Precursor Metabolite in Plasma

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A liquid chromatographic/mass spectrometric (LC/MS) assay was developed for the simultaneous determination of a pro-drug (Ro 48-3657), its active metabolite (platelet inhibitor, Ro 44-3888) and precursor metabolite (Ro 48-3656) in human, dog and rat plasma, utilizing on-line column-switching solid-phase extraction (SPE) for clean-up and high-performance liquid chromatography (HPLC) for separation of the analytes, with on-line detection by ionspray (pneumatically assisted electrospray) tandem mass spectrometry in the selected reaction monitoring (SRM) mode. The assay was validated for the quantification of all three analytes. The method involves protein precipitation with perchloric acid, enrichment of the analytes on a standard bore trapping column (i.d. 4.6 mm) and separation on a narrow-bore analytical column (i.d. 2 mm). Except for the plasma precipitation step, the assay was fully automated, allowing unattended operation. The lower limits of quantification were 0.20 ng ml^{-1} (Ro 48-3657, Ro 44-3888) and 0.50 ng ml^{-1} (Ro 48-3656) using a 0.5 ml plasma aliquot. The mean inter-assay precision and accuracy derived from quality control samples were 5.3% and 101%, respectively, utilizing the calibration range 0.2–200 ng ml^{-1}. Using the unique features of column-switching HPLC combined with MS/MS, it was possible to develop the method in a short period of time. The method has been successfully applied to map complete concentration–time courses for the kinetic evaluation of the drug and its metabolites in man, dog and rat. This LC/MS assay is sensitive, specific, accurate, precise and robust.

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INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) coupled with atmospheric pressure ionization (API) tandem mass spectrometry (MS/MS) has become an increasingly powerful analytical tool for confirmatory and quantitative analysis of drugs in biological fluids.[1–3] Owing to the unique properties of LC/MS/MS, fast method development and very sensitive quantitative analysis of drugs and their metabolites have become feasible.[4–7]

Although atmospheric pressure ionspray MS/MS provides excellent selectivity, along with ultimate sensitivity, the direct injection of biological fluids such as human plasma on to an analytical column, or even flow injection, is not a practicable approach. This is mainly due to ion suppression effects occurring during the ion evaporation process in the ionspray interface caused by proteins or other extraneous endogenous compounds and salts which dramatically compromise sensitivity and also the ruggedness of the assay. Therefore, plasma samples have to be cleaned-up prior to analysis. Column-switching on-line solid-phase extraction (SPE) has long been used for sample preparation as a highly automated methodology combined with HPLC, along with conventional detection modes,[8–10] but this approach has rarely been applied for quantitative analysis of drugs from biological fluids by LC/MS,[11–16] in contrast to environmental applications for the analysis of polar pesticides.[17] This is probably due chiefly to the longer analytical run time cycle[18] compared with the combination of off-line clean-up techniques with LC/MS with selected reaction monitoring (SRM). Nevertheless, when using ionspray MS/MS for detection, acceptable analysis times (6–9 min) can be achieved with a single column-switching system. The time-limiting steps are the loading of the sample on to the trapping column and the transfer of the trapped

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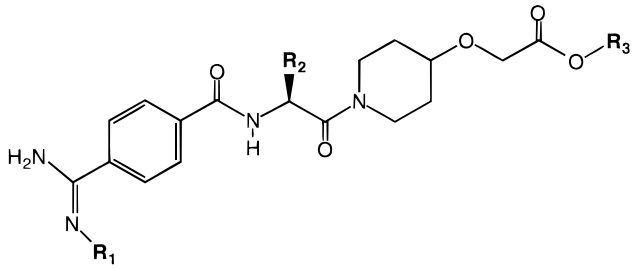
Table 1. Structural formulae of the compounds studied



Compound	Type[a]	R_1	R_2	R_3	M_r (Da)
Ro 48-3657	Parent drug	OH	CH_3	CH_2CH_3	420.5
Ro 48-3657-d_3	IS1	OH	CD_3	CH_2CH_3	423.5
Ro 48-3656	Metabolite	OH	CH_3	H	392.4
Ro 48-3656-d_3	IS2	OH	CD_3	H	395.4
Ro 44-3888	Metabolite	H	CH_3	H	376.4
Ro 44-3888-d_3	IS3	H	CD_3	H	379.4
Ro 48-7624	IS4	OH	CH_2CH_3	H	406.4
Ro 48-7324	IS5	H	CH_2CH_3	H	390.4

[a] IS = internal standard.

analytes on to the analytical column.[7,16] However, by using a dual trapping column-switching system,[8,16] the run time cycle can be further reduced by 33%.

This paper describes an LC/SRM/MS assay employing an ionspray interface for the simultaneous determination of a drug and two of its metabolites, which exhibit prominent differences in polarity, by on-line column-switching SPE combined with MS/MS detection.

Ro 48-3657 [ethyl (*Z*)-(*S*)-{[1-(2{[4-(aminohydroxyiminomethyl)benzoyl]amino}-1-oxopropyl)-4-piperidinyl]oxy} acetate] is a double protected pro-drug of the potent, specific, selective and reversible fibrinogen receptor antagonist Ro 44-3888 (Table 1). This drug is being developed for secondary prevention of arterial thrombosis, i.e. following unstable angina and stroke.[19–21] After oral administration to man, the pro-drug Ro 48-3657 (amidoxime ethyl ester) undergoes biotransformation to the precursor metabolite Ro 48-3656 (amidoxime free acid) and is then partially transformed into the active metabolite Ro 44-3888 (amidine free acid, platelet inhibitor).

In support of pharmacokinetic and pharmacodynamic profiling of this drug in human volunteers under current clinical trial evaluation, a sensitive quantification method was required for mapping complete concentration–time courses of the pro-drug and its metabolites.

EXPERIMENTAL

Chemicals and reagents

Ro 48-3657, Ro 48-3656 and Ro 44-3888 and their trideuterated labelled structural analogues used as internal standards (Ro 48-3657-d_3, Ro 48-3656-d_3 and Ro 44-3888-d_3), and also the subsidiary internal standards Ro 48-7624 and Ro 48-7324, were synthesized and supplied by Dr Th. Weller (Hoffmann-La Roche, Basle, Switzerland). The structures of the compounds are summarized in Table 1.

Methanol, ethanol, acetonitrile, tetrahydrofuran, formic acid (>98%), perchloric acid (70%) and ammonium formate (all of analytical-reagent grade) were purchased from Merck (Darmstadt, Germany) and used without further purification. Ammonium formate buffer (5 mM) was prepared from 0.315 g of ammonium formate dissolved in deionized water, adjusted to pH 3.6 using formic acid and made up to 1000 ml. Dichlorvos (2,2-dichlorovinyldimethyl phosphate) was obtained from Riedel-de Haën (Bender and Hobein, Zürich, Switzerland). Human blood was obtained from the Blutspendezentrale (Basle, Switzerland). The plasma was isolated by centrifugation of EDTA-treated blood. Dichlorvos was used for inhibition of the esterase activity of EDTA plasma. A 25 mg amount of dichlorvos was dissolved in 2.5 ml of tetrahydofuran and further diluted with water to 25 ml (1 mg ml^{-1}). A 4 ml volume of this solution was added to 400 ml of plasma to yield a final dichlorvos concentration of 10 μg ml^{-1}. Pure nitrogen (>99.999%) and ultrapure argon (>99.9997%) were supplied by Carbagas (Basle, Switzerland).

Liquid chromatographic and mass spectrometric instrumentation

The HPLC system consisted of two pumps, L-6200A (pump A), equipped with a low-pressure gradient module and L-6000A (pump B) from Merck/Hitachi (Darmstadt, Germany), for supplying the trapping and analytical column with eluent, respectively, an AS-4000 autosampler (Merck) equipped with a 2 ml sample loop and a Rheodyne 7000 E high-speed switching valve (LabSource, Basle, Switzerland). The six-port switching valve 7000E was controlled from the 'timed events' output of the L-6200A pump, and the accessory pump L-6000A was directly controlled by the L-6200A pump.

A SCIEX (Thornhill, ON, Canada) API III^{plus} triple-quadrupole mass spectrometer equipped with a laboratory-made ionspray interface was used.

Preparation of calibration and QC samples

Individual stock solutions containing Ro 48-3657, Ro 48-3656 and Ro 44-3888, each at a concentration of 1 mg ml^{-1}, were prepared by weighing 10.0 mg of each compound into a 10 ml volumentric flask, dissolving Ro 48-3656 and Ro 44-3888 with aqueous 5 mM ammonium formate (pH 3.6) and Ro 48-3657 with methanol, and further dilution to volume. Individual stock solutions of the trideuterated internal standards Ro 48-3657-d_3, Ro 48-3656-d_3 and Ro 44-3888-d_3, along with the subsidiary internal standards Ro 48-7624 and Ro 48-7324, were prepared in the same way at 0.1 mg ml^{-1}.

A series of working standard solutions containing Ro 48-3657, Ro 48-3656 and Ro 44-3888 in aqueous 5 mM ammonium formate were prepared in the range 4–4000 ng per 50 μl. For the preparation of calibration samples, a 50 μl aliquot of the respective working standard solution was diluted to 20 ml in a volumetric flask with

dichlorvos-pretreated drug-free human plasma. The resulting plasma concentrations covered the calibration range 0.20–200 ng ml^{-1} in serial steps. The quality control samples were prepared by the same procedure as used for the calibration samples, but by a different analyst using independently prepared stock and working standard solutions. The concentrations of the quality control samples were 1, 10 and 50 ng ml^{-1} for each of the analytes. All samples fortified with Ro 48-3657, Ro 48-3656 and 44-3888 were stored in 1 ml portions in capped micro test-tubes from Eppendorf (Hamburg, Germany) and immediately frozen at −20 °C after preparation until used.

An internal standard solution of Ro 48-3657-d_3, Ro 48-3656-d_3 and Ro 44-3888-d_3 was prepared in aqueous 5 mM ammonium formate at 10 ng per 25 µl. The corresponding solution for the subsidiary internals standards Ro 48-7624 and Ro 48-7324 were prepared in the same manner as outlined above. These solutions could be stored at 4 °C for at least 3 months without any evidence of degradation.

Sample work-up

Aqueous internal standard solution (50 µl) was added to plasma (0.500 ml) in a 2 ml disposable micro test-tube (Eppendorf) and vortex mixed. For protein precipitation, 0.5 M perchloric acid (0.5 ml) was added and the mixture again briefly vortex mixed. The samples were then centrifuged at ~3360 *g* for 10 min. An aliquot of the liquid phase (0.6 ml) was transferred into a tapered sample vial (1.5 ml) and diluted with 1 M ammonium formate (0.6 ml), adjusting the pH of the sample to ~3.6. Following centrifugation at about 3360 *g* for 10 min, a 1 ml aliquot of the sample was injected with an autosampler.

Chromatography and column-switching set-up

The chromatographic system (Fig. 1) consisted of a trapping column of Supelcosil LC-ABZ (20 × 4.6 mm i.d.) from Supelco (Gland, Switzerland) and an analytical column, Superspher C_{18}, 5 µm (125 × 2.0 mm i.d.), from Stagroma (Wallisellen, Switzerland), which were connected by the switching valve. The mobile phases were 5 mM aqueous ammonium formate (E1, pH 3.6, pump A), and 1% aqueous formic acid–methanol (60:40, v/v) (E2, pump B). The mobile phase E3 (pump A) consisted of aqueous 5 mM ammonium formate (pH 3.6) and ethanol (10:90, v/v). The mobile phases E1 and E3 were always used with the trapping column at a flow rate of 2.5 and 3.5 ml min^{-1}, respectively and E2 with the analytical column at 0.2 ml min^{-1}. All columns were operated at ambient temperature.

The set-up of this single trapping column switching system is displayed in Fig. 1. After injection of a 1 ml aliquot of the sample by the autosampler into the 2 ml sampling loop, the sample was transferred to the trapping column at a flow rate of 2.5 ml min^{-1}. After flushing of the trapping column for 2 min with eluent E1, the automatic switching valve was turned to the alternate position, so that the trapping column and the analytical column were in-line. By using the backflush mode, mobile phase E2 transferred the enriched analytes from the trapping on to the analytical column. After a 3 min transfer time, the switching valve was turned back to the starting position to disconnect the trapping column from the analytical column and to allow the former to be flushed with eluent E3 to remove strongly retained endogenous compounds, and then re-equilibrated with mobile phase E1. The analytes were eluted from the analytical column with eluent E2 and the effluent was passed without splitting to the ionspray interface.

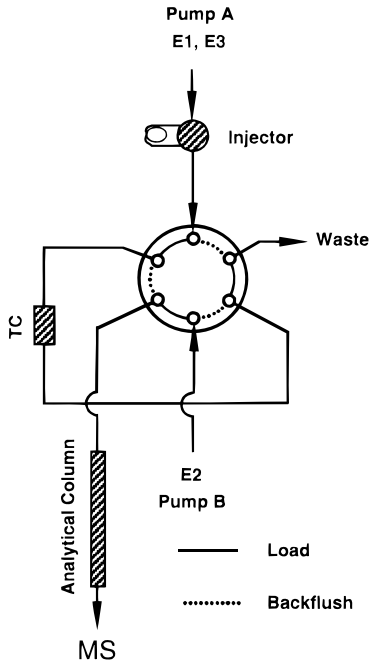
Under these conditions, Ro 48-3657 eluted after ~7.7 min and Ro 48-3656 co-eluted with Ro 44-3888 after ~5.1 min. As expected, the corresponding trideuterium-labelled internal standards also co-eluted with their structural analogues. The total analytical run time cycle lasted 9 min.

Mass spectrometry

A laboratory made ionspray (pneumatically assisted electrospray) interface was used with the triple-quadrupole mass spectrometer. The metal sleeve of the sprayer capillary completely wrapped the fused-silica capillary, which accommodated the whole effluent from the HPLC column (200 µl min^{-1}). The sprayer voltage was kept at +5600 V and the orifice potential was +50 V. Positive ions produced at atmospheric pressure were transmitted into the vacuum chamber of the quadrupole mass analyser by the forces of the applied electric field. While the temperature of the sprayer capillary was held at ambient temperature, the counterflowing curtain gas was set at ~60 °C to assist in the evaporation of the sprayed droplets. An orifice potential of 50 V proved to be a good compromise between efficient declustering of the ions and up-front collision-induced dissociation (CID) of the protonated molecules. Ultrapure nitrogen was used as the curtain and nebulizing gas at a flow rate of 1.8 and 3 l min^{-1}, respectively. The mass calibration of the mass spectrometer was tuned daily up to 600 Da at unit mass resolution by infusion of a standard solution of quaternary alkylammonium salts in acetonitrile (200 fmol μl^{-1}), utilizing the ionspray interface.

Prior to each occasion of an analytical run, the first mass-analysing quadrupole (Q1) was retuned, using a standard solution of Ro 48-3657, Ro 48-3656 and Ro 44-3888, to yield a resolution of 0.6 Da at peak half-height for the precursor ion mass peak of the protonated molecules at *m/z* 421, 393 and 377, and also for the internal standards Ro 48-3657-d_3, Ro 48-3656-d_3 and Ro 44-3888-d_3 at *m/z* 424, 396 and 380, respectively. The same procedure was repeated with the second mass-analysing quadrupole (Q3), using the product ion scan mode. The resolution on Q3 was tuned to be 0.6 Da at peak half-height for the product ions of Ro 48-3657, Ro 48-3656 and Ro 44-3888, along with their internal standards Ro 48-3657-d_3, Ro 48-3656-d_3 and Ro 44-3888-d_3, using the following fragmentation pathways: *m/z* 421 → 188, *m/z* 393 → 160, *m/z* 377 → 190 and *m/z* 424 → 188, *m/z* 396 → 160, *m/z* 380 → 193, respectively.

The mass spectrometer was used in the MS/MS mode, utilizing CID of the protonated molecules in Q_2.



Load: Transfer of 1-ml sample volume from 2-ml loop to TC

Backflush: TC and AC connected. Backflush of trapped components to AC

Flush: Flushing of TC with eluent E3 to remove endogenous components

Trapping column: (TC)

Supelcosil LC-ABZ, 5 μm, 20 x 4.6 mm

Analytical column: (AC)

Supersper C18, 5 μm, 125 x 2.0 mm

Eluents:

E1: 5 mM ammonium formate (pH=3.6)

E2: 1 % formic acid/methanol = 60/40; (v/v)

E3: 5 mM ammonium formate (pH=3.6)/ethanol=10/90; (v/v)

———	·····	———		Position of switching valve
E1	E2	E3	E1	Eluent of TC
2.5	0.2	3.5	2.5	Flow rate (ml/min) of TC
Load	Backflush	Flush	Equilibration	Process trapping column (TC)
Idling	Transfer	Separation		Process analytical column (AC)
0.2				Flow rate (ml/min) of AC
E2				Eluent of AC

0 2 5 6 9 (min)

One analytical run lasts 9 min.

Figure 1. Set-up and time sequence of a single trapping column-switching system.

The collision gas was ultrapure argon at a collision gas thickness of $\sim 2.8 \times 10^{15}$ molecules cm^{-2} and a collision energy of 20 eV was used. For detection of the compounds, selected reaction monitoring (SRM) was used. Two periods for data acquisition were employed for adaptation of the dwell time. During the first period, the reactions monitored were m/z 393 → 160 (Ro 48-3656), m/z 377 → 190 (Ro 44-3888), along with m/z 396 → 160 (Ro 48-3656-d_3) and m/z 380 → 193 Da (Ro 44-3888-d_3). The dwell time was set at 70 ms. During the second period, the target ions of Ro 48-3657 and its internal standard Ro 48-3657-d_3 were used with the transitions m/z 421 → 188 and m/z 424 → 188, respectively, at a dwell time of 400 ms. A pause time of 50 ms was used. In order to optimize the position of the sprayer needle relating to the orifice under the conditions used for analysis of the samples (flow rate 200 μl min^{-1}), flow injection of a standard solution containing the aforementioned compounds was carried out. The sprayer needle position yielding the best signal-to-noise ratio was maintained throughout the analysis of study samples.

A Macintosh Quadra 650 computer was used for instrument control, data acquisition and data processing. Data acquisition and integration of SRM chromatograms were performed running the proprietary software packages RAD (Routine Acquisition and Display program) and MACQUAN from PE SCIEX, respectively. Calibration graphs were established by linear least-squares regression, weighted to the quadratic reciprocal of concentration ($1/x^2$), *vs.* the measured peak area ratios of the respective analyte/internal standard using MACQUAN. Concentrations of study and quality control (QC) samples were calculated using the resulting linear calibration equation.

Validation procedures

This LC/MS assay was validated for the recovery of the analytes from plasma, stability in plasma from various species, linearity of calibration, inter-assay accuracy and precision, quantification limit in routine use and specificity of the methodology. The accuracy and precision of

the method were assessed by analysing replicates of QC samples spiked at different concentrations for each analyte in the respective plasma. The concentrations were determined using the calibration equation.

RESULTS AND DISCUSSION

Sample clean-up and liquid chromatography

In a continuous effort to develop a sensitive and selective assay for the simultaneous quantification of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in plasma, a clean-up procedure was selected which was efficient, straightforward and time-saving as well as suitable for automation. After denaturation and precipitation of the plasma proteins with perchloric acid,[22] and separation of the aqueous liquid supernatant by centrifugation, the sample had to be diluted with 1 M ammonium formate to adjust the pH to 3.6. The last step of the clean-up procedure was only required for preventing cleavage of the ester group of Ro 48-3657 owing to the low pH of the precipitation medium. A further benefit was that the useful lifetime of the trapping column could be also increased. All other steps for concomitant analysis of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in plasma were completely automated. The time needed for the transfer of an aqueous 1 ml sample to the standard bore trapping column, removal of endogenous compounds and extraneous salts from this trapping column was 2 min.

An alternative approach, using a narrow-bore (2 mm i.d.) trapping column instead of a standard-bore column was not advantageous because of a four-fold longer sampling time. The reduction in the transfer time from a narrow-bore trapping to an analytical column of the same internal diameter could not readily compensate this loss. In contrast to this approach, the combination of a standard-bore trapping and analytical column allows a reduction in the transfer time from 3 to 1 min, but ultimate sensitivity was compromised owing to the concentration-sensitive behaviour of the ionspray interface.[23] If the highest priority is speed of analysis, a standard-bore trapping and analytical column should be used, and the flushing step could be omitted. This resulted in a run time cycle of 5–6 min, but at the expense of sensitivity and a shorter lifetime of the trapping column. For all these reasons, a standard-bore trapping column in combination with a narrow-bore analytical column was considered to be the best compromise relating to speed of analysis, sensitivity and ruggedness of the assay. One trapping column could be loaded with more than 300 ml of plasma equivalents without affecting its efficiency or that of the analytical column. The recovery proved to be better than 72%, demonstrating the efficacy of the enrichment process. The Superspher C_{18} narrow-bore analytical column can be replaced with Inertsil ODS-3 (GL Science, Tokyo, Japan) or Supelcosil ABZ$^+$Plus (Supelco, Bellefonte, PA USA) without losing chromatographic performance.

In order to achieve short retention times of the analytes on the analytical column, the elution of Ro 48-3656 and Ro 44-3888 was driven to a low capacity factor ($k' < 1$). The whole analytical run time cycle lasted 9 min, but the effective retention times comprising the transfer time from the trapping to the analytical column (including the retention on the analytical column) were ~3.1 min for Ro 48-3656, Ro 44-3888 and their internal standards and ~5.7 min for Ro 48-3657 and its co-eluting internal standard (Fig. 2). The run cycle time was not optimized to its full potential. The retention times of the analytes could be further reduced by using an analytical column of 3–5 cm length.

Although a longer analysis cycle was involved with on-line SPE compared with off-line approach, the main benefit of a considerable reduction in the labour-intensive clean-up readily compensated this shortcoming, owing to a high degree of automation. More than 150 consecutive injections of plasma samples could

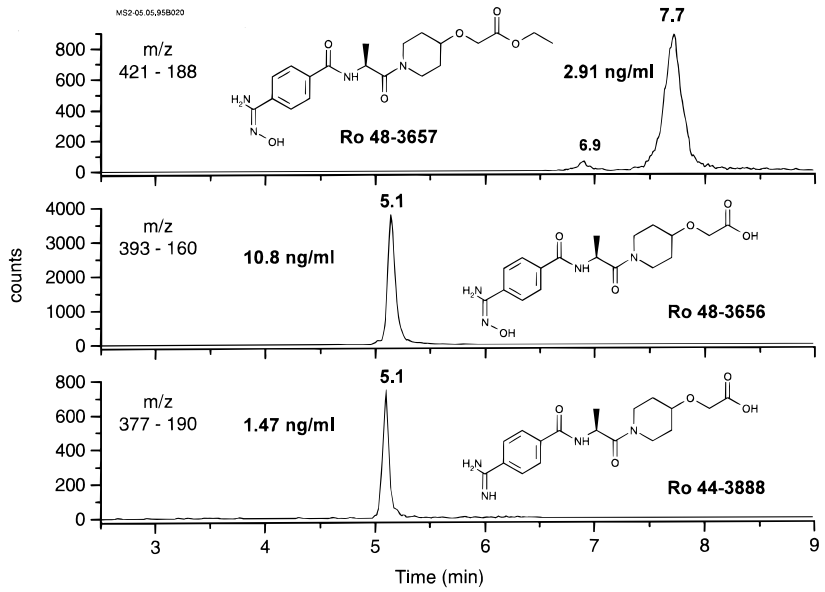


Figure 2. SRM chromatograms of a plasma sample taken 2 h after oral administration of the pro-drug Ro 48-3657 to a human subject. The respective SRM transitions are displayed in the upper left-hand corner.

be made without any evidence of deterioration of the system's performance.

When using a dual trapping column switching system, the analytical run time cycle could be further reduced from 9 to 6 min. This approach allowed the analysis of more than 150 samples in 16 h.[16] For quantifying Ro 48-3656 and Ro 44-3888, it was not necessary to separate them chromatographically. Nevertheless, the method offers the opportunity to separate all analytes employing gradient elution, to backflush the trapped compounds from the precolumn and separate them on the analytical column. This is particularly useful when screening of metabolites in biological fluids is required or metabolic profiles are to be mapped.

Figure 3(A) depicts the SRM chromatogram of Ro 48-3657 and its metabolites obtained following injection of a 1 ml sample aliquot on to the precolumn, and backflushing of the trapped analytes on to the analytical column using gradient elution compared with direct injection of the same amount of compounds on to the analytical column [Fig. 3(B)]. The peak profiles indicated that no apparent peak broadening occurred during the transfer of the analytes from the standard-bore (4.6 mm i.d.) on to the narrow-bore (2.0 mm i.d.) analytical column. A shortcoming of the gradient elution *vs.* the isocratic approach was the longer run time cycle due to the re-equilibration time needed.

Mass spectrometric considerations

Heated nebulizer (APCI). Initial trials, using the heated nebulizer interface, indicated extensive thermal fragmentation of Ro 48-3656 and Ro 44-3888, ruling out sensitive detection and accurate quantification. For Ro 48-3657, the extent of thermal degradation was much less pronounced, but losses of oxygen and NH_3 could be clearly confirmed. Moreover, the extent of degradation in the interface caused by heat and catalytically induced decomposition, mediated by active sites, was strongly dependent on the concentration. For all these reasons, the heated nebulizer interface was not suitable for reliable and sensitive analysis of these compounds.

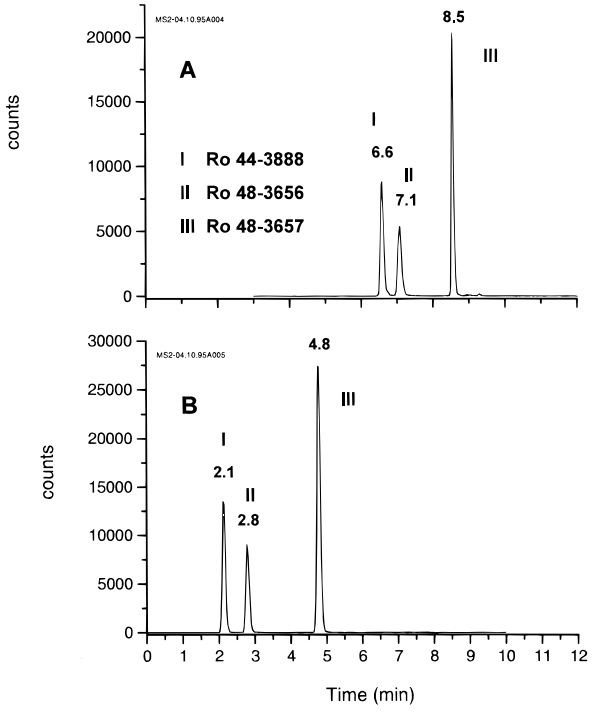


Figure 3. (A) SRM chromatogram following injection of a 1 ml sample (2 ng ml^{-1}) on to a trapping column and gradient elution on the analytical column. Eluents: (A) 5 mM ammonium formate–20% methanol; (B) 5 mM ammonium formate–80% methanol; gradient profile; proportion of eluent A increased from 20% to 80% in 4 min. Flow rate, 0.2 ml min^{-1}. (B) SRM chromatogram following injection of 5 µl (2 ng per 5 µl) on to the analytical column and gradient elution. Same gradient profile as above.

Ionspray. Ro 48-3657, Ro 48-3656 and Ro 44-3888 are readily ionized in the aqueous solutions used as eluents for separation of these compounds by reversed-phase HPLC. Therefore, the ionspray interface was considered to be well suited for efficient transmission of the ions from the condensed to the gas phase and subsequent extraction by electric forces into the mass spectrometer. Positive charge ionization was preferred to negative ionization, since all three compounds could be simultaneously detected with comparably good sensitivity.

Figure 4 depicts the positive full-scan product ion spectra of Ro 48-3657, Ro 48-3656 and Ro 44-3888 using the protonated molecules $[M + H]^+$ at m/z 421, 393 and 377, respectively. The spectra were obtained by the infusion of a standard solution (1 ng μl^{-1}) at 20 µl min^{-1}, using acetonitrile–1% formic acid (50:50) as solvent. The compounds undergo extensive fragmentation in the collision cell. Ro 48-3657 and its major metabolites, together with their trideuterated structural analogues, yielded the most abundant fragment ion peaks at a collision energy of 20 eV. The transitions used for detection were selected to yield maximum sensitivity. Whereas for Ro 48-3657 and Ro 48-3656 the product ions selected for SRM formally correspond to the y_n series of peptide fragmentation, the most abundant product ion for Ro 44-3888 corresponds to the a_n series.[24] When using the trideuterated structural analogues as internal standards, the same product ions were used as for the non-labelled compounds. Therefore, the possible carry-over in the collision cell had to be checked carefully to rule out any pitfalls.

Ion suppression. Ion suppression due to matrix effects during the ionization process is a parameter which should be investigated in more detail, if ultimate sensitivity is required. Ro 44-3888 in particular was extremely prone to this effect, probably chiefly because this compound was hardly retained on the analytical column, and eluted with the whole bulk of unretained endogenous compounds. This effect was strongly dependent on the analytical column used and the elution strength of the eluent. Under the LC/MS conditions described, a decrease in sensitivity by a factor of ~ 2.5 was found equally for all three compounds when plasma extracts spiked with the same amount of compounds were compared with a pure solution containing the same concentrations.

Inter-assay precision and accuracy

The inter-assay precision and accuracy were evaluated by assaying QC samples made up in human plasma on

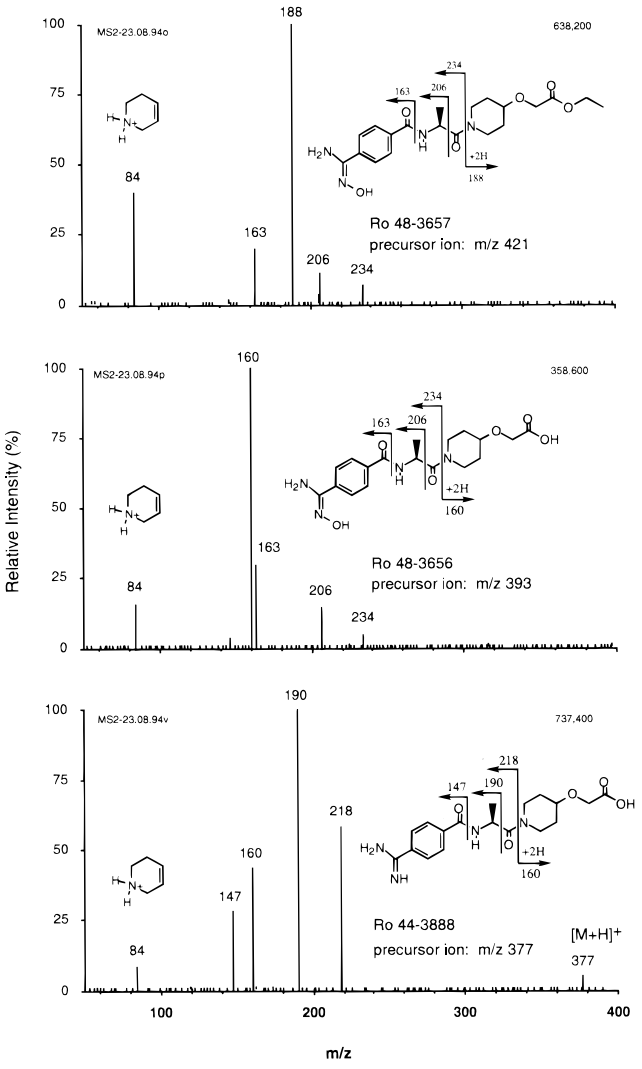


Figure 4. Product ion spectra of Ro 48-3657, Ro 48-3656 and Ro 44-3888 with specified fragment ions. Positive ion-mode; collision gas, argon; collision energy, 20 eV; collision gas thickness, 2.8×10^{15} molecules cm^{-2}.

six different days covering a period of 6 weeks. Table 2 displays the data for Ro 48-3657 and its metabolites in human plasma in the concentration range 0.2–100 ng ml^{-1}. The mean accuracies for Ro 48-3657, Ro 48-3656 and Ro 44-3888 were found to be 99.6, 102.9 and 100.8%, respectively, and the mean precisions were 4.2, 5.4 and 6.4%, respectively. Table 3 displays the respective data for Ro 48-3656 and Ro 44-3888 in the concentration range 0.5–100 ng ml^{-1}, using the subsidiary internal standards. The mean precisions for Ro 48-3656 and Ro 44-3888, including the precision at the QL, were found to be 9.7 and 5.1%, respectively, and the respective mean accuracies were 100.5 and 98.3%. The decrease in the precision using the subsidiary rather than the deuterated internal standards demonstrates the utility of the stable isotope-labelled internal standards, which has long been recognized. In particular, a useful feature of this type of internal standard is the compensation of matrix effects.

It was noteworthy that the accuracy was not affected by the change from the deuterated to the conventional internal standards, emphasizing the ruggedness of the assay procedure. This finding underlines that at the beginning of the method development a conventional internal standard might be sufficient when a stable isotope-labelled internal standard is not readily available. Although gradient elution of the analytes from the analytical column was not required for their simultaneous determination, the data in Table 4 indicate that the precision and accuracy were comparable to those of the isocratic approach. Obviously, the continuous change in solvent composition during gradient elution did not upset the ion evaporation process in the ionspray interface, or else the response fluctuations were readily compensated for by the stable isotope-labelled internal standard.

Linearity and limit of quantification

The calibration range was selected according to the concentrations expected in the samples to be analysed. The concentration range 0.20–200 ng ml^{-1} proved to be sufficient for the determination of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in human plasma samples. For all three compounds, excellent linearity was found in this concentration range, covering three orders of magnitude. The intercept of the calibration graph did not differ significantly from zero, confirming the excellent selectivity of the assay and the absence of the non-labelled compound in the trideuterated structural analogues used as internal standards.

The lower quantification limit for Ro 48-3657 and Ro 44-3888 was 0.20 ng ml^{-1}, and for Ro 48-3656 it was set at 0.50 ng ml^{-1} to meet the precision criterion of $\leqslant$15% when using a plasma aliquot of 0.5 ml (Table 2). The precision and accuracy for Ro 48-3656 and Ro

Table 2. Inter-assay precision and accuracy with the isocratic column-switching system using deuterated internal standards for determination of the analytes in human plasma ($n = 5$)

	Ro 48-3657 IS1: 48-3657-d_3			Ro 48-3656 IS2: 48-3656-d_3			Ro 44-3888 IS3: 44-3888-d_3		
Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)
0.200	0.204	102.2	7.6	—	—	—	0.213	106.5	12.4
0.500	0.473	94.5	5.0	0.559	111.8	8.3	0.501	100.2	10.6
1.00	1.01	100.8	4.1	1.03	103.0	6.4	0.972	97.2	4.7
10.0	10.5	105.3	2.1	10.1	101.4	2.2	10.6	105.5	1.6
100.0	95.2	95.2	2.1	95.3	95.3	4.8	94.7	94.7	2.5

Table 3. Inter-assay precision and accuracy with the isocratic column-switching system using subsidiary internal standards for the determination of the analytes in human plasma ($n = 5$)

	Ro 48-3656 IS4: Ro 48-7624			Ro 44-3888 IS5: Ro 48-7324		
Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)
0.500	0.516	103.3	18.5	0.516	103.3	5.0
1.00	1.09	109.0	6.1	0.993	99.3	5.6
10.0	9.87	98.7	8.1	9.85	98.5	4.8
100.0	90.9	90.9	5.9	91.5	91.5	5.1

Table 4. Inter-assay precision and accuracy with the gradient column-switching system using deuterated internal standards for the determination of the analytes in human plasma ($n = 4$)

	Ro 48-3657 IS1: 48-3657-d_3			Ro 48-3656 IS2: 48-3656-d_3			Ro 44-3888 IS3: 44-3888-d_3		
Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)	Concentration found (ng ml^{-1})	Accuracy (%)	RSD (%)
1.00	1.07	107.0	7.7	1.04	104.1	3.9	0.935	93.5	2.5
50.0	52.3	104.6	2.5	48.4	96.8	0.7	48.1	96.3	0.3

44-3888 were found to be unacceptable at concentrations below 0.5 ng ml^{-1} using the subsidiary internal standards (Table 3).

Should a lower quantification limit be required, the conditions of the assay could easily be adapted to achieve this goal, by using a 1 ml plasma aliquot and/or allocation of a longer dwell time to the respective transition, at the expense of others, to improve the signal-to-noise ratio. An investigation of the gradient elution approach showed that the same quantification limits could be achieved for the respective analytes, but with 0.25 instead of 0.5 ml of plasma sample, compared with the isocratic version. This was chiefly due to the small peak width of the analytes in the gradient elution SRM chromatogram, which exploited the concentration-dependent response behaviour of the ionspray interface to effect an increase in sensitivity.

Recovery

The overall recovery for Ro 48-3657 and its metabolites from human plasma samples was found to be between 72.1 and 82.1% with a precision $\leq 6.3\%$, covering the concentration range 2–100 ng ml^{-1}. The recovery comprised the extraction of the analytes following protein precipitation and allowed for the losses occurring during the sampling on to the trapping column, along with the losses due to incomplete transfer of the analytes from the trapping column to the analytical column. The recoveries of the trideuterated internal standards were not investigated. The recovery was assumed to be equivalent to their non-labelled structural analogues. The data are summarized in Table 5.

Stability in plasma and precipitation medium

An investigation of the stability of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in human plasma was performed.[25] In contrast to Ro 48-3656 and Ro 44-3888, Ro 48-3657 was found to be unstable in human plasma at room temperature for periods longer than 0.5 h if no esterase inhibitor was used (Table 6). Decreases in concentration of 18% and 8.9% were found following storage of a 10 ng ml^{-1} plasma sample at ambient temperature (22 °C) for 1 h and at −20 °C for 48 h, respectively. When using dichlorvos as esterase inhibitor (10 μg ml^{-1}), no degradation of Ro 48-3657 (10 ng ml^{-1}) in human plasma could be found after storage at ambient temperature for 2 h. All other stability investigations with human plasma were performed using dichlorvos (10 μg ml^{-1} plasma) for stabilization. Ro 48-3657, Ro 48-3656 and Ro 44-3888 demonstrated stability over three freeze–thaw cycles (−20/+22 °C) in human plasma, and after storage at −20 °C for 5 months (Table 7).

During the usual time-scale of the protein precipitation procedure, Ro 48-3657 underwent only minor cleavage of the ester moiety. The occurrence of the

Table 5. Recovery of Ro 48-3657, Ro 48-3656 and Ro 44-3888 from human plasma following protein precipitation ($n = 5$)

Concentration (ng ml^{-1})	Ro 48-3657 (%)	RSD (%)	Ro 48-3656 (%)	RSD (%)	Ro 44-3888 (%)	RSD (%)
2.00	72.6	6.0	82.1	6.3	76.1	4.0
100	72.1	2.1	79.4	2.3	77.7	0.8

Table 6. Stability of Ro 48-3657 in human plasma samples

Storage period (h)	Storage temperature (°C)	Esterase inhibitor dichlorvos (μg ml^{-1})	Concentration added (ng ml^{-1})	DEV[a] (%)	LL[a] (%)	UL[a] (%)	n[a]	Evaluation
1	22	—	10	−18	−21	−14	5	Unstable
2	22	—	10	−30	−30	−32	5	Unstable
24	22	—	10	−96	−97	−96	5	Unstable
48	−20	—	10	−8.9	−13	−5.2	5	Unstable
2	22	10	10	0.9	−3.1	5.1	5	Stable

[a] DEV corresponds to the deviation from the concentration added; LL and UL refer to the lower and upper limits of the 90% confidence interval; n = number of determinations.

Table 7. Stability of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in human plasma after 3 freeze/thaw cycles at −20 °C

Compound	Concentration added (ng/ml)	DEV[a] (%)	LL[a] (%)	UL[a] (%)	n	Evaluation
Ro 48-3657	5.0	−3.2	−5.7	−0.7	6	stable
Ro 48-3656	5.0	−5.4	−10.7	0.3	6	stable
Ro 44-3888	5.0	2.0	−3.6	8.0	6	stable

[a] Dichlorvos (10 μg/ml plasma) was added as esterase inhibitor. DEV corresponds to the deviation of the concentration added; LL and UL refer to the lower and upper limits of the 90% confidence interval.

cleavage product Ro 48-3656 was found to be below 0.05%, which could be neglected.

Sample analysis and specificity

This assay was used for concomitant determination of Ro 48-3657 and its major metabolites (Ro 48-3656 and Ro 44-3888) in human plasma samples from clinical studies and in dog plasma samples from toxicity and kinetic studies. Ro 48-3657 was also determined in plasma samples for residue analysis from a 26 week toxicity study in rats.

Figure 2 shows SRM chromatograms from a plasma sample following oral administration of a 5 mg dose of Ro 48-3657 to a human subject. The concentrations of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in the sample were found to be 2.91, 10.8 and 1.47 ng ml^{-1}, respectively. As can be expected, with chromatographic and two stages of mass selectivity, the SRM traces of the corresponding predose sample were clear of any relevant interferences (Fig. 5). The small peaks at the expected retention times of the drug and its metabolites were produced by the non-labelled compounds present as minor impurities of the trideuterium-labelled components.

A cross-validation was performed between an HPLC/UV assay[26] and this LC/MS assay, where Ro 48-3656 and Ro 44-3888 were determined in samples from a clinical study with each of the methods. The results indicated that there were no significant differences between the assays in the concentration range investigated (>1 ng ml^{-1}).

CONCLUSION

The LC/SRM/MS assay provided the requisite sensitivity to define accurately the pharmacokinetic profiles of Ro 48-3657, Ro 48-3656 and Ro 44-3888 in man from the first clinical study, and in dog and rat from toxicity and kinetic studies. This straightforward methodology could be successfully used for compounds with a broad

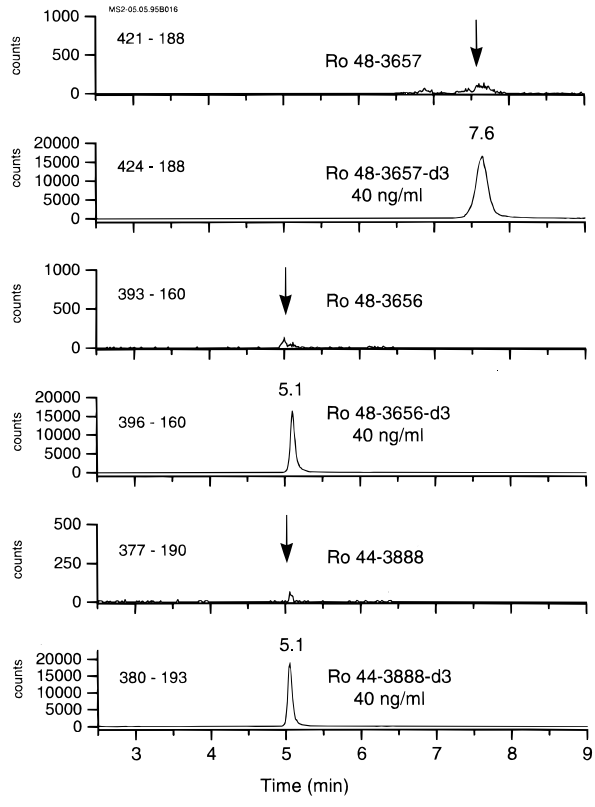


Figure 5. SRM chromatograms of a human plasma sample taken prior to oral administration of the pro-drug Ro 48-3657. The arrows indicate the retention times of the pro-drug and its metabolites.

range of polarity, which are often difficult to clean up simultaneously by off-line procedures. The longer run time cycles compared with off-line methods were readily compensated by the superior performance and robustness of the assay, along with the reduced efforts required for sample clean-up. Except for the plasma precipitation step, the assay was fully automated, allowing unattended operation. Under routine conditions more than 100 plasma samples could be analysed on a daily basis. The method has proved to be sensitive, specific, accurate, precise and robust in routine use.

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