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Development and validation of a solid-phase extraction and high-performance liquid chromatographic assay for a novel fluorinated 2-nitroimidazole hypoxia probe (SR-4554) in Balb/c mouse plasma

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Abstract

N-(2-Hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide, a novel 2-nitroimidazole, is currently being developed as a non-invasive probe for tumour hypoxia. A sensitive (minimum quantifiable level = 25 ng/ml; C.V. = 6.01%) and selective assay has, therefore, been developed for the analysis of this compound in mouse plasma. The assay employed a solid-phase extraction followed by a rapid (10 min) HPLC analysis with UV-photodiode-array detection. No drug-related metabolites were observed in plasma when mice were treated with 180 mg/kg of the drug. The assay has proved to be suitable for studying the plasma pharmacokinetics of this fluorinated 2-nitroimidazole in mice.

1. Introduction

Tumour hypoxia is a well known phenomenon which is primarily created by an inadequate and non-uniform vascular network within the tumour mass [1–3]. This property has been found to be responsible, at least in part, for the resistance of many tumours to conventional radiotherapy and chemotherapy [2,4,5]. The existence of such resistance mechanisms has led to intensive research to identify and eradicate this sub-population of resistant cells in tumour tissues. In particular, this would allow the selection of most patients likely to benefit from hypoxia-targeted therapies.

Existing technology for the detection of hypoxic tumour cells has often yielded an average value for large numbers of neighbouring cells and/or are surgically invasive. The exception to this, however, are techniques that employ 2-ni-

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troimidazoles containing appropriate labels [6–10]. The nitroimidazoles undergo nitroreduction to form stable covalent adducts within hypoxic tissues [11]. The introduction of a ^{19}F label will then permit its localization by non-invasive techniques such as magnetic resonance spectroscopy (MRS) and positron emission tomography (PET) [7,8]. The successful design of nitroimidazoles as hypoxic probes will enable the selection of cancer patients who may benefit from treatments aimed at eradicating hypoxic cells. Factors which are considered to be important in the design of these probes include the toxicity (which is influenced by lipophilicity) and pharmacokinetics of the agents, as well as their susceptibility to detection by non-invasive techniques such as MRS [12–14]. Such factors have been considered in the design and synthesis of N-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide as a novel non-invasive fluorinated 2-nitroimidazole MRS probe for tumour hypoxia.

The aim of this work was to develop an assay suitable for studying the pharmacokinetics of this compound in mice. Levels of 2-nitroimidazoles in body fluids have previously been assessed by techniques such as ultraviolet spectroscopy [15], gas chromatography [16], high-performance liquid chromatography (HPLC) [16,17], and polarography [15]. However, some of the reported techniques are limited by lack of sensitivity or selectivity, while others are not suitable for routine analysis. This paper, therefore, describes a sensitive and selective method for the routine detection of N-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide in mouse plasma using solid-phase extraction and HPLC. The application of this technique to the analysis of other 2-nitroimidazoles is also discussed.

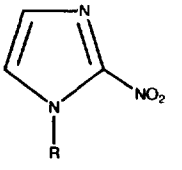
2. Experimental

2.1. Chemicals, reagents and animals

N-(2-Hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide (SR-4554), I, and N-(2-hydroxyethyl)-2-(2-nitro-1-imidazolyl) acetamide (Etanidazole; SR2508), II, were synthesised and supplied by SRI International, Menlo Park, CA, USA. All other 2-nitroimidazoles including 1-(2-nitro-1-imidazolyl)-3-methoxy-2-pro-panol (Misonidazole; Ro 07-0582), III, 1-(2-nitro-1-imidazolyl)-2,3-propandiol (Desmethylmisonidazole; Ro 05-9963), IV, 1-(2-nitro-1-imidazolyl)-3-chloro-2-propanol (Ro 07-0269), V, and 1-(2-nitro-1-imidazolyl)-3-fluoro-2-propanol (Ro 07-0741), VI, were synthesised and supplied by Roche Products, Welwyn Garden City, Herts, UK. The compounds (Fig. 1) were assessed for chromatographic purity and used without further purification. All other reagents were HPLC grade or analytical reagent grade. Female Balb/c mice and mouse plasma were obtained from Harlan Olac, Oxon, UK. The mice were allowed laboratory chow and water ad libitum and weighed between 18 and 22 g.

2.2. Animal studies

Female Balb/c mice were injected intravenously with compound I in 0.9% saline at a dose of 180 mg/kg. Control mice received 0.9% saline. The mice were sacrificed at various time points (5, 10, 15, 30, 45, 60, 90, 120, 240 and 360 min) and whole blood removed by cardiac puncture



R-substituent	Compound
$-CH_2CONHCH_2CH(CF_3)OH$	SR-4554; (I)
$-CH_2CONHCH_2CH_2OH$	Etanidazole; SR-2508; (II)
$-CH_2CH(OH)CH_2OCH_3$	Misonidazole; Ro 07-0582; (III)
$-CH_2CH(OH)CH_2OH$	Desmethylmisonidazole; Ro 05-9963; (IV)
$-CH_2CH(OH)CH_2Cl$	Ro 07-0269; (V)
$-CH_2CH(OH)CH_2F$	Ro 07-0741; (VI)

Fig. 1. Structural formulae of 2-nitroimidazoles.

into heparinized syringes. The blood samples were immediately centrifuged for 3 min (1000 *g*) to obtain plasma, which was frozen on solid carbon dioxide and stored at −70°C prior to analysis.

2.3. Preparation of calibration curve and validation standards

Compound I was weighed and dissolved in 1 m*M* Tris–HCl buffer (pH 7.4) to give a 2 mg/ml solution. Other standard stock solutions were prepared by serial dilution in the same buffer. Calibration standards were prepared by adding 20 μl of the standard stock solutions to 230 μl of mouse plasma to give final drug concentrations of between 0.025 and 160 μg/ml. Each concentration was prepared as a single sample (n = 13), except for the lowest (0.025 μg/ml), intermediate (10 μg/ml) and highest (160 μg/ml) concentrations, which were prepared in triplicate.

Efficiency of the extraction process for compound I from plasma was evaluated using recovery standards prepared in 1 m*M* Tris–HCl buffer (pH 7.4). The recovery standards also contained 1 μg/ml of the internal standard (compound V). The overall extraction efficiency was calculated as the ratio of the slope of the plasma standard to recovery standard calibration curves.

Quality control (QC) samples containing compound I were prepared in mouse plasma as follows: QC1 blank; QC2 0.025 μg/ml; QC3 0.1 μg/ml; QC4 10 μg/ml; QC5 100 μg/ml; QC6 160 μg/ml. Aliquots (250 μl) of the QC samples were stored at −70°C until analysis. Six quality control samples at each concentration level were thawed and analysed together with the calibration standards on day to assess the within-assay variability at the various levels. The between-day variability was evaluated on five separate occasions by assaying single QC samples from all the concentration levels together with fresh calibration standards. Within-assay and between-assay variability were calculated as the C.V. (%) of the QC samples. In addition, triplicates of QC samples prepared freshly as well as those subjected up to three cycles of freezing (−70°C) and thawing were analysed to determine the ability of compound I to withstand multiple cycles of freezing and thawing. The long-term stability of compound I in mouse plasma was also assessed by analysing QC samples on day 1 and after storage for 32 days at −70°C.

2.4. Extraction of plasma samples and standards

Aliquots (250 μl) of Tris–HCl buffer (pH 8.5) containing 1 μg/ml of the internal standard (compound V) were added to plasma samples or standards (250 μl) and vortexed. The buffered plasma samples and standards were then applied to Bond Elut C_{18} cartridges (100 mg, 1 ml capacity; Varian, Harbor City, CA, USA), preconditioned with 1 ml of methanol and 2 ml of 1 m*M* Tris–HCl buffer (pH 8.5). The samples were drawn through the cartridges under vacuum using a Vac Elut SPS24 (Varian) system. Plasma contaminants were eluted to waste with 1 ml of 1 m*M* Tris–HCl buffer (pH 8.5) and the columns dried for 5 min under vacuum. The analytes were then eluted with 1 ml of methanol–HCl (199:1).

The extracts were taken to dryness using a vortex-evaporator (Haakebuchler Instruments, Saddle Brook, NJ, USA), at a temperature of 50°C and under vacuum. The dried extracts were reconstituted in 250 μl of HPLC mobile phase, clarified by centrifugation to remove any contaminating silica resin (8 min, 2500 *g*, at 20°C) and transferred into autosampler glass inserts. Aliquots (200 μl) of these samples were then injected into the chromatograph. All solvents used for the extraction of the plasma samples and standards were filtered (0.45 μm Teflon PTFE–polypropylene filter membranes) and degassed with helium.

2.5. HPLC methodology

All extracts were analysed using a Waters chromatographic system (Millipore, Milford, MA, USA) consisting of a WISP Model 712 autosampler, a Model 660 gradient controller with a quaternary HPLC pump, a Model 991 photodiode-array detector and a NEC Power-

mate SX/16 personal computer running Water 991 photodiode-array software.

Separation of analytes were performed on a μ-Bondapak C_{18} (10 μm, 300 × 3.9 mm) analytical column (Millipore) at ambient temperature. The analytical column was protected from contamination by a pellicular ODS pre-column (Anachem, Luton, UK). The mobile phase which comprised methanol–water (15:85), was filtered through a 0.45-μm Teflon PTFE–polypropylene filter membrane, degassed with helium, and delivered isocratically at a flow-rate of 2 ml/min. Column effluents were monitored by UV-photodiode-array detection at 324 nm and the peak-area ratio of compound I to compound V used for conversion of the detector response to concentration estimates. The column performance in the assay was determined by measuring the efficiency (heights equivalent to theoretical plates), the symmetry factor, and the resolution between drug and internal standard using standard equations [18].

2.6. Mass spectral (MS) analysis

Prior to analysis by MS, plasma samples containing compound I were separated on a column as above, and the peak corresponding to compound I collected using a Waters fraction collector (Millipore). Both compound I fractions evaporated to dryness under N_2 gas, as well as authentic samples of compound I, were dissolved in a minimum amount of dichloromethane–methanol mixture (5:1). Aliquots (20 μl) were injected into a JEOL JMS-AX505HA (JEOL, Tokyo, Japan) MS in direct inlet mode. The mass spectrometer was operated at an accelerating potential of 3 kV (voltage 200 eV) in the positive ion chemical ionisation mode using methane gas.

2.7. Statistical analysis

The statistical analysis of the results were generated using Minitab Release 9 (Minitab, State College, PA, USA) and Microsoft Excel Version 4.0 (Microsoft, Redmond, WA, USA). The assay precision was the coefficient of variation C.V. (%) for each concentration level. This is defined as the ratio of the standard deviation to the mean expressed as a percentage. The accuracy was defined as the mean difference deviation (%) from the given or nominal concentration. The minimum quantifiable level (MQL) was defined as the lowest concentration of analyte which could be accurately and reproducibly measured.

The following acceptance criteria were used to evaluate the data: (1) p-values for all statistical tests be <0.05; (2) the within/between assay variability be within 15%; (3) 75% of all QC samples analysed be within 15% of their respective nominal values; (4) the correlation coefficient of calibration curves be 0.98. In addition the C.V. for the lowest, intermediate and highest levels within individual calibration curves be within 15%.

3. Results

3.1. Chromatography

Analysis of plasma samples obtained from mice by HPLC showed that compound I could be detected after a dose of 180 mg/kg. Typical chromatograms of compound I and internal standard (compound V) are illustrated in Fig. 2. No drug-related metabolites or interfering peaks were observed on analysis of both drug-treated and control plasma samples. The chromatographic analysis was rapid allowing separation of compound I and internal standard within 10 min. The retention times of compounds I and V were 5.8 and 7.4 min, respectively, and the two peaks were completely resolved (resolution, $R = 1.4$). Typical column efficiencies (HETP) were 0.28 mm and 0.14 mm for compound I and internal standard (compound V), respectively. In addition symmetry factors were calculated to be 1.09 and 1.12, respectively. Both compound I and the internal standard (compound V) had similar UV-spectral properties with a λ_{max} (for both compounds) of 324 nm (Fig. 3). In addition to UV-spectra, the identity of compound I, extracted from plasma, was confirmed by MS (Fig. 4). The protonated molecular ion peak (M + 1) as well

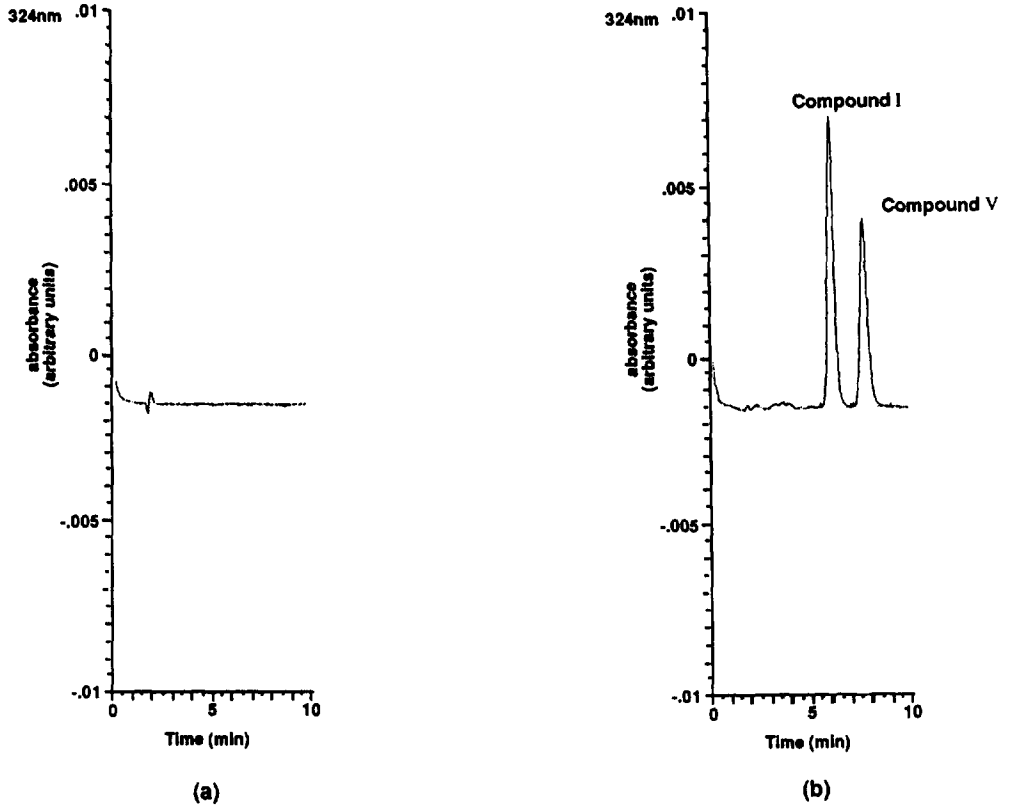


Fig. 2. Typical chromatograms of plasma extracts obtained from (a) control, and (b) compound I treated mice. Plasma samples were extracted using a C_{18} solid-phase cartridge and analysed by HPLC as described in Section 2.

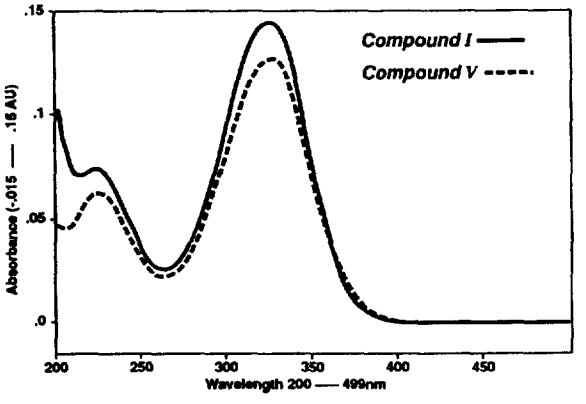


Fig. 3. UV-spectra of compound I and internal standard (compound V) in HPLC mobile phase using photodiode array detection. Spiked plasma samples of compound I and compound V were extracted, as described in Section 2, and the UV-spectra of the compounds obtained using a Waters photodiode-array detection. The λ_{max} for both compounds was found to be 324 nm.

as other fragments and methane adducts of compound I were observed.

3.2. Validation

The assay was reproducible at the at all concentration levels (Table 1), with all C.V.-values within the accepted limit of 15%. The MQL was validated at 25 ng/ml (on-column concentration of 5 ng) with a coefficient of variation of 6.01%.

Weighted calibration curves (weight $= 1/y^2$) for compound I were found to be linear over the concentration range 25–160 μg/ml and had near-zero intercepts. For instance, the calibration curve for the peak area ratio of compound I to internal standard (compound V) vs concentration of compound I used in the determination of within assay variability had a slope of 0.601 and an intercept of 0.013 ($r = 0.999$; S.D. $= 0.028$).

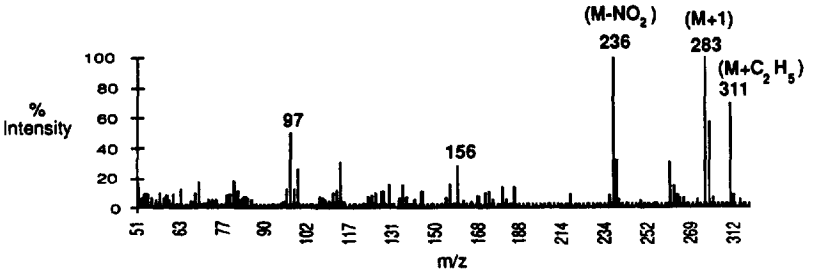


Fig. 4. Positive ion chemical ionisation spectrum of compound I. The spectrum was determined as described in Section 2, and shows the protonated molecular ion peak (M + 1), as well as, other peaks corresponding to ($M - NO_2$), and methane adduct ($M + C_2H_5$).

The mean recovery of compound I from four determinations was found to be 81.1 ± 6.9 (C.V. = 8.6%), whilst the recovery of the internal standard at a single concentration of 1 μg/ml was 80.6 ± 3.8 (C.V. = 4.7%).

Compound I was found to be stable up to three cycles of freezing and thawing as well as to storage at −70°C for 32 days (Table 2).

3.3. Pharmacokinetic profile

The assay was used to study the absorption and disposition of compound I in female Balb/c mice. Fig. 5 shows the pharmacokinetic profile of compound I in Balb/c mice. Compound I was detectable in plasma from 5 min up to 6 h post-injection.

4. Discussion

A rapid and sensitive HPLC method has been developed for the determination of N-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide (compound I) in small volumes

Table 1
Within- and between-day variability of the HPLC assay at six different concentration levels

QC	Given concentration (μ/ml)	Calculated concentration		
		Mean (μg/ml)	C.V. (%)	Deviation (%)
Within-day variability				
1	Blank	–	–	–
2	0.025	0.025	6.01	1.20
3	0.1	0.107	4.01	6.67
4	10	10.190	3.34	1.90
5	100	96.430	3.31	−3.57
6	160	148.90	1.52	−6.94
Between-day variability				
1	Blank	–	–	–
2	0.025	0.024	11.86	−3.84
3	0.1	0.111	3.89	11.20
4	10	9.790	6.51	−2.93
5	100	97.067	6.34	−2.93
6	160	147.174	3.77	−8.02

Plasma samples were determined in each case as described in Section 2, and the MQL was validated at 0.025 μg/ml (within-assay variability).

Table 2
Freeze–thaw and long-term stability of compound I in mouse plasma

	Mean concentration (μg/ml)	Deviation (%)	C.V. (%)
Freeze–thaw stability			
Fresh	10.28	2.8	1.45
Cycle 1	10.11	1.1	2.44
Cycle 2	10.27	2.7	1.60
Cycle 3	10.19	1.9	3.88
Long-term stability			
Day 1	10.59	5.9	2.50
Day 32	11.49	14.9	1.94

(250 μl) of mouse plasma. The method employs a semi-automated solid-phase extraction procedure followed by a fully automated HPLC assay. A run time of 10 min allows several samples to be routinely analysed over a relatively short period of time. Both the drug and internal standard had similar UV-spectra, which made it possible to monitor the elution of analytes at a single wavelength of 324 nm, representing the λ_{max} of both compounds. The sensitivity of the assay described in this paper (25 ng/ml) is much better than those for most 2-nitroimidazoles reported to date, and is also highly specific and sufficiently reproducible for routine use [15–17].

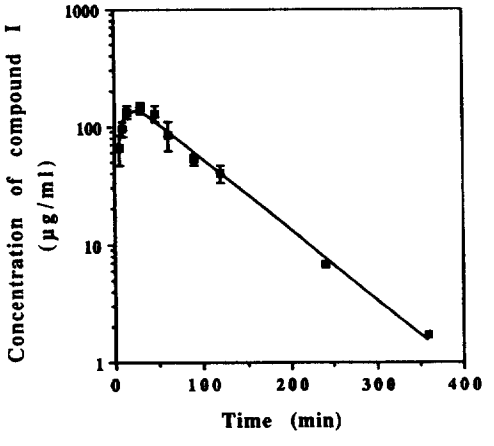


Fig. 5. Plasma pharmacokinetic profile of compound I (180 mg/kg) in female Balb/c mice showing rapid absorption and elimination phases. Plasma from individual mice were analysed separately. The error bars represent standard deviations from three such determinations.

The specificity of the assay was confirmed by UV-spectra and mass spectral analysis.

The assay was used to measure drug levels in plasma samples of mice treated with compound I. Although the parent drug was readily detected, it should be noted that no metabolites of compound I were observed in the plasma samples. This assay is specific for 2-nitroimidazoles which have an intact nitroimidazole chromophore. However, the analysis of samples at other wavelengths and with MS, did not give any indication of the presence of any compound I metabolites. This is an important property with respect to the use of compound I as a hypoxic probe by MRS, since the side chain containing the fluorine label is stable to in vivo metabolic processes. The drug and internal standard, the only analytes observed, were completely resolved ($R > 1.0$). Due to the slight peak tailing, however, peak areas instead of peak heights were used in the quantitation of the drug and internal standard peaks [17]. The recovery of the assay for both compound I and internal standard (compound V) from plasma was found to be good (81.1 and 80.6%, respectively) and reproducible. In addition the within- and between-day variability of the assay, as well as the accuracy of determining compound I, were found to be within 15%. The results of the study also show that plasma samples of compound I were stable for up to three cycles of freezing and thawing, as well as to storage at −70°C for up to 32 days.

In an attempt to develop a suitable method for the analysis of compound I in mouse plasma, other techniques were employed. One of such methods which involved a methanol extraction step offered good sample clean-up but had low sensitivity (MQL = 200 ng/ml). A solid-phase extraction method was, therefore employed. Various sorbents such as C_{18}, C_8, propylsulfonic acid (PRS) and benzenesulfonic acid (SCX) were tested for their ability to retain and release drug at various pH values in Tris–HCl buffer and other solvents such as methanol and acetonitrile. C_{18} columns proved superior to other sorbents due to good sample retention, recovery and low background interference, without resorting to solvents which affect the stability of the drug or sorbent. The reconstitution of the extract in the

mobile phase prior to analysis by HPLC also allowed a high injection volume to be used without significantly compromising the efficiency of the chromatographic separation.

The HPLC method in this paper has proved to be suitable for studying the pharmacokinetics of compound I in mice. With slight modifications in mobile-phase composition, this assay has also been applied to the analysis of compound III and its metabolite (compound IV), compound VI and compound II in our laboratory. We would expect the assay to form the basis of the analysis method for the novel fluorinated 2-nitroimidazole N-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide (compound I) during the anticipated clinical use of this drug as a non-invasive MRS probe for tumour hypoxia.

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