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Short Communication

A sensitive high-performance liquid chromatographic determination of 6-(pyridin-3-yl)-quinolin-2(1H)-one and its *N*-oxide metabolite in rat plasma and urine

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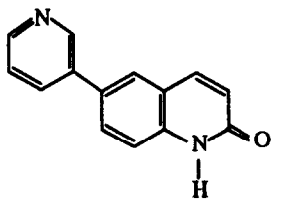
Introduction

6-(pyridin-3-yl)-quinolin-2(1H)-one (UK-57, 400, Fig. 1) is a prototype compound from a series of 2-quinolinone cardiac stimulants which possess inotropic and vasodilatory properties [1–3]. This class of compounds was developed for the treatment of congestive heart failure, one of which 6-(2,4 dimethyl-1H-imidazol-1-yl)-8-methyl-2(1H)-quinolinone progressed further into clinical evaluation [4]. These agents increase myocardial contractility with little tachycardia.

In dogs, UK-57,400 undergoes extensive first pass metabolism to the pyridyl-*N*-oxide [5]. In order to conduct detailed pharmacokinetic and disposition studies in rats, we have developed an improved method for simultaneous quantitation of the drug and its more polar *N*-oxide metabolite (UK-59,572) in biomatrices. The HPLC–UV assay for dog plasma [6] was inadequate for the rat plasma for two main reasons. Firstly, the sensitivity of the assay could not cope with a much smaller volume of plasma available from rat compared to the dog, and secondly, the presence of other unidentified metabolites, detected with fluorescence monitoring of the eluent, which occur at higher doses of UK-57,400, necessitated a modified separation method.



UK-57,400

Figure 1
Structure of 6-(pyridin-3-yl)-quinolin-2(1H)-one.

Materials and Methods

Reagents and chemicals

Acetonitile and tetrahydrofuran (Romil, Loughborough, Leics, UK) were HPLC grade. Water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Potassium dihydrogen phosphate, AR grade, was purchased from British Drug Houses (Poole, Dorset, UK). Tetrabutyl ammonium bromide (TBA) of HPLC grade was supplied by Fisons (Loughborough, Leics, UK). Orthophosphoric acid was obtained from Fluka (Glossop, Derbyshire, UK). Compounds UK-57,400 and 8-methyl-6-(pyridin-3-yl)-quinolin-2(1H)-one (UK-59,669) internal standard were supplied

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by Pfizer Central Research (Sandwich, Kent, UK). The pyridyl *N*-oxide metabolite UK-59,572 was prepared by a method described elsewhere [7].

For the mobile phase, phosphate buffer (0.010 M) containing TBA (0.005 M) was prepared by dissolving 1.36 g l^{-1} potassium dihydrogen phosphate hydrate and 1.6 g TBA in Milli-Q water, and the pH was adjusted to 5.5 with orthophosphoric acid. Acetate buffer (0.2 M) was prepared by dissolving 16.406 g sodium acetate in 1 l of distilled water and the pH was adjusted to 4.5 with glacial acetic acid.

Reference solutions and internal standard

Stock solutions of UK-59,572 (1 and 10 μg ml^{-1}), UK-57,400 (2 and 20 μg ml^{-1}) and the internal standard UK-59,669 (2 and 20 μg ml^{-1}) were prepared by dissolving each compound in methanol. Standard solutions were stored refrigerated at 4°C and remained stable for at least 3 months.

Sample preparation

Plasma samples. A 0.1 ml volume of rat plasma samples were transferred to 10 cm × 1 cm borosilicate glass tubes to which 0.4 ml of 10 mM phosphate buffer (pH 7.4) and 10 μl (20 ng) of the internal standard solution were added, and then mixed well. Bond-Elut® CH cartridges were conditioned with 1 ml of methanol followed by 1 ml of water. The plasma samples were then transferred to the preconditioned cartridge via Pasteur pipettes. The cartridges were centrifuged at 1000 rpm for 5 min and then washed with water (500 μl). Analytes were desorbed by 1 ml of methanol under gravity. Eluates were evaporated to dryness under nitrogen at 37°C and reconstituted in 50 μl of mobile phase; 40 μl of this concentrated extract was injected onto the chromatograph.

Urine samples. Urine samples were diluted 1 to 100 with acetate buffer. To 1 ml aliquots of these diluted urines was added 10 μl (20 ng) of internal standard UK-59,669 and the samples were then vortexed. These samples were applied to previously conditioned Bond-Elut CH cartridges as described above, and centrifuged at 1000 rpm for 5 min. The cartridges were washed with 1 ml of a mixture of water–methanol (80:20, v/v) which gave a clean extract without compromising the extractability. UK-59,572 and UK-59,669 were eluted with 1 ml of methanol, under gravity. The methanol eluates were evaporated under nitrogen to dryness at 37°C. The residues were reconstituted in 100 μl of mobile phase and 60 μl injected onto the HPLC column.

Chromatographic systems

The method was developed on a liquid chromatograph which consisted of a Constametric 3000 solvent delivery system (Milton Roy®, Stone, Staffs, UK), a LKB-Pharmacia autosampler (Milton Keynes, Bucks, UK) an Upchurch® pre-column (2 mm × 2 cm) packed with Co-Pell® ODS (Whatman, Maidstone, Kent, UK), a Spherisorb® 5 μm phenyl column (12.5 cm × 4.6 mm) (Hichrom, Berks, UK), a Hitachi–Merck® F1000 HPLC fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) and CI-4000 Milton Roy (Stone, Staffs, UK) integrator. The excitation and emission wavelengths for all compounds were at 340 nm and 420 nm, respectively (sensitivity 10, time constant 0.3).

The solvent system consisted of 0.005 M TBA in phosphate buffer (pH 5.5)–acetonitrile–THF (88:10:2, v/v/v). The mobile phase was filtered through a 0.2 μm membrane filter (Sartorius, G-B Belmount, Surrey, UK) and degassed, and delivered at a flow rate of 0.9 ml min^{-1}.

Calibration

Calibration samples were prepared by spiking control plasma (0.1 ml) with UK-59,572 (1, 2, 4, 6 and 10 ng) and UK-57,400 (2, 4, 8, 12 and 20 ng). Considering the difficulty in handling very small plasma sample volumes (100 μl), the higher calibration ranges (100–1000 ng ml^{-1} for UK-59,572 and 200–2000 ng ml^{-1} for UK-57,400) were intended for earlier time points following kinetic studies of higher intravenous doses of UK-57,400 (>5.0 mg kg^{-1}), thus eliminating the need for dilution of the sample to fit the lower calibration curves. Earlier pilot experiments showed that assay using high calibration curve gave the same result with that obtained by lower calibration curve after dilution of the sample. For the higher ranges, calibration curves were constructed by spiking 0.1 ml control plasma with UK-59,572 (10, 20, 40, 60 and 100 ng) and UK-57,400 (20, 40, 80, 120 and 200 ng). Samples were processed as described above, and the ratios of peak height of the analytes to the peak height of the internal standard were calculated.

Calibration curves were constructed by linear regression analysis.

The inter-assay variability was determined at three concentrations (10, 50 and 100 ng ml^{-1} UK-59,572; and 20, 100 and 200 ng ml^{-1} UK-57,400) on five occasions. The intra-assay variability was determined at different concentrations in the ranges 10–100 ng ml^{-1} UK-59,572 and 20–200 ng ml^{-1} UK-57,400 on at least six occasions. Similar procedures were employed for the validation of the higher range calibration curve.

Blank control urine samples were used to construct the urine calibration curves in similar manner as for the plasma calibration. The urine calibration curve was constructed between 10 and 100 ng ml^{-1} of UK-59,572.

Results and Discussion

Although, there is a method for the quantitation of UK-57,400 and UK-59,572 in dog plasma by HPLC with UV detection, this method lacks the sensitivity required for pharmacokinetic studies in rats using serial blood sampling. The main problems encountered during HPLC–fluorescence determination of plasma UK-57,400 and UK-59,572 concentrations concerned the handling of much smaller quantities of the analytes, and the presence of unknown metabolite peaks in the plasma, which were observed from preliminary experiments in rats.

The dilution of the plasma sample with phosphate buffer made the sample handling easier and at the same time reduced the transference losses. The low speed centrifugation step (1000 rpm) was introduced to drive the sample through the extraction column, instead of the high vacuum generated by VacElut®, since it was found that recovery of nanograms of analytes were irreproducible owing to drastic and uncontrollable variations in vacuum pressures. For low level of analytes in plasma, the washing solvent was altered to water only since the presence of methanol caused significant losses in extractibility of compounds at these low ranges. Because of the presence of the unknown metabolite peaks which were only partially resolved from UK-59,572 and UK-57,400 with our previously reported assay [6], a new HPLC mobile phase was also required.

Figure 2 shows representative chromatograms of the spiked rat plasma standards and plasma extracts after dosing with UK-57,400. Under the chromatographic conditions used, UK-59,572, UK-57,400 and UK-59,669 all gave sharp peaks and were well separated from the unknown metabolites. The response was linear in the concentration ranges 10–100 ng ml^{-1} UK-59,572 and 20–200 ng ml^{-1} UK-

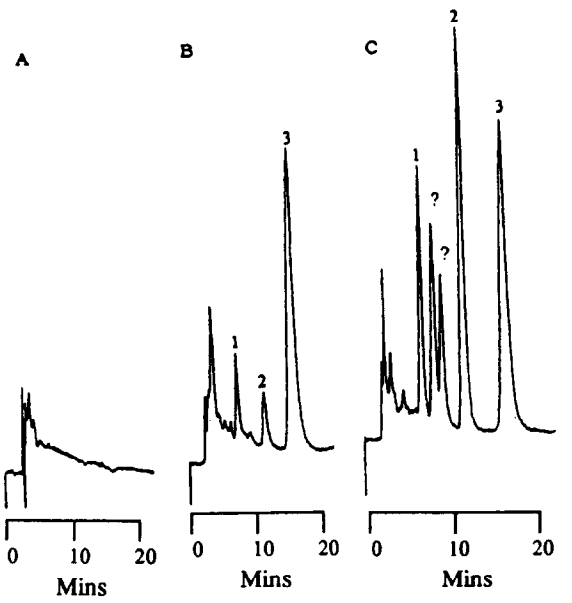

Figure 2
HPLC–fluorescence chromatograms of extracts of (A) drug-free rat plasma, (B) rat plasma spiked with 20 ng ml^{-1} UK-59,572; 40 ng ml^{-1} UK-57,400 and 200 ng ml^{-} UK-59,669 (internal standard), and (C) plasma of a rat dosed with UK-57,400 (5 mg kg^{-1}, IV). (1) UK-59,572, R_t = 7 min; (2) UK-57,400, R_t = 12 min; (3) UK-59,669, R_t = 16 min; and (?) unknown metabolite(s).

Table 1
Linearity and between-assay precision of the low and high range HPLC–fluorescence calibration curves for UK-59,572 and UK-57,400 (n = 6)

(a) **Low range**

	UK-59,572			UK-57,400		
	Slope × 10^{-2}	Intercept × 10^{-3}	r	Slope × 10^{-3}	Intercept × 10^{-3}	r
Mean	1.31	1.39	0.9999	4.83	−6.92	0.9998
SD	0.035	4.66		0.045	1.87	
RSD	2.67%			0.93%		

(b) **High range**

	UK-59,572			UK-57,400		
	Slope × 10^{-2}	Intercept × 10^{-3}	r	Slope × 10^{-3}	Intercept × 10^{-3}	r
Mean	1.845	−0.112	0.9998	2.451	−0.046	0.9998
SD	0.023	0.058		0.062	0.057	
RSD	1.25%			2.53%		

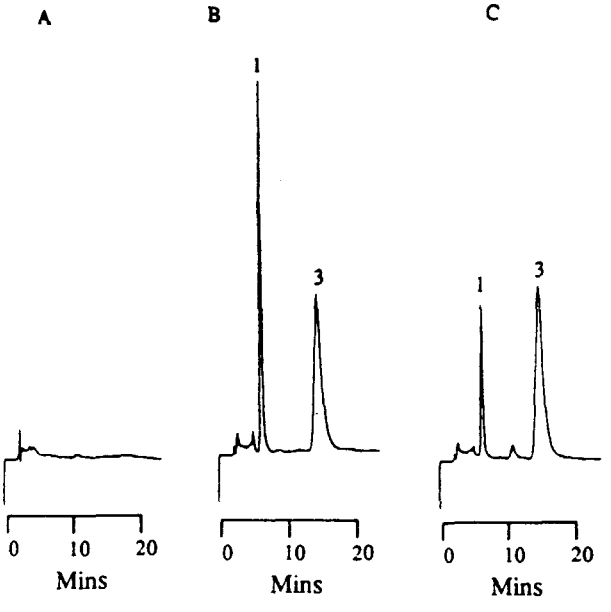

Figure 3
HPLC–fluorescence representative chromatograms of extracts of (A) blank control urine, (B) a spiked standard at concentration of 100 ng ml^{-1}, and (C) urine from a rat dosed with UK-57,400 (5.0 mg kg^{-1}, IV). (1) UK-59,572, R_t = 7 min; (3) UK-59,669, R_t = 16 min.

57,400 with a correlation coefficient of at least 0.9998 (Table 1).

Results were compared with those obtained following extractions of blank control rat plasma. The methanolic eluates were spiked with appropriate amounts of standards and compared with extracts of spiked plasma to determine recovery. The extraction yields were (mean ±SD, n = 4): UK-59,572, 98 ± 4%; UK-57,400, 96 ± 2%; and UK-59,669, 97 ± 1%.

The intra-assay relative standard deviation (RSD) for UK-57,400 was 8% at 20 ng ml^{-1} and 2% at 200 ng ml^{-1}. For UK-59,572 the intra-assay RSD was 13% at 10 ng ml^{-1} (n = 6) and 4% at 100 ng ml^{-1} (n = 6). The inter-assay RSD varied from 6% at 10 ng ml^{-1} to 2% at 100 ng ml^{-1} for UK-59,572, and from 7% at 20 ng ml^{-1} to 1% at 200 ng ml^{-1} for UK-57,400 (Table 2). It may be concluded that the lowest concentration of UK-59,572 and UK-57,400 that can be quantified accurately was 5

Table 2
Accuracy and precision data for the low range HPLC–fluorescence assay of UK-59,572 and UK-57,400

Conc. (ng ml^{-1})	Mean conc. found* (ng ml^{-1})	RSD (%)	Accuracy† (%)
UK-59,572			
100	105.51 ± 2.51	2	106
50	49.39 ± 0.76	2	99
10	10.29 ± 0.59	6	103
UK-57,400			
200	205.79 ± 2.10	1	103
100	96.53 ± 0.97	1	97
20	20.61 ± 1.45	8	103

*Results given are mean ±SD, $n = 6$.

†Accuracy (%) $= \frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$.

and 10 ng ml^{-1}, respectively, depending on the quantity of the plasma.

The method was also adapted for urine analysis. Typical chromatograms are shown in Fig. 3. Since UK-57,400 was not detected in urine of rats following administration of UK-57,400, the calibrations were constructed for UK-59,572 only. The slope of the curve displays an RSD of 7%. The within-run reproducibility shows an RSD of 8% at the lowest concentration tested (10 ng ml^{-1}).

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