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Development of a high-performance liquid chromatographic–mass spectrometric assay for the specific and sensitive quantification of Ro 64-0802, an anti-influenza drug, and its pro-drug, oseltamivir, in human and animal plasma and urine

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Abstract

Oseltamivir phosphate (Ro 64-0796/002) is a pro-drug of the anti-influenza neuraminidase inhibitor, Ro 64-0802, and as Tamiflu™, has been developed for the treatment of both A and B strains of the disease. This paper describes an HPLC–MS–MS assay for both compounds in plasma and urine which fulfils all of the criteria for a good analytical method. It is sensitive with limits of quantification of 1 and 10 ng/ml for the pro-drug and active neuraminidase inhibitor, respectively. It is both accurate and precise with typical coefficients of variation from some 5000 quality control samples of approximately ±3 and ±6%, respectively. Extensive stability studies have demonstrated the absence of significant problems associated with the decomposition of either compound, although ex vivo hydrolysis of Ro 64-0796 to Ro 64-0802 in rodent plasma has to be prevented by the use of the esterase inhibitor, dichlorvos. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ro 64-0802; Oseltamivir

1. Introduction

Influenza A and B viral infections continue to be a serious health concern. Yearly epidemics are responsible for significant morbidity among the general population and mortality among very young children, the elderly, and patients with chronic health problems. The available options for the control of influenza are limited. Vaccines currently serve as the primary defence against influenza but, due to the rapid change in viral antigenic determinants, annual vaccination is required and is not always effective. Increased understanding of the molecular aspects of the life cycle of the influenza virus has led to the identification of several potential targets for antiviral

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intervention. One of the most promising of these is the viral neuraminidase.

Ro 64-0802 (GS4071) is a potent and highly selective inhibitor of the neuraminidases from the A and B strains of the influenza virus [1]. These enzymes are essential for the replication of the virus and the drug has shown potent antiviral activity against all laboratory strains tested in tissue culture [2]. The ethyl ester pro-drug (Ro 64-0796, GS4104, oseltamivir) has been developed as its phosphate salt, Tamiflu™, for the treatment of patients suffering from influenza [3], and has recently been registered in a number of countries, including the USA. Ro 64-0796 is rapidly hydrolysed in vivo to Ro 64-0802 (Fig. 1) and provides excellent bioavailability of the active neuraminidase inhibitor in animals [4] and approximately 80% in man [5]. Animal studies have shown that the drug is well distributed, with high levels in the lung (the primary target site), and anti-viral concentrations have been demonstrated in other organs likely to be infected by influenza, such as the nasal mucosa, trachea and middle ear [6]. Ro 64-0796 has exhibited efficacy in models of influenza in the ferret and mouse [2] as well as activity in the treatment and prevention of the disease in man [3].

Since both pro-drug and active enzyme-inhibitor circulate in plasma, an assay for the simultaneous measurement of the two compounds was required in order to define the safety and efficacy of the drug. Ro 64-0796 and Ro 64-0802 are excreted in urine and so measurement of urinary levels of the two compounds was also needed.

As the development of Ro 64-0796 progressed, different requirements were placed on the analytical methods needed to measure plasma levels of the pro-drug and active neuraminidase inhibitor. Initially a bio-assay, based on neuraminidase inhibition, was used [2,4], and this was followed by an HPLC/fluorescence method with pre-column derivatisation [7]. However, neither assay was suitable for large numbers of human samples and a more sensitive, selective and robust method was required. HPLC with tandem mass spectrometric detection was the most attractive option for the simultaneous assay of Ro 64-0796 and Ro 64-0802 in plasma and urine.

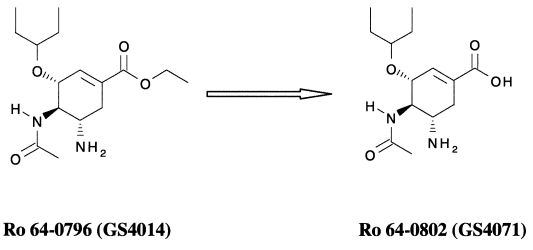


Fig. 1. The structures of Ro 64-0796 and Ro 64-0802.

Ro 64-0796 is an ethyl ester and so susceptible to esterases circulating in plasma. Ex vivo hydrolysis of esters can take place, especially in rodent plasma, making it difficult to measure plasma levels of both pro-drug and active compound in these species. However, drawing blood directly into collection tubes containing an esterase inhibitor, such as dichlorvos, allows the concentrations of both drug and pro-drug to be reliably determined.

2. Experimental

2.1. Conditions

2.1.1. Chemicals

Ro 64-0796/002, lot 4104-02-B-1, Ro 64-0802/000, lot 1031-121, and the internal standards, GS 4723 (Ro 64-0802/001), lot 1179-30, and GS 5209 (Ro 64-0796/902), lot 1150-77, were synthesised by Gilead Sciences. EDTA human plasma was supplied by Interstate Blood Bank. Dichlovos was supplied by Reidel-deHaen.

2.1.2. Instrumentation

For automated extraction, 96-well mixed phase cationic extraction blocks were used with a Tecan Genesis RSP100 Automated Sample Processor.

Mass spectrometric detection was carried out using four instruments: a Finnigan TSQ 7000 tandem quadrupole mass spectrometer with an electrospray ionisation source and ICIS version 8.2.1 control software, a Finnigan TSQ 700 tandem quadrupole mass spectrometer with an electrospray interface and ICIS 7.2 control software and PE-Sciex API 3 or API 365 mass spectrometers with Turbo Ionspray interfaces and MacQuan processing software.

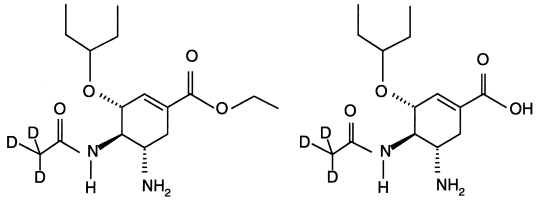
2.1.3. Stock solutions

Stock solutions of Ro 64-0796 and Ro 64-0802 were prepared in distilled water at 1.00 mg/ml. These were then diluted with blank EDTA human plasma to give calibration and quality control samples. The ranges of the calibration curves for Ro 64-0796 and Ro 64-0802 were 1.00–250 and 10.0–10 000 ng/ml, respectively, and nine duplicate standards were used in each batch. Quality control samples were normally made up at 5, 100 and 200 ng/ml for Ro 64-0796 and 50, 4000 and 8000 ng/ml for Ro 64-0802.

The standards were prepared and stored at −20°C except in the case of Ro 64-0796 in rat and mouse plasma, where they were prepared freshly in dichlorvos-treated plasma. Quality control samples containing both Ro 64-0796 and Ro 64-0802 were prepared and stored at −20°C except for rodent plasma, when the quality control samples were made up separately, Ro 64-0802 being prepared in advance and Ro 64-0796 freshly.

For analysis in urine, the stock solutions were diluted with blank urine to give samples for the calibration curves with ranges for Ro 64-0796 and Ro 64-0802 of 5.00–1000 and 30.0–30 000 ng/ml, respectively; again nine duplicate standards were used in each batch. Quality control samples were generally made up at 25, 400 and 800 ng/ml for Ro 64-0796 and 150, 12 000 and 24 000 ng/ml for Ro 64-0802.

The trideuterated internal standards (Fig. 2) were also prepared at 1.00 mg/ml and were then diluted with distilled water to give a combined working solution containing 50 ng/ml of Ro 64-0796/902 and 2000 ng/ml of Ro 64-0802/001.



Ro 64-0796/902 (GS5209)
(hydrochloride salt)
Ro 64-0796/003
(phosphate salt)

Ro 64-0802/001 (GS4723)
(Hydrated free base)
Ro 64-0802/004
(hemi-tartrate salt)

Fig. 2. The structures of the deuterated internal standards of Ro 64-0796 and Ro 64-0802 used in the HPLC–MS–MS assay.

2.1.4. Extraction

The methods used for the extraction of plasma and urinary samples were identical. Sample aliquots (100 μl) were centrifuged if necessary to eliminate clots or suspended matter. The combined internal standard working solution (50 μl) was added, followed by 5 m*M* ammonium acetate, pH 3.5 buffer (1.0 ml). Solid phase extraction (SPE) disc cartridges (Empore Mixed Phase Cation-MPC, 7 mm/3 ml) were conditioned by rinsing with methanol (1 ml), followed by 9:1 methanol–50 m*M* aqueous ammonium acetate buffer (3×3 ml) and then 5 m*M* ammonium acetate, pH 3.5 buffer (1 ml). The diluted plasma or urine was drawn through the cartridges and the sample tube rinsed with water (1 ml). The SPE cartridges were washed with the rinse, followed by methanol (1 ml), and then 90% methanol–10% water (1 ml). They were then dried under vacuum and the analytes eluted into glass tubes with 9:1 methanol–50 m*M* aqueous ammonium acetate buffer (1 ml) and evaporated to dryness with nitrogen at ~50°C.

The dried extracts were reconstituted in 150 μl of deionised water and centrifuged briefly at >500 rpm. The reconstituted samples were transferred to the appropriate glass autosampler vial insert in a randomised order and 100 μl injected onto the LC–MS–MS apparatus.

2.1.5. Separation and detection

The drug and pro-drug were separated by reversed-phase HPLC using a Nova-Pak CN HP cartridge (5×100 mm, 4 μm, Waters) housed in a RCM 8×10 radial compression module (Waters). The mobile phase was 50% MeOH–50% 80 m*M* aqueous formic acid, pH 3. The flow-rate was 0.5 ml/min and the retention times for the two compounds were approximately 3.5 (Ro 64-0802) and 5 min (Ro 64-0796).

The analytes were introduced into the mass spectrometer via the appropriate interface and the protonated molecular ions fragmented by collision with argon (nitrogen for PE-Sciex API 365). The resultant daughter ions (see below; M^+-88 amu, i.e., without the pentyloxy side-chain, Fig. 3) were then detected.

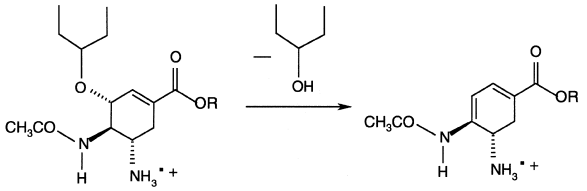


Fig. 3. Mass spectral fragmentation of Ro 64-0796 and Ro 64-0802 used in the HPLC–MS–MS assay.

Compound	Protonated molecular ion	Daughter ion
Ro 64-0796/000	$m/z=313$	$m/z=224$
Ro 64-0796/902 (GS 5209)	$m/z=316$	$m/z=227$
Ro 64-0802/000	$m/z=285$	$m/z=198$
Ro 64-0802/001 (GS 4723)	$m/z=288$	$m/z=201$

A weighted quadratic or linear regression line was used for comparing the peak area ratios of 'unknowns', quality control samples (at least three concentrations in duplicate per batch) and standards.

2.2. Validation

The method was validated in both human plasma and urine and then cross-validated in animal plasma and urine.

For human plasma, duplicate standards of Ro 64-0796 (nominal range 1–250 ng/ml) and Ro 64-0802 (nominal range 10–10 000 ng/ml) were used. Three or five concentrations of each compound were used as quality control samples, three within the calibration range (Ro 64-0796: 5, 100 and 200 ng/ml, $n=26$; Ro 64-0802: 50, 4000 and 8000 ng/ml, $n=22$), and two which had been diluted 4- or 10-fold ($n=4$). For cross-validation studies in plasma, the concentrations of Ro 64-0796 and Ro 64-0802 in quality control samples made up in animal and human plasma were compared, using calibration standards prepared in human plasma. Quality control samples were made up at three concentrations of both Ro 64-0796 and Ro 64-0802.

For human urine, duplicate standards of Ro 64-0796 (nominal range, 5–1000 ng/ml) and Ro 64-0802 (nominal range, 30–30 000 ng/ml) were used. Three concentrations of each compound were again used as quality control samples ($n=6$, Ro 64-0796: 25, 400 and 800 ng/ml; Ro 64-0802: 150, 12 000 and 24 000 ng/ml). In addition, two samples which had been diluted 10-fold ($n=4$, 800 ng/ml for Ro 64-0796 and 24000 ng/ml for Ro 64-0802) or 100-fold ($n=4$, 50 000 ng/ml for Ro 64-0796 and 500 000 ng/ml for Ro 64-0802) were assayed. For cross-validation studies in urine, the concentrations of Ro 64-0796 and Ro 64-0802 in quality control samples made up in animal urine were assayed with calibration standards prepared in human urine. Quality control samples were made up at three concentrations of both Ro 64-0796 and Ro 64-0802.

Cross-validation was also undertaken between the automated extraction equipment (Tecan Genesis System) and the manual technique, and between the Finnigan MAT TSQ 700 and the Sciex API 365 mass spectrometers.

Accuracy was calculated as the percentage difference between the nominal (n) and actual (a) concentrations. Precision (coefficient of variation, CV) was calculated as the ratio of the standard deviation of the actual concentrations from the mean (SD) divided by that mean (m) and expressed as a percentage: i.e.

$$\text{Accuracy} = \frac{n-a}{n}\% \quad \text{Precision} = \frac{\text{SD}}{m}\%$$

'Recoveries' were determined in two ways. Firstly, the measured peak areas of quality control samples (six samples each at 5, 100 and 200 ng/ml for Ro 64-0796; 50, 4000 and 8000 ng/ml for Ro 64-0802) worked up in the usual manner were compared with those of unextracted aqueous solutions of similar final extract concentrations (six samples at each concentration). Secondly, the response from a range of standards (1–5000 ng/ml for Ro 64-0796 and 10–10 000 for Ro 64-0802) prepared in plasma and extracted as usual was compared with that from identical samples made up in previously extracted blank plasma.

The specificity of the method was tested by examining six samples each of blank EDTA human and animal plasma and blank human and animal urine. Sample carryover was assessed by interspers-

ing increasing concentrations of calibration samples with blank human plasma.

2.3. Stability

A large programme of stability testing was carried out with Ro 64-0796 and Ro 64-0802 in plasma from man, rat, mouse, marmoset, rabbit and ferret (22, 4, −20°C) and in human blood (22, 4°C) and urine (22, 4, −20°C). This was particularly necessary because Ro 64-0796 can theoretically decompose to form Ro 64-0802 and so distort the results for both compounds.

Duplicate human plasma samples (5.00 and 200 ng/ml for Ro 64-0796 and 50.0 and 8000 ng/ml for Ro 64-0802) were incubated at room temperature for up to an hour. Quintuplicate or quadruplicate samples at the same concentrations were stored at 4°C for up to a week and at −20°C for up to 6 months. Human urinary samples were treated in the same way as those in plasma, but nominal concentrations of 25.0 and 800 ng/ml for Ro 64-0796 and 150 and 24 000 ng/ml for Ro 64-0802 were employed. Quality control samples in both human and animal plasma were subjected to three freeze–thaw cycles.

Quintuplicate samples in human blood of the same concentrations as in plasma, except that at 4°C the 200 ng/ml sample was replaced by one at 2000 ng/ml, were stored for up to 4 h both at room temperature and at 4°C. Low concentration samples (5 ng/ml for Ro 64-0796 and 50 ng/ml for Ro 64-0802) were also prepared in blood which was transferred into Vacutainer tubes containing EDTA and gently mixed for 5 min at room temperature. The samples were then transferred to either polypropylene or glass tubes, the blood separated by centrifugation (15 min) and the plasma stored in glass tubes prior to analysis. The duration of this experiment was approximately 20 min.

Stability studies were also carried out with Ro 64-0796 in dichlorvos-treated plasma. The blood or plasma was treated with a solution of dichlorvos in acetonitrile at 100 and 200 μg/ml of biological fluid and incubated with Ro 64-0796 (5 μg/ml) at 37°C for 10 (plasma) or 60 (blood) min. A further study was carried out over 2 months to determine the extent of hydrolysis of Ro 64-0796 to Ro 64-0802 in dichlorvos-treated rat plasma at −20°C. A stable solution was defined as one showing less than a 15% decrease from nominal concentrations of Ro 64-0796 and Ro 64-0802, or less than 15% formation of Ro 64-0802 from Ro 64-0796.

3. Results

The mass spectra of Ro 64-0796 and Ro 64-0802 (Fig. 4) are characterised by a strong M-88 peak which corresponds to loss of the isopentyl side-chain (Fig. 3), forming the daughter ions used in the assay. Representative chromatograms of Ro 64-0796 and Ro 64-0802 are shown in Fig. 5. The retention times of the two analytes were dependent on the individual column and were also affected by the ionic strength of the mobile phase. Thus doubling the molarity of the buffer from 20 to 40 m*M* reduced the retention times of Ro 64-0796 and Ro 64-0802 by 30 and 37%, respectively.

3.1. Measurement of recoveries

The recoveries for Ro 64-0796 and Ro 64-0802 from plasma and urine are shown in Table 1. The recoveries were determined in two ways: relative to stock solutions of the two compounds in water and relative to solutions in extracted blank plasma. Those calculated by the former method include apparent losses caused by suppression of the mass spectral signal by endogenous plasma components and were 40–50%; however, comparable responses of the internal standards compensated for this. Comparison with solutions in extracted plasma showed that the actual recoveries were high (85–90%) for both compounds (Table 1).

3.2. Validation

The lower limits of quantification were 1 ng/ml for Ro 64-0796 and 10 ng/ml for Ro 64-0802 in plasma and 5 ng/ml for Ro 64-0796 and 30 ng/ml for Ro 64-0802 in urine.

For validation in human plasma, both the precision and the accuracy of the results were excellent (Table 2) with typical values for both calibration standards and quality control samples of approximately ±5%. Samples of both drugs were diluted by up to 10

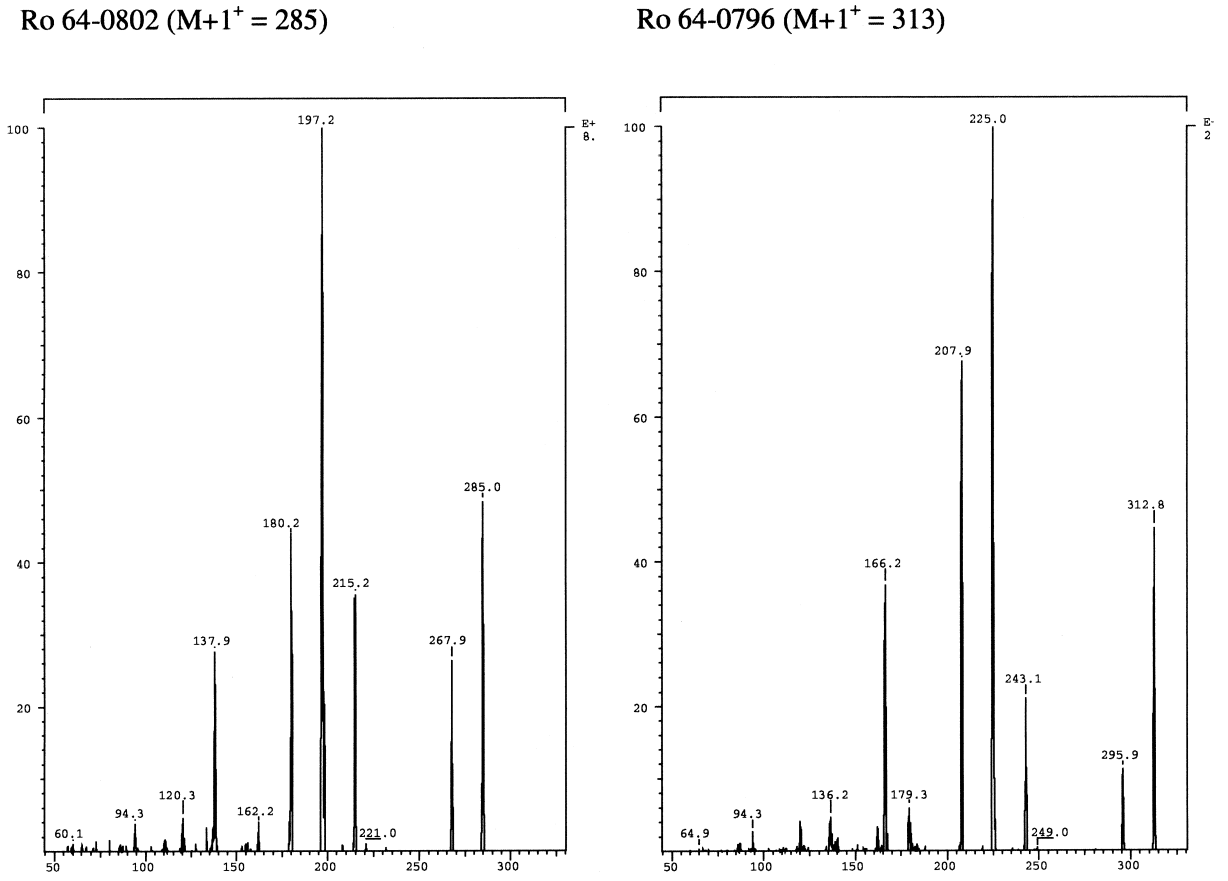


Fig. 4. Product ion mass spectra of Ro 64-0796 and Ro 64-0802.

times with blank plasma with no loss of performance (accuracy and precision of the diluted samples were for Ro 64-0796: −3.6 and ±5.1% and for Ro 64-0802: +3.2 and ±0.5%).

Duplicate standards of Ro 64-0796 (nominal range 1–250 ng/ml) and Ro 64-0802 (nominal range 10–10 000 ng/ml) in human plasma were used in the cross-validation exercises in animal plasma. Cross-validation data are included in Table 3. Accuracy and precision were generally excellent with most mean values being <±5%.

For validation in human urine, both the precision and the accuracy of the results were again very good (Table 4) with typical figures for both standards and quality control samples of approximately ±5%. Samples of the two compounds were diluted by up to 100-fold with 5 m*M* aqueous ammonium acetate with no diminution of performance (accuracy and precision were −3.2 and ±1.1% for Ro 64-0796 and −3.2 and ±1.2% for Ro 64-0802).

Duplicate standards of Ro 64-0796 (nominal range, 5–1000 ng/ml) and Ro 64-0802 (nominal range, 30–30 000 ng/ml) in human urine were used in the cross-validation exercises in marmosets and rats. The precision of the quality control samples was <±10% in both animals for the two analytes (data not shown). The measured concentrations of Ro 64-0796 in the rat and of both drugs in the marmoset were found to be significantly higher than the nominal values in the cross-validation exercises. It is

(A) blank human plasma sample

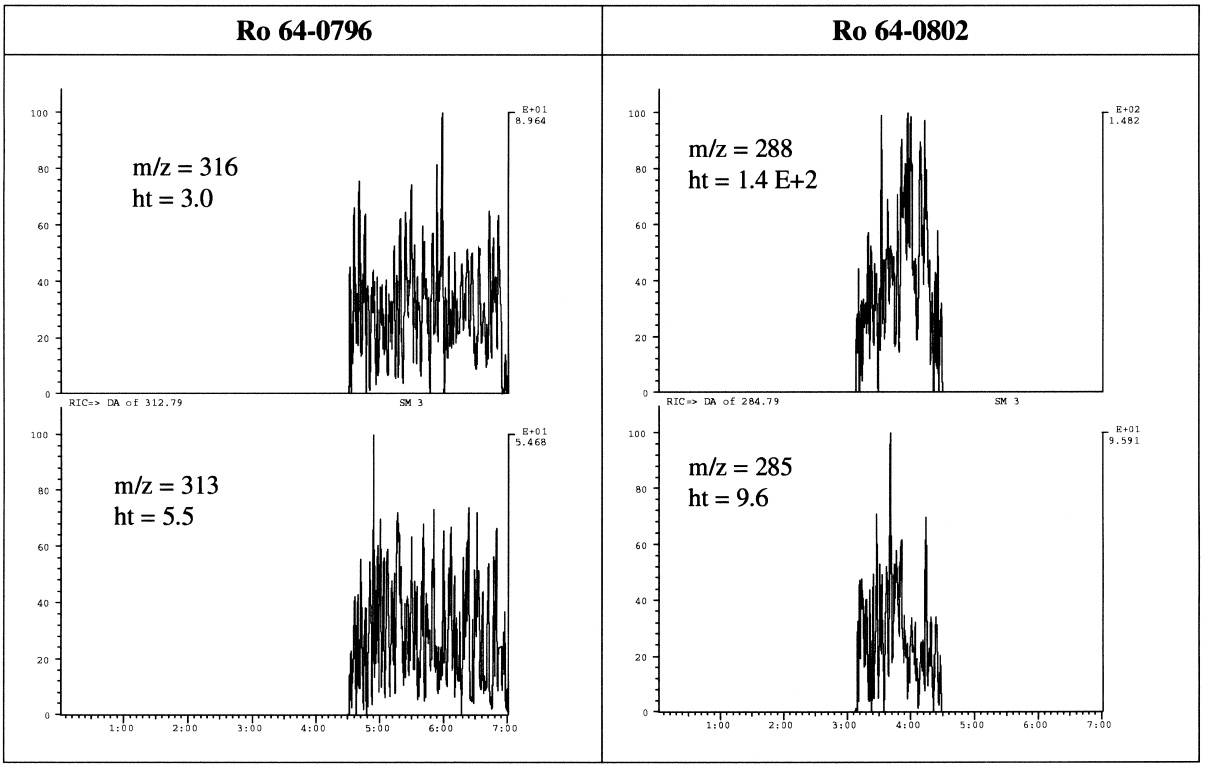


Fig. 5. Representative ion chromatograms of: (A) blank human plasma sample; (B) lowest calibration standards in human plasma; (C) typical human plasma samples; (D) typical rat plasma sample; (E) lowest calibration standards in human urine; (F) typical human urine sample; (G) typical rat urine sample blank human plasma sample.

probable that this was caused by an analytical error as further quality control samples assayed during experimental work gave accuracies within ±10%.

3.2.1. Specificity of the method

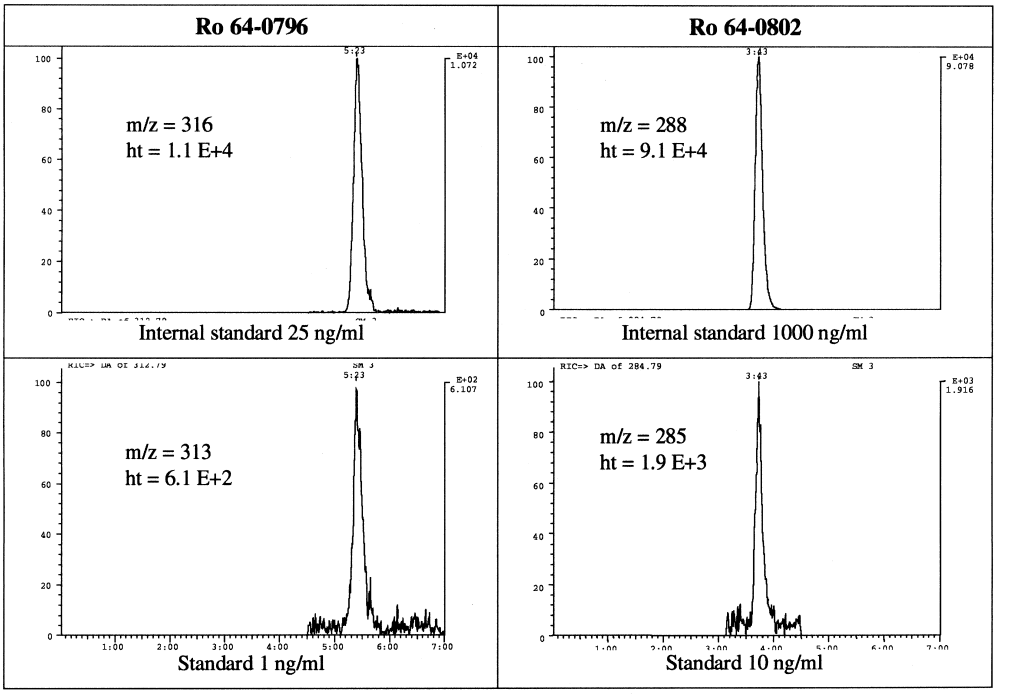
There were no interfering peaks at the retention time of Ro 64-0796 in human plasma. Although one sample showed a small peak at the retention time of Ro 64-0802 and its internal standard, it was not large enough to interfere with the assay.

Similar specificity checks were carried out with plasma from other species. In the chicken occasional interfering peaks of about half the size of the lowest standard were observed for both compounds, whereas those in the ferret (Ro 64-0796) and mouse (Ro 64-0802) were only 10–20% of the limit of quantification. Possible interference of Ro 64-0796 in marmosets and Ro 64-0802 in rats up to the level of the lowest standard (1 ng/ml Ro 64-0796 in the marmoset and 8 ng/ml Ro 64-0802 in the rat) could also have occurred.

Four samples of human urine showed small interfering peaks corresponding to Ro 64-0796 and two to Ro 64-0802. In no cases were the peaks large enough to interfere with the assay. No peaks were observed in rat or marmoset urine and the detection limit was considerably less than the limit of quantification (5 ng/ml).

During the development of the method, compounds with peaks corresponding to the ions of Ro

(B) Low calibration standards in human plasma



(C) Typical human plasma sample

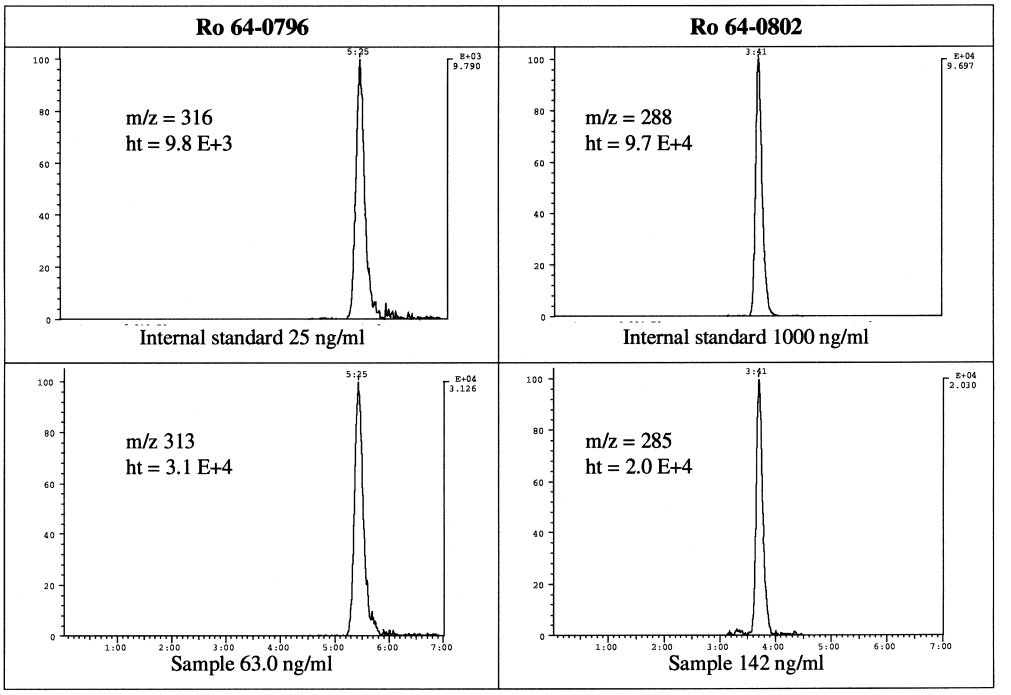
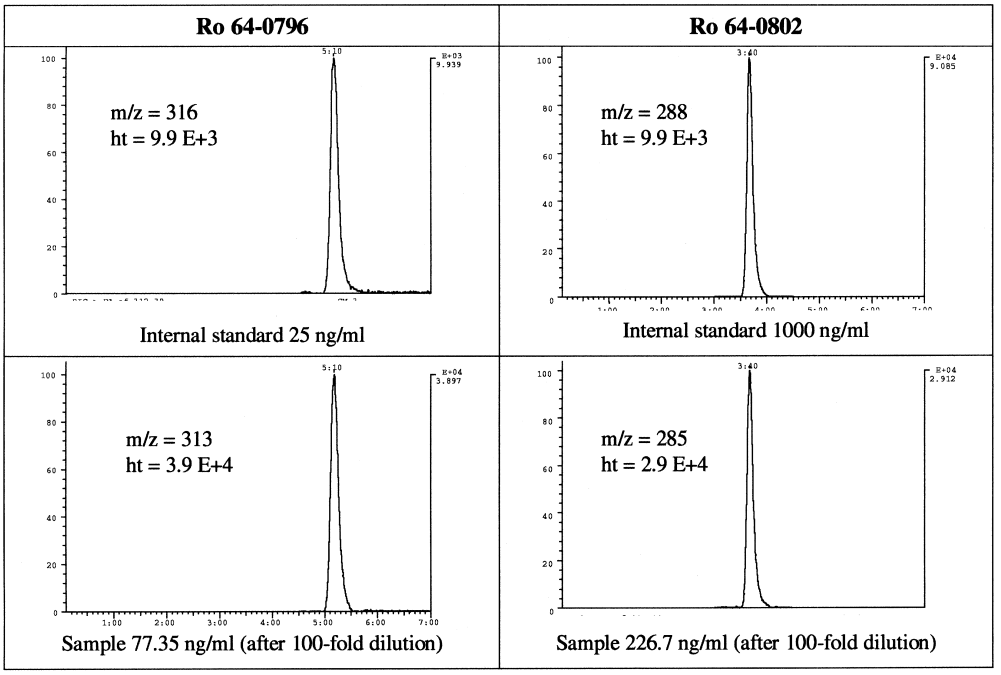


Fig. 5. (*continued*)

(D) Typical rat plasma sample (with dichlorvos)



(E) Lowest calibration standards in human urine

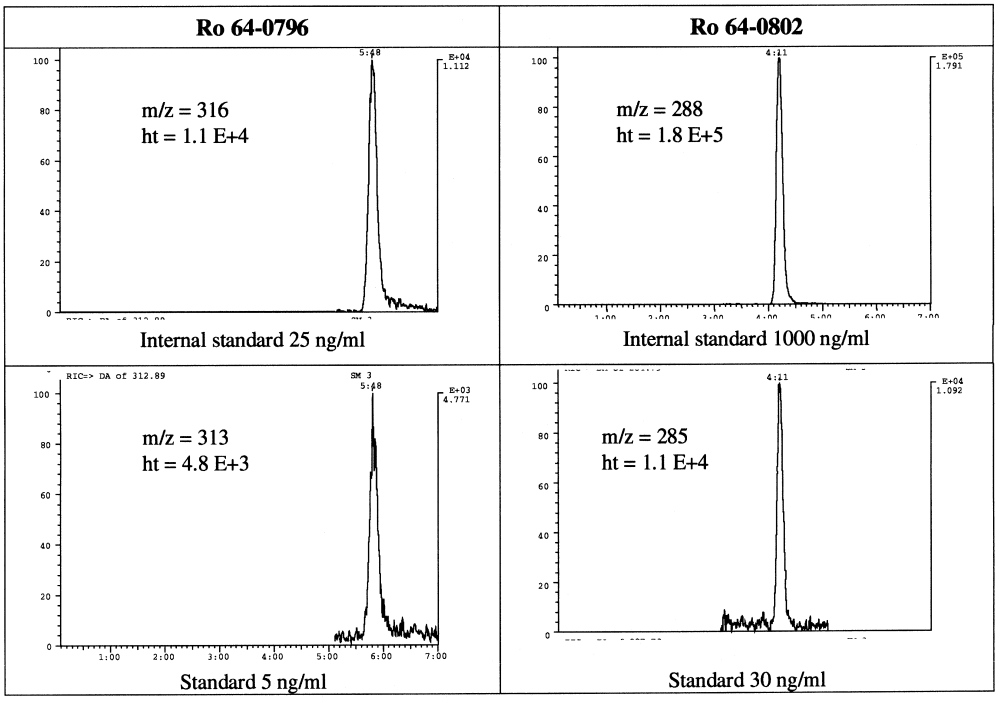
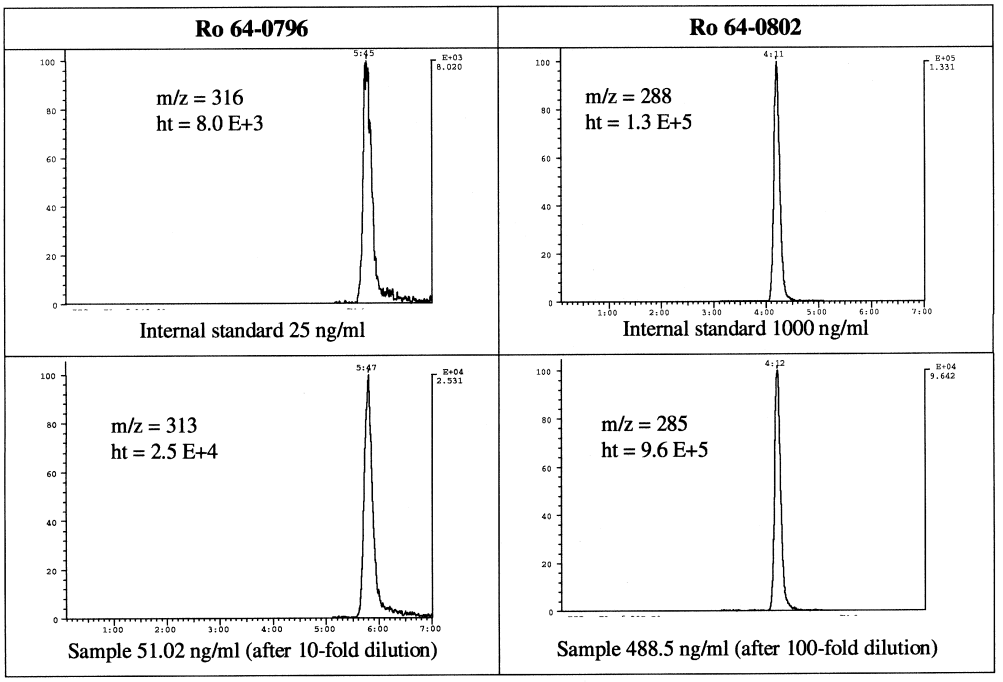


Fig. 5. (*continued*)

(F) Typical human urine sample



(G) Typical rat urine sample

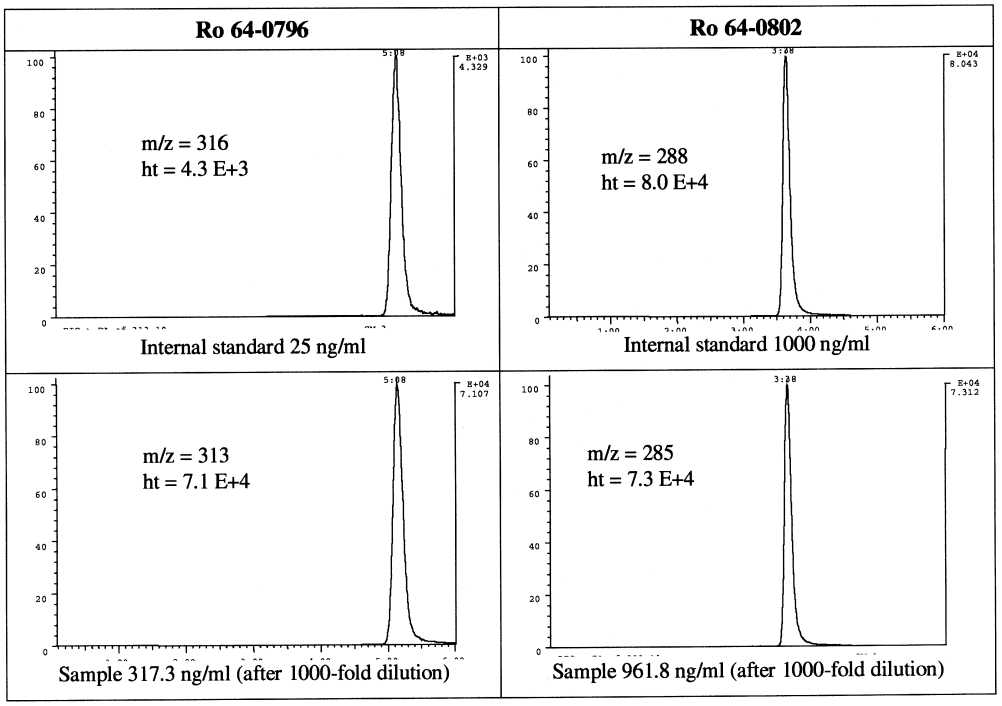


Fig. 5. (*continued*)

Table 1
Recoveries of Ro 64-0796 and Ro 64-0802 from plasma after solid-phase extraction

	Plasma		Urine	
	Compound	I.S.	Compound	I.S
Ro 64-0796 (%)	84.8±19.3[a]			
Ro 64-0802 (%)	95.4±5.5[a]			
Ro 64-0796 (%)	65.6[b]	64.4[b]	50.7[b]	60.0[b]
Ro 64-0802 (%)	51.1[b]	52.6[b]	38.6[b]	39.8[b]

[a] Calculated relative to solutions in extracted plasma.
[b] Calculated relative to solutions in water.

64-0796 and Ro 64-0802 were sometimes eluted from the solid-phase extraction cartridges. These peaks were not observed to change the values of low quality control samples with either compound and were presumably suppressed by the drugs. Nevertheless, as a result of this observation, the cartridges are now washed with 3×3 ml of elution solvent rather than 1 ml. Typically, with the more thorough washing system, any extraneous peaks were well below the limit of quantification.

Table 2
Validation of the HPLC–MS–MS assay in human plasma as shown by accuracy and precision of the calibration standards and quality control samples

	Analytical standards		Quality control samples	
	Ro 64-0796	Ro 64-0802	Ro 64-0796	Ro 64-0802
No. of batches	5	4	5	4
No. of samples	89	70	86	74
CV (%)	±6.5	±3.5	±6.0	±4.0
CV (lowest standard) (%)	±11.7	±4.8		
Accuracy (lowest standard) (%)	−2.7	−3.2		
Mean accuracy (%)			−1.83	+1.12

Table 3
Cross-validation of the HPLC–MS–MS assay in animal plasma as shown by accuracy and precision of quality control samples

	Rabbit	Chicken	Ferret	Marmoset	Mouse[a]	Rat[a]
Ro 64-0696						
Precision (%)	±3.7	±4.0	±1.5	±1.1	±4.7	±4.6
Accuracy (%)	+4.1	+6.6	+7.8	+12.2	−1.2	+6.2
No./conc.	6	6	6	6	20	4
Ro 64-0802						
Precision (%)	±3.5	±3.0	±1.1	±1.7	±3.0	±4.8
Accuracy (%)	−1.9	+4.1	+1.3	+1.5	+2.7	+7.4
No./conc.	6	6	6	6	18	4

[a] Dichlorvos-treated plasma.

Table 4
Validation of the HPLC–MS–MS assay in human urine as shown by accuracy and precision of the calibration standards and quality control samples

	Analytical standards		Quality control samples	
	Ro 64-0796	Ro 64-0802	Ro 64-0796	Ro 64-0802
No. of batches	4	4	4	4
No. of samples	72	72	26	26
CV (%)	±2.9	±2.1	±2.4	±3.5
CV (lowest standard) (%)	±5.4	±3.7		
Accuracy (lowest standard) (%)	+0.5	−0.2		
Mean accuracy (%)			−4.5	−3.0

Table 5
Performance data for the measurement of Ro 64-0796 and Ro 64-0802 in plasma in pharmacokinetic studies

	Limit of quantification				All standards		Quality control samples		
	Conc. (ng/ml)	*n*	CV%	Error %	*n*	CV%	*n*	CV%	Error %
Ro 64-0796 (preclinical)	1	218	±7.33	+0.12	2204	±4.05	832	±8.20	+3.26
Ro 64-0802 (preclinical)	10	307	±6.90	+0.50	2884	±4.68	1043	±5.14	+0.68
Ro 64-0796 (clinical)	1	451	±6.72	+0.13	4228	±4.21	1450	±5.92	−2.40
Ro 64-0802 (clinical)	10	455	±5.66	−0.17	4199	±3.54	1432	±5.83	+2.41

3.2.2. Validation of different instruments

The performance of the duplicate set of standards used to validate the PE Sciex API 365 Mass Spectrometer was excellent with figures for average precision and accuracy of $<\pm 5\%$. The performance of the quality control samples was also generally good except for the lowest (5 ng/ml) Ro 64-0796 samples (accuracy −19%). Automated extraction with a Tecan Genesis instrument was also satisfactory although the precision of the lowest standard was found to be ±24%.

3.3. Use of the assay for the measurement of Ro 64-0796 and Ro 64-0802 in clinical and pre-clinical pharmacokinetic studies

The performance of the methods used for the measurement of plasma and urinary levels in actual pre-clinical and clinical pharmacokinetic studies is tabulated in Tables 5 and 6. Typical figures for precision and accuracy were $<\pm 7$ and $<\pm 5\%$, respectively, for around 7000 standards and 2500 quality control samples in plasma and 1700 standards and 600 quality control samples in urine.

3.4. Stability in biological fluids

3.4.1. Stability of Ro 64-0802 in plasma, urine and blood

Ro 64-0802 was stable in plasma and processed extracts for up to 24 h both at 4°C and at room temperature, as well as through three freeze–thaw cycles. The average coefficient of variation of the different quadruplicate and quintuplicate aliquots stored at 4 and −20°C was ±2.8% and in no cases were any of the mean values outside ±15% of the nominal figures, demonstrating the stability of the compound. There was no indication of any decomposition of the drug in any of the matrices (Table 7).

Measured concentrations of Ro 64-0802 in blood

Table 6
Performance data for the measurement of Ro 64-0796 and Ro 64-0802 in urine in pharmacokinetic studies

	Limit of quantification				All standards		Quality control samples		
	Conc. (ng/ml)	*n*	CV%	Error %	*n*	CV%	*n*	CV%	Error %
Ro 64-0796 (preclinical)	5	30	±6.6	−0.3	275	±4.9	108	±6.5	−2.7
Ro 64-0802 (preclinical)	30	26	±3.8	−0.4	245	±3.1	96	±4.1	+0.8
Ro 64-0796 (clinical)	5	190	±6.0	−0.3	1733	±4.5	600	±5.6	+0.9
Ro 64-0802 (clinical)	20/30	157	±7.7	−0.9	1548	±4.3	548	±4.2	+3.4

Table 7
Percentage of Ro 64-0796 or Ro 64-0802 (relative to nominal) remaining after storage of analytical samples at different temperatures

	Ro 64-0802				Ro 64-0796			
	Conc. (ng/ml)	+22°C 60 min	+4°C 168 h	−20°C 26 weeks	Conc. (ng/ml)	+22°C 60 min	+4°C 168 h	−20°C 26 weeks
Human plasma	8000	101.3	100	101.3	200	91.1	56.9	98.3
	50	92.6	99.2	91.6	5	107.9	54.3	92.7
Human urine	24 000	100.3	101.7	112.9	800	114.4	99.5	106.8
	150	102.5	101	108.6	25	98.5	93.1	112.5
Human blood	8000	123.2	128.6		200	78.8	77.8	
	50	109.5	115.2		5	78.5	99.9	
Ferret plasma	8000	94	101.1	101.2	200	104.1	105.7	104.5
	50	84	99.6	81.7	5	76.5	97.6	80.2
Rat plasma[a]	8000	95.6	98.3	102.7	200	11.8[c]		
	50	137.9	89.8	81.2	5	20.3[b]		
Mouse plasma[a]	8000	100.6	97.8	99.7	200	53.3[c]		
	50	91.8	92.1	88.8	5	56.1		
Rabbit plasma	8000	99.4	102.8	110.7	200	97.4	84.1	115.9
	50	94.7	106.3	110.6	5	72.6	87.9	107.8
Marmoset plasma	8000	95.2	102.2	103.8	200	106.5	106.7	112.3
	50	102.2	97.9	93.5	5	100.7	119.2	121.5

[a] No dichlorvos added (the percentages of Ro 64-0796 remaining in dichlorvos-treated rat plasma stored at −20°C for 2 months were 80% (5 ng/ml), 94% (100 ng/ml) and 92% (200 ng/ml)).
[b] Forty minutes.
[c] See also Fig. 6.

were consistently higher than the theoretical values, with average deviations of about +23%, because the compound does not enter erythrocytes to a significant extent. Ro 64-0802 was quite stable in human blood at 4°C but there appeared to be some decomposition after storage for 4 h at room temperature. Thus the '50 ng/ml' samples dropped by an average of 38%, but those at '8000 ng/ml' were unchanged. There were no effects of the collection devices on the plasma levels of the drug.

3.4.2. Stability of Ro 64-0796 in plasma and urine

Ro 64-0796 was stable in human plasma at 4°C for up to 24 h but at room temperature the lower concentrations were decreasing after 16 h and had dropped by 15–20% after 24 h. Slow hydrolysis of Ro 64-0796 to Ro 64-0802 was observed in human plasma stored at 4°C for long periods (Table 7). Thus, both high and low concentrations showed a reduction of between 40 and 50% after a 7-days storage, and Ro 64-0802 was also detected at both the 24-h (8%) and 7-day (40%) time-points. A first-order equation fitted to the data gave rate constants of −0.0039 and −0.0033 h^{-1}, equivalent to half-lives of 7.4 and 8.8 days for the 5 and 200 ng/ml samples, respectively. Ro 64-0796 was stable in human plasma stored at −20°C for up to 6 months and at room temperature for 60 min.

After three freeze–thaw cycles, concentrations of Ro 64-0796 were within the ±15% acceptance limits although there was a suggestion of a small decrease in concentration. The processed extracts were stable at room temperature for 24 h. The average coefficient of deviation of the quadruplicate and quintuplicate aliquots stored at 4 and −20°C was ±2.9% and in no cases were any of the mean values outside ±15% of the nominal figures. No instability was observed in human urine, or ferret, rabbit or marmoset plasma (Table 7). Ro 64-0802 was not detected in any of these high concentration (800 ng/ml for urine and 200 ng/ml for plasma) samples of Ro 64-0796, showing that degradation was $<5\%$, as the lower limit of quantification of Ro 64-0802 was 30 ng/ml for urine and 10 ng/ml for plasma.

Ro 64-0796 was rapidly hydrolysed in rat and mouse plasma at room temperature (Fig. 6). The rate constants calculated from the decay of the pro-drug at 5 and 200 ng/ml and the formation of the active

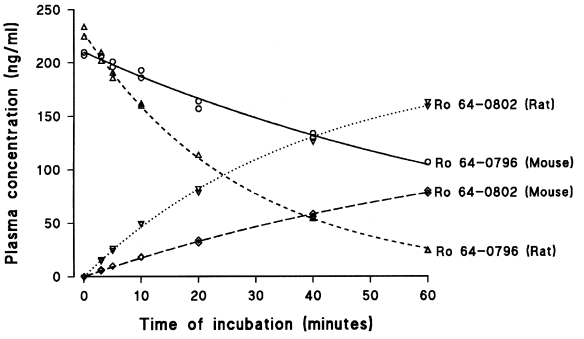


Fig. 6. Decay of Ro 64-0796 and appearance of Ro 64-0802 in rodent plasma incubated at 22°C.

compound from the latter concentration gave half-lives of about 20 and 60 min for rats and mice, respectively. However, treatment with dichlorvos stabilised the compound (Section 3.4.4).

3.4.3. Stability of Ro 64-0796 in human blood

Concentrations of Ro 64-0796 at zero time were consistently lower than the theoretical figures, with a typical deviation of −20%, because the affinity of the compound is rather greater for erythrocytes than for plasma.

Slow but significant decomposition of Ro 64-0796 occurred in human blood at both room temperature and at 4°C, as shown by the formation of Ro 64-0802, with its concentration at 4 h being about 25% (room temperature) and 13% (4°C) of the nominal values of Ro 64-0796. There was no suggestion of any instability in the various collection devices examined.

3.4.4. Stability of Ro 64-0796 and Ro 64-0802 under other conditions

Blood from rodents is routinely treated with dichlorvos to inhibit esterase-catalysed ex vivo hydrolysis of Ro 64-0796. The efficacy of this inhibitor was demonstrated in pooled samples of rat blood and plasma incubated with Ro 64-0796. The Ro 64-0802:Ro 64-0796 peak area ratios from blood and plasma were 12.6 and >70, respectively, in the absence of dichlorvos and this was reduced to <0.0004 in its presence. There was no effect on potassium ion or lactate dehydrogenase concentrations showing that haemolysis was not increased by treatment of whole blood with dichlorvos. Similarly, no changes in plasma protein or albumin concentrations arose from the experiments with dichlorvos in rat plasma.

The stability of Ro 64-0796 (5, 100, 200 ng/ml) in dichlorvos-treated rat plasma was also tested at −20°C. No decomposition was found after storage for up to 1 month, but at 2 months 15–20% of the pro-drug had been converted to Ro 64-0802.

4. Discussion

The analytical method involves the initial extraction of Ro 64-0796 and Ro 64-0802, together with their respective tri-deuterated internal standards (Fig. 2), from plasma or urine using mixed phase cationic extraction discs or cartridges. The analytes are eluted from the cartridges, separated by reversed-phase HPLC and detected mass spectrometrically after atmospheric pressure ionisation. The protonated molecular ions of the two compounds are selected by the first mass analyser to undergo collisionally activated dissociation in a collision cell pressurised with argon or nitrogen. Specific product ions which result are then selected by a second mass analyser for detection.

4.1. Extraction

Ro 64-0796 and Ro 64-0802 both contain a weakly basic amino group but Ro 64-0802 also has a carboxylic acid and is, therefore, a zwitterion. Ro 64-0796 is fairly polar (Log $P = 0.36$ at pH 7.4) and Ro 64-0802 always ionised and highly polar (Log $P = -2.1$) [8]. Extraction of these basic, polar compounds was best achieved by adsorption onto a cationic ion-exchange medium. Use of an acidic buffer, which protonates both compounds, and a non-polar mixed phase cartridge, improved extraction. The actual recoveries of Ro 64-0796 and Ro 64-0802 from plasma were roughly 90%, but the signals from both analytes appeared to be partially suppressed by endogenous components of plasma (Table 1). The use of deuterated internal standards to compensate for this effect was very important.

Extraction of the analytes was satisfactorily accomplished using manual and automated techniques.

4.2. Validation

The assay used for the measurement of Ro 64-0796 and Ro 64-0802 in all clinical and the majority of non-clinical pharmacokinetic studies is robust, specific and sensitive. Toxicokinetic studies gave values of maximum concentrations up to 39400 ng/ml for Ro 64-0796 and 93200 ng/ml for Ro 64-0802. The K_i for Ro 64-0802 against viral neuraminidases is typically 1 ng/ml, and hence concentrations of 10 ng/ml are approximately equal to the IC 90. This figure defines the lower limit of quantification of Ro 64-0802 and minimum plasma levels in patients receiving the recommended 75 mg b.i.d regimen are about 150–200 ng/ml. The assay of high concentrations was successfully achieved by dilution of samples into the calibration range. The limit of quantification of 1 ng/ml for Ro 64-0796 is ample, and that for Ro 64-0802 (10 ng/ml) has only occasionally been insufficient for the analysis of all samples.

The method has been validated for use in plasma from eight species. The relative levels of the two compounds varies considerably between species, with the amount of Ro 64-0796 being between 5 and 10% of that of Ro 64-0802 in man and marmoset, through ~30% in rats, mice and rabbits up to 100% in ferrets and chickens. This meant that any conversion of Ro 64-0796 to Ro 64-0802 ex vivo would, in general, be a greater problem in determining accurate levels of Ro 64-0796 than Ro 64-0802.

This assay has been set up successfully in three laboratories for the simultaneous assay of Ro 64-0796 and Ro 64-0802 in plasma and it was possible to assay up to 800 samples a week. The assay has also proved itself to be reliable for the measurement of urinary concentrations of Ro 64-0796 and Ro 64-0802 in human, rat and marmoset urine. The lower limits of quantification for Ro 64-0796 and Ro 64-0802 of 5 and 30 ng/ml, respectively, have provided sufficient sensitivity for all studies. The performance of the analytical method has been generally good with typical figures for precision and inaccuracy of $<\pm 7\%$ in all matrices.

4.3. Stability

Ro 64-0802 was quite stable in human blood for 2 h and no decomposition is to be anticipated when using standard collection devices. Although some hydrolysis of Ro 64-0796 to Ro 64-0802 does take place, negligible losses should occur if the blood is cooled and the plasma harvested within 2 h of collection. Even if the blood were not cooled before centrifugation, negligible conversion of Ro 64-0796 to Ro 64-0802, or degradation of Ro 64-0802, is likely within an hour of collection.

Both Ro 64-0796 and Ro 64-0802 were stable when stored at -20°C for up to 6 months in plasma from man, rabbits, marmosets and ferrets as well as in human urine. In addition, Ro 64-0802 was stable in rat and mouse plasma under the same conditions. Freeze–thawing did not appear to cause decomposition of either compound.

High concentrations of esterases circulate in rodents and the ready hydrolysis of Ro 64-0796 in rat plasma has been well established [4]. However, treatment of fresh blood with dichlorvos is effective in preventing the ex vivo decomposition of the pro-drug. Dichlorvos-treated rodent plasma should be stored at -80°C as significant decomposition can occur within 2 months at -20°C. The use of dichlorvos to inhibit the plasma esterases of rodents does not pose a problem to the assay and has no effect on red blood cells or plasma proteins.

5. Conclusions

A robust, sensitive, accurate and precise HPLC–MS–MS analytical method for the quantitative measurement of concentrations of the pro-drug, Ro 64-0796, and the active influenza neuraminidase inhibitor, Ro 64-0802, has been developed. The assay can be used for both plasma and urine and has been validated in human samples and in those of several animal species (six in plasma and two in urine). The limits of quantification have been satisfactory for the measurement of both compounds in pharmacokinetic and toxicokinetic studies and valid data have been generated to support the use of Tamiflu™ in the treatment of influenza.

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