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Determination of U-89968E, a $5HT_{1a}$ agonist in rat plasma using solid-phase extraction, precolumn derivatization and reversed-phase high-performance liquid chromatography

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Abstract

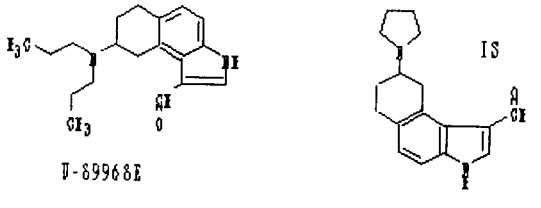
A selective and sensitive HPLC method was developed for the determination of U-39968E in rat plasma. The assay involved solid-phase extraction of the analyte and the internal standard and precolumn derivatization with cyclohexane-1,3-dione reagent before injection on to the HPLC column. The samples were chromatographed on a Spherisorb S5 CN column (25 cm × 4.6 mm i.d.) with a mobile phase containing acetonitrile–trifluoroacetic acid-water (17:0.2:83, v/v/v) at a flow rate of 1.5 ml min^{-1}. The column eluent was monitored by flourescence detection with excitation at 272 nm and emission at 320 nm. The assay is linear over the range 4-759 ng ml^{-1}. The relative standard deviation at the limit of quantification, 4 ng ml^{-1}, was 7.1%. This method was successfully applied to the determination of U-89968E in rat plasma during pharmacokinetic studies.

Keywords: $5HT_{1a}$ agonist; Plasma; Precolumn derivatization; Reversed-phase high-performance liquid chromatography; Solid phase

1. Introduction

Pharmacia and Upjohn, in collaboration with the Department of Pharmacology at the University of Göteborg, Sweden, is currently evaluating a series of 8-hydroxy-2-(Di-*n*-propylamino)-tetralin (8-OH DPAT) analogues. These compounds are potent $5HT_{1a}$ agonists [1] and U-89968E (Fig. 1) is one of a series of heterocyclic analogues of 8-OH DPAT.

U-89968E has a poor UV response and lacks native fluorescence. Therefore, derivatization was considered in order to measure this compound in biofluid samples. U-89968E possesses three possi-



U-89968E

Fig. 1. Structures of I and internal standard.

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ble reactive functional groups: two tertiary amines and an aldehyde.

Methods reported for the derivatization of tertiary amino groups include precolumn formation of a fluorescent carbamate with naphthyl chloroformate [2] and post-column ion-pair extraction of the amine as a 9,10-dimethoxyanthracene-2-sulphonate ion-pair complex [3]. However, the precolumn reaction can produce more than one reaction product with complex molecules and/or matrices. The reported sensitivities obtained with the post-column ion-pair extraction system did not appear to be adequate to meet project requirements.

One report for aldehyde-containing compounds describes the photoreduction and subsequent fluorescence detection with anthraquinone [4]. The reagent is added to the HPLC mobile phase and a high organic content is required to dissolve the anthraquinone salt. This, however, was incompatible with the HPLC of U-89968E. Another method reported precolumn derivatization with a reagent synthesized from cyclohexane-1,3-dione [5]. This reagent had increased aqueous solubility, which facilitated synthesis of the reagent, and the reaction did not need high reaction temperatures, unlike that of the earlier analogue 5,5-dimethylcyclohexane-1,3-dione [6].

This paper describes the development, validation and application of an assay using the cyclohexane-1,3-dione-derived reagent [5] to determine U-89968E (**I**) in low-volume plasma samples.

2. Experimental

2.1. Reagents

Compound **I** and the internal standard (Fig. 1), a close structural analogue, were obtained from Pharmacia and Upjohn (Kalamazoo, MI, USA). HPLC-grade acetonitrile (CH_3CN), methanol (MeOH) and trifluoroacetic acid (TFA) and AR-grade toluene were purchased from Fisons Scientific Equipment (Loughborough, Leics., UK). Cyclohexane-1,3-dione was purchased from Aldrich Chemical (Gillingham, Dorset, UK). Hydrochloric acid (sp.gr. 1.18) and ammonium acetate (Analar-grade) were purchased from BDH (Poole, Dorset, UK). HPLC-grade water was produced in-house with a Milli-Q filter system (Millipore (UK), Watford, Herts., UK). Glassware was silanized with a 10% (v/v) solution of chlorotrimethylsilane (Aldrich Chemical) in toluene.

2.2. Instrumentation

The HPLC system consisted of a Waters 600 Multisolvent Delivery System (Millipore (UK)), a Shimadzu CTO-2A column oven (Dyson Instruments, Hetton, UK), a Waters WISP Model 712 (Millipore (UK)) and a Perkin-Elmer LS 40 fluorescence detector (Perkin-Elmer, Beaconsfield, Bucks. UK). The HPLC column, a Spherisorb S5 CN (25 cm × 4.6 mm i.d.) (Hichrom, Reading, Berks., UK), was maintained at 35–40°C. Analyses were performed with a mobile phase consisting of CH_3CN–TFA–H_2O (17:0.2:83, v/v/v). The flow rate was 1.5 ml min^{-1}. The column eluent was monitored by fluorscence detection with excitation at 271 nm and emission at 320 nm. Chromatograms were recorded on an SP4290 computing integrator (Spectra-Physics, Hemel Hempstead, Herts., UK). Quantitation was based upon peak-height ratios (**I**/internal standard). A weighted ($1/x^2$) least-squares regression was fitted to each individual calibration curve (SAS Institute, Cary, NC, USA). The fitting algorithim does not force the curve through zero.

2.3. Standard solutions

Compound **I** and the internal standard were dissolved in H_2O to give stock solutions of approximately 100 μg ml^{-1} free base equivalents. The stock solution of **I** was further diluted with H_2O to 20, 4, 1, 0.4 and 0.1 μg ml^{-1}, and likewise for the internal standard, to 0.05 μg ml^{-1}, to provide solutions for spiking aliquots of control plasma. All standard solutions were stored at 4°C.

2.4. Preparation of plasma

Blood samples (ca. 0.3 ml) were taken from the superior vena cava, collected in disposable syringes containing K_2EDTA as anticoagulant and

transferred to 0.5 ml Eppendorf microtest-tubes (BDH). Plasma was prepared by centrifugation at room temperature at 1000*g* for 10 min and stored at −20°C prior to analysis by HPLC. Aliquots of plasma (0.05 ml) were transferred to 75 × 10 mm i.d. neutral borosilicate test-tubes (Fisons Scientific Equipment), spiked with an aliquot of the internal standard (0.5 ml of 0.05 μg ml^{-1} solution) and diluted with 1 ml of water. After thorough mixing, the samples were extracted within 30 min.

2.5. Preparation of calibration curve samples

Plasma calibration samples were prepared daily over the concentration range 4–759 ng ml^{-1} **I** free base equivalents. Aliquots (0.25 ml) of control rat plasma were spiked with a small volume (0.01 ml) of standard solution After thorough mixing, three 0.05 ml aliquots were removed and prepared as for study samples.

2.6. Preparation of quality control samples

Quality control (QC) samples were prepared in bulk at a low (33 ng ml^{-1}) and a high (329 ng ml^{-1}) concentration to monitor the performace of the assay. Control rat plasma was spiked with a small volume (to a maximum of 0.5% of the total volume) of standard solution and thoroughly mixed. Aliquots (0.25 ml) were transferred to 1.5 ml Eppendorf microtest-tubes (BDH) and stored at −20°C prior to analysis. One vial each of the low and high QCs were removed for each assay, thawed and prepared as described for study samples.

2.7. Solid-phase extraction (SPE)

The required number of Varian Vac-Elut boxes (Varian, Walton-on-Thames, UK) were loaded with Varian Bond Elut carboxylic acid 100 mg/1 ml cartridges (Varian). The cartridges were primed successively with 2 ml of CH_3CN, 2 ml of CH_3CN–H_2O–TFA (60:40:0.5, v/v/v) (the eluting solution) and 2 ml of H_2O. Prepared plasma samples were drawn slowly through the primed cartridges with the minimum of applied vacuum (10 kPa). The cartidges were rinsed with 2 ml of H_2O followed by 2 ml of MeOH–H_2O (1:1, v/v). The vacuum was then increased to 50–68 kPa for 15–20 s to dry out the sorbent bed partially. Compound **I** and the internal standard were eluted into silanized 75 × 10 mm i.d. neutral borosilicate test-tubes (Fisons Scientific Equipment) with 1 ml of the eluting solution. The eluates were reduced to dryness under vacuum (90 min at 40°C) using a Gyrovap centrifugal evaporator (VA Howe, London, UK).

2.8. Preparation of the derivatization reagent

This was prepared in bulk as described by Stahovec and Mopper [5]. The reagent was partially purified by passing it through a 'piggy-backed' 100 mg Varian Bond Elut C_8 and a 1 g Sep-Pak C_{18} cartridge (Millipore (UK)), previously primed with a 2 ml of MeOH and 2 ml of H_2O. The reagent was stored until required in an amber-glass bottle at −7°C for up to 3 weeks.

2.9. Derivatization of plasma extracts

An aliquot (0.2 ml) of the derivatization reagent was added to each extract, thoroughly mixed, transferred using extended fine-tipped pastettes (Alpha Laboratories, Eastleigh, UK) to 0.3-CVG autosampler vials (Chromacol, Welwyn Garden City, UK) and crimped capped with a silicon/Teflon seal (Chromacol). The vials were then placed in a Reactitherm III heater/stirrer (Pierce and Warriner (UK), Chester, UK) for 2 h at 120°C. After cooling, the derivatized extracts were transferred to 0.3 ml Microsert WISP inserts (Jones Chromatography, Hengoed, UK) for injection.

3. Results and discussion

3.1. Optimization of chromatography

From the initial column screen (Table 1), it appeared that the Zorbax CN gave the most efficient chromatography for derivatized **I**. However, when chromatographing derivatized ex-

Table 1
Summary of typical chromatographic parameters of **I** derivative achieved during initial column screen[a]

Stationary phase	Mobile phase	k	Efficiency (plates/colunm)	Skew[b]
Spherisorb ODS-2	CH_3CN-H_2O–TFA (32:68:0.5)	4.1	5225	Poor
	CH_3CN-H_2O–TFA (34:66:0.2)	4.6	2542	Poor
	CH_3CN-H_2O–TFA (37:63:0.1)	3.9	2916	1.7
Zorbax ODS	CH_3CN-H_2O–TFA (32:68:0.25)	3.4	—	Poor
	THF–H_2O–TFA (15:85:0.2)	2.8	2180	3.0
Zorbax C_8	CH_3CN-H_2O–TFA (43:57:0.2)	6.0	3920	2.3
	THF–H_2O–TFA (18:82:0.2)	2.8	6155	2.0
Zorbax Rx-C_8	CH_3CN-H_2O–TFA (30:70:0.2)	3.8	9661	1.4
Zorbax Phenyl	CH_3CN-H_2O–TFA (40:60:0.5)	4.3	10295	1.5
	CH_3CN-H_2O–TFA (45:55:0.2)	3.8	8291	1.4
Zorbax CN	CH_3CN-H_2O–TFA (27:73:0.2)	3.2	16000	1.3
	THF–H_2O–TFA (18:72:0.2)	4.6	11400	1.3
Spherisorb S5-CN	CH_3CN-H_2O–TFA (18:72:0.2)	4.0	9656	0.9
Zorbax TMS	CH_3CN-H_2O–TFA (20:80:0.2)	3.3	4627	2.0

[a]For all columns: mobile phase compositions are given by volume; all columns were obtained from Hichrom (Reading, UK); column dimensions were 25 cm × 4.6 mm i.d., particle size 5 μm, flow rate 1.5 ml min^{-1}, column oven temperature 35–45°C and detection was by fluorescence with excitation at 271 nm and emission at 320 nm.
[b]Skew was calculated by measuring the ratio of peak widths at 10% of the analyte peak height.

tracts, peaks running close to and underlying the derivatized **I** peak were observed. These were apparent in extracts of derivatized aqueous standards. Changing the mobile phase conditions failed to resolve the derivatized **I** from interferences. However, silanization of the test-tubes in which the SPE eluates were reduced to dryness decreased the level of interference, and changing the analytical column to a Spherisorb CN, although reducing chromatorgraphic efficiency, effected resolution of the derivatized analyte from the interferences (Fig. 2).

3.2. Optimization of derivatization procedure

Stahovec and Mopper [5] suggested the use of a 3 g Bond Elut C_{18} cartridge fitted to a 1 g Sep-Pak C_{18} cartridge to remove by-products of the reagent synthesis. However, a 100 mg Bond Elut C_8 cartridge fitted 'piggy-backed' style to a Sep-Pak C_{18} cartridge appeared to provide a cleaner reagent solution and this method of reagent preparation was used throughout.

Fig. 3 shows the effect of temperature and reaction time on the formation of the derivative of **I**. The highest responses were seen following derivatization at 100°C for 120 and 150 min. At 80°C, the response appeared still to be increasing after 150 min. A similar response was seen after derivatization at 120°C for 150 min, although the initial rate of product formation was greater. As there was concern about the integrity of the vial seals when derivatizing at 120°C, a reaction time of 120 min at 100°C was chosen as it combined an adequate derivative yield with a reaction time that was practical for routine analysis. The formation of the **I** derivative and the disappearance of **I** was also confirmed chromatographically by monitoring the column eluent by diode-array detection at 250 nm, the optimium absorbance wavelength for **I**, and at 270 nm, the optimum wavelength for the **I** derivative.

Derivatized spiked plasma extracts showed acceptable stablity for at least 24 h after storage at room temperature in a WISP autosampler (Table 2). Although a small decrease in the derivative peak height was observed during overnight analysis, there was no significant change in peak-height ratio, as any loss in analyte response was compensated for by a concommitant decrease in internal standard peak height.

The effect of temperature and light on the derivatizing reagent during storage was assessed by measuring the chromatographic detector re-

sponse for the **I** derivative. No apparent differences between light or dark storage or storage at room temperature, −7 or 20°C were observed (Fig. 4). As a precaution, the reagent was stored at −7°C and allowed to equilibrate to room temperature before use. The effect of prolonged reagent storage was not formally investigated, although in routine use no apparent decrease in analyte derivative response was observed.

The pH of the freshly prepared derivative varied from 5.2 to 6.4. Batch to batch comparison of the reagent was made, but no significant difference in the derivatized analyte response was observed.

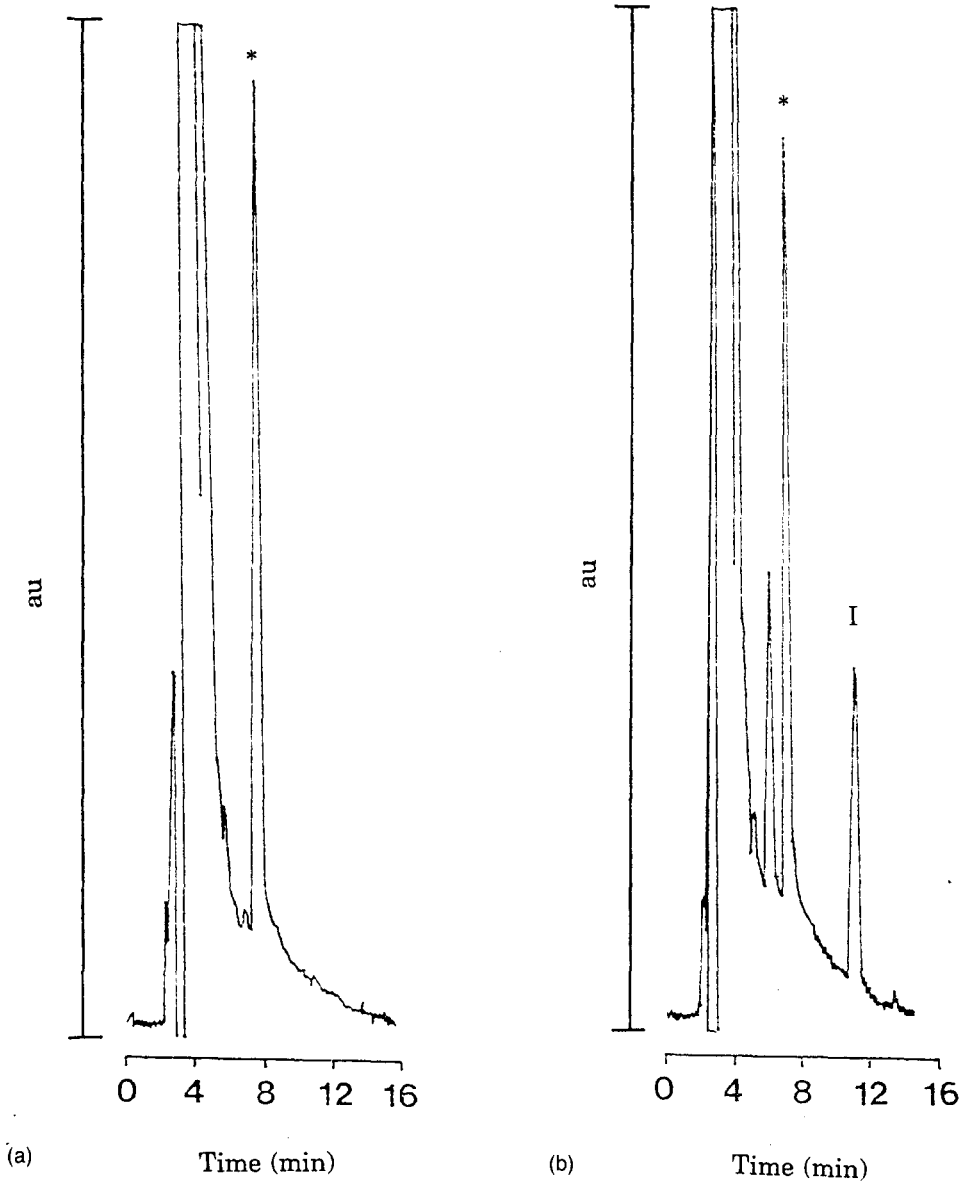

Fig. 2.

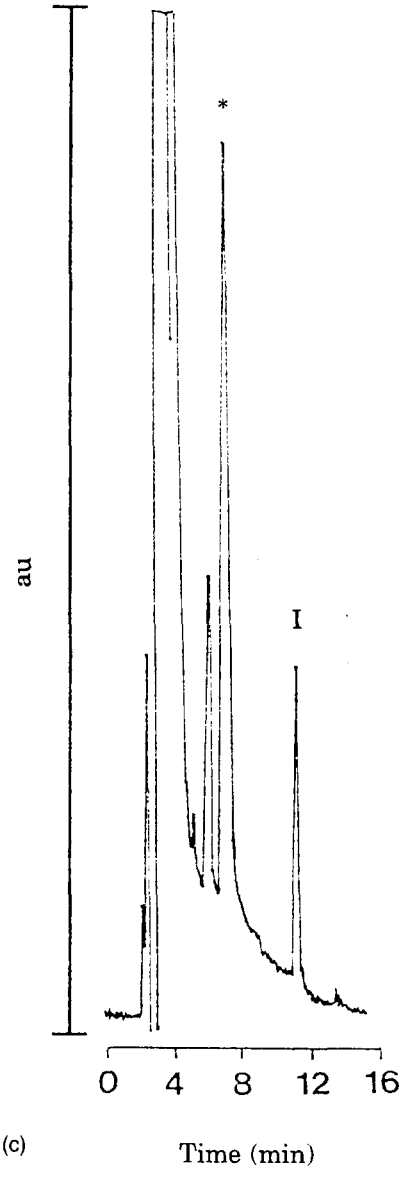

Fig. 2. Chromatograms of extracted and derivatized (a) control plasma, (b) plasma spiked at 53 ng/ml of **I** and (c) plasma from a rat taken 0.5 h after receiving an oral dose of 6 mg kg^{-1} of **I** with a calculated concentration of 30 ng ml^{-1}. The asterisk denotes the internal standard. Analytical conditions as described in the Experimental section.

3.3. Fluorescence detection

The UV spectrum of the **I** derivative, as assessed by diode-array detection, showed a maximum absorbance at 271 nm. Fluorescence spectroscopy of a sample containing concentrated **I** derivative revealed excitation maxima at 236 and 271 nm with emission at 320 nm. However, the emission response achieved at an excitation wavelength of 271 nm was greater than that at 236 nm. The higher excitation wavelength may also provide better selectivity from possible endogenous derivatives.

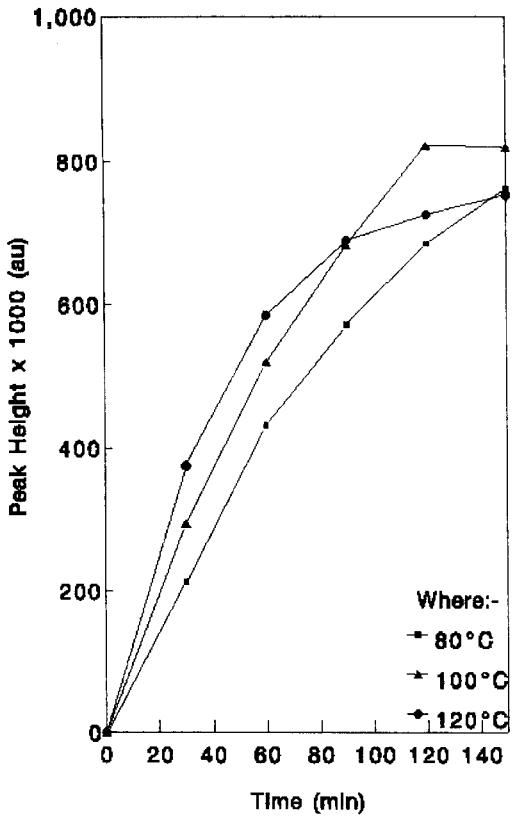

Fig. 3. Effect of temperature and reaction time on the rate of formation of the **I** derivative. Analytical conditions as described in the Experimental section.

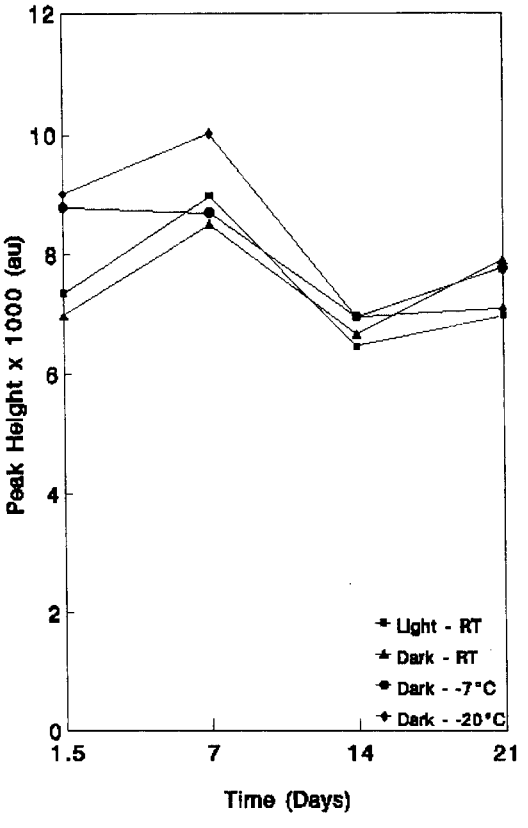

Fig. 4. Effect of storage conditions on the reagent and its ability to derivatize **I**. Analytical conditions as described in the Experimental section.

3.4. Stability of *I*

Stability of the analyte at −20°C was not formally investigated. However, inter-assay variation for the QC samples stored at −20°C, was acceptable.

3.5. SPE extraction efficiency

Control rat plasma was spiked at with **I** at 2 μg ml^{-1} and the drug extracted as described in the Experimental section. These samples were assayed using chromatographic conditions reported else-

Table 2
Stability of derivatized plasma extracts after overnight storage at room[a] temperature

Sample	Peak height (au)	Mean ± S.D.	RSD (%)	Recovery (%)
Day 1	27737	26643	3.7	—
	26413	±999		
	25779			
Day 2	25717	24366	5.0	91.4 ± 0.01
	24019	±1216		
	23361			

[a]Analytical conditions as described in Experimental section.

Table 3
Efficiency of extraction of **I** spiked control plasma samples[a]

Sample	Peak height (au)	Recovery (%)
Spiked control plasma	$x = 624 \pm 12$ ($n = 5$, RSD = 1.9%)	101.3 ± 0.01
Equivalent aqueous recovery standard	$x = 616 \pm 19$ ($n = 5$, RSD = 3.1%)	—

[a]Analytical conditions: column, Zorbax Rx-C_8 (Hichrom) (25 cm × 4.6 mm i.d.) obtained from Hichrom; mobile phase, CH_3CN–H_2O–TFA (25:75:0.1, v/v/v); flow rate, 1.5 ml min^{-1}; temperature, ca. 35°C; UV detection at 250 nm.

where [7] with UV detection at 250 nm. The extraction efficiency was calculated by comparison of the detector response of the extracted analyte with that of a known aqueous standard. The average extraction efficiency was repeatably 101% (Table 3).

3.6. Recovery of extracted and derivatized I

Control rat plasma was spiked at a known concentration with **I** (53 and 527 ng ml^{-1}) or the internal standard (270 ng). Aliquots were extracted and then derivatized with the cyclohexane-1,3-dione reagent as described in the Experimental section. The sample was then chromatographed with a derivatized aqueous standard, as no authentic sample of either derivative was available. The apparent reaction efficiency is greater than 100% (Table 4), perhaps owing to the difficulties of ensuring equivalent volumes of final reaction solution. Another possibility is that the derivization procedure is more effective with extracts owing to the presence of co-extractants.

3.7. Assay precision and accuracy

Analysis of calibration curve data obtained from the assay of spiked plasma samples showed linearity from 4 to 459 ng ml^{-1}. The correlation coefficient (r) of the slope of the calibration curves was > 0.992 in all cases. The relative standard deviation (RSD) of the slopes ($n = 8$) was 7.3%, which was acceptable in routine application. The limit of quantification was 4 ng ml^{-1} (RSD = 7.1%). However, the sensitivity of the assay was not limited by co-chromatographing interferences but by the absolute sensitivity of the detector. Sensitivity might be improved by microbore chromatographic techniques.

QC samples, spiked at 33 and 329 ng ml^{-1}, were included in each assay. The mean inter-day recovery obtaind for the QC samples were 33 ± 2 (RSD = 3.7%, $n = 22$) at 33 ng ml^{-1} and 332 ± 21 (RSD = 4.5%, $n = 20$) at 329 ng ml^{-1}. The intra- and inter-day variability were acceptable as assessed by triplicate analyses of spiked control plasma (Table 5).

3.8. Applicability of the assay

The preliminary pharmacokinetics of **I** were assessed in male Sprague–Dawley rats. Six animals, divided equally into two groups, were dosed either with 6 mg kg^{-1} of **I** orally or 2 mg kg^{-1} intravenously (i.v.). Plasma concentrations of **I**

Table 4
Recovery of extracted and derivitized **I** and the internal standard from rat plasma[a]

Sample	Mean peak height ± S.D. (au)[b]	RSD (%)	Recovery (%)
Spiked control plasma containing 270 ng internal standard	166 715 ± 9917	6.0	128.7
Equivalent aqueous recovery standard	129 523 ± 12103	9.5	—
Spiked control plasma containing 53 ng ml^{-1} **I** fbe[c]	330 ± 8	2.3	135.1
Equivalent aqueous recovery standard	244 ± 7	2.3	—
Spiked control plasma containing 527 ng ml^{-1} **I** fbe[c]	3651 ± 94	2.6	145.5
Equivalent aqueous recovery standard	2520 ± 101	4.0	—

[a]Analytical conditions as described in Experimental section.
[b]$n = 3$.
[c]fbe = free base equivalents.

Table 5
Precision and accuracy data over the range 4–759 ng ml^{-1} of **I** in rat plasma[a]

Target concentration of **I** (ng ml^{-1} fbe[b])	Actual concentration of **I** on occasion 1 (ng ml^{-1})		Actual concentration of **I** on occasion 2 (ng ml^{-1})		Actual concentration of **I** on occasion 3 (ng ml^{-1})		Inter-assay RSD (%)
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	
4	4	7.0	4	12.5	4	4.6	7.1
15	14	7.7	15	1.7	16	4.8	7.2
38	41	4.8	39	1.4	39	2.1	3.7
152	144	4.8	150	1.6	149	7.1	4.5
759	796	1.6	752	4.3	743	5.6	4.8

[a]Analytical conditions as described in Experimental section. Mean actual concentrations were determined from triplicate analyses.
[b]fbe = free base equivalents.

were measured using the described HPLC methodology and data from an animal dosed i.v. and another dosed orally are shown in Fig. 5. Compound **I** was detected in plasma samples from animals dosed i.v. up to 8 h post-dose and for 12 h after oral dosing.

4. Conclusion

A sensitive HPLC assay has been developed to determine **I** in rat plasma. The method involves SPE of the plasma samples, precolumn derivatization of the eluates with a reagent synthesized from cyclohexane-1,3-dione and reversed-phase HPLC followed by fluorescence detection of the derivatized compound. The method was applied to the determination of preliminary pharmacokinetics of **I** in the rat.

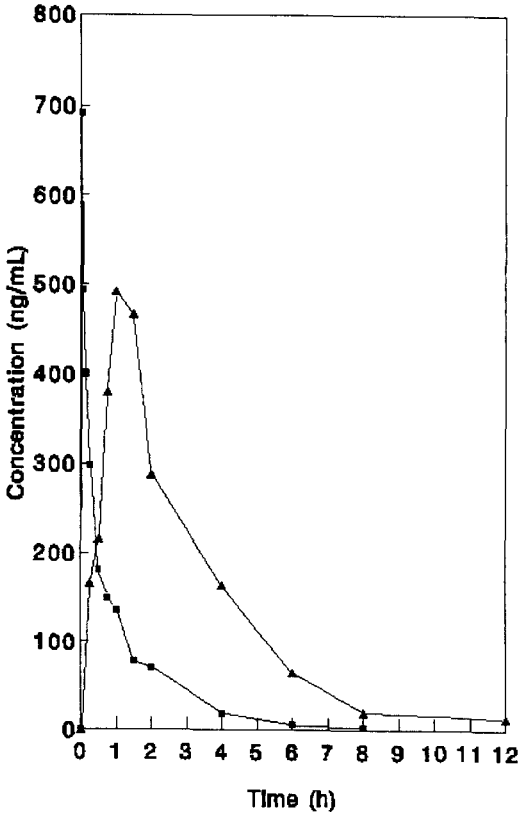

Fig. 5. Concentration–time profile of **I** after (■) an intravenous dose of 2 mg kg^{-1} or (▲) an oral dose of 6 mg kg^{-1}.

Acknowledgements

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