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Quantitative high-performance liquid chromatographic method for pharmacokinetic studies of the potent mast cell inhibitor 4-(4′-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P131)

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Abstract

The novel quinazoline derivative 4-(4′-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P131) has recently been identified as a potent mast cell inhibitor capable of preventing IgE/antigen induced cutaneous as well as systemic fatal anaphylaxis in mice. Here we describe a sensitive high-performance liquid chromatography (HPLC)-based quantitative detection method for measurement of WHI-P131 levels in plasma as well as in target mast cells. The average extraction recovery for WHI-P131 was 88.4% for plasma and 75.7% for RBL-2H3 mast cell lysates. Good linearity ($r>0.999$) was observed throughout the concentration range of 0.1–20 μM in plasma and 0.01–5 nmol in $5\cdot10^6$ cells (0.5–238 μM per cell) for WHI-P131. Intra- and inter-assay variabilities were <7% and the lowest detection limit of WHI-P131 was 0.05 μM in plasma and 0.005 nmol in 5 million cells, respectively, at a signal-to-noise ratio of ~2. The practical utility of this new HPLC method was confirmed in a pilot pharmacokinetic study in BALB/c mice as well as in a cellular drug uptake and disposition study in RBL-2H3 mast cells. After intraperitoneal administration of a non-toxic 40 mg/kg bolus dose of WHI-P131, the estimated maximum plasma concentration was 92.7 μM, which is approximately 1-log higher than the effective in vitro mast cell inhibitory concentrations of WHI-P131. The drug absorption was rapid with an absorption half-life of only 2.9 min and the estimated time to reach the maximum plasma concentration was 8.3 min. WHI-P131 was cleared with an apparent systemic clearance rate of 2586 ml/h/kg and an elimination half-life of 1.8 h. An intracellular exposure level (AUC) of 55 $\mu M\cdot$h was obtained after in vitro treatment of RBL-2H3 mast cells with WHI-P131 at a 33.6 μM final concentration in culture medium. The availability of the described quantitative HPLC detection method for WHI-P131 provides the basis for further development of WHI-P131 as an anti-allergic drug through detailed pharmacodynamic studies in preclinical animal models.

Keywords: 4-(4′-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline

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1. Introduction

Mast cells have been recognized as the major effector cells of the allergic or type 1 hypersensitivity reactions by virtue of their high affinity receptors for IgE. These allergic reactions are mediated by the activation of mast cells through the cross-linking of their high affinity surface IgE (FcϵRI) receptors [1,2]. This IgE receptor cross-linking initiates the release of preformed granule-associated proinflammatory mediators (e.g. histamine, serotonin, proteases), newly synthesized arachidonic acid metabolites [e.g. leukotriene (LT) C_4, prostaglandin D_2 and platelet activating factor], and a number of proinflammatory cytokines [e.g. Tumor necrosis factor (TNF)α, interleukin (IL) -1, -3, -4, -5, -6,-8, -9, -13] [3]. The released mediators alone or in combination are responsible for the clinical signs and symptoms of allergic responses [4].

In a systematic search for effective mast cell inhibitors, we have recently discovered that the novel quinazoline derivative 4-(4′-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P131) is a potent inhibitor of the IgE receptor-mediated mast cell responses. WHI-P131 inhibited degranulation and proinflammatory mediator release from mouse, rat, as well as human mast cells after IgE receptor/FcϵRI cross-linking [5]. In vivo administration of WHI-P131 prevented mast cell degranulation and development of cutaneous as well as systemic fatal anaphylaxis in mice [5]. Therefore, WHI-P131 may be useful for treatment as well as prevention programs against mast cell mediated allergic reactions.

Further development of WHI-P131 will require detailed pharmacodynamic studies in preclinical animal models. Currently, there are no analytical methods available for detecting WHI-P131 in biological fluids or in cells. Therefore, we set out to establish a sensitive and accurate detection method for WHI-P131. Here, we first describe a high-performance liquid chromatography (HPLC)-based quantitative detection method which allows the measurement of WHI-P131 levels in plasma as well as in cells. The practical utility of this method for detecting WHI-P131 is demonstrated in a pilot pharmacokinetic study in mice as well as a cellular drug uptake and disposition study in RBL-2H3 mast cells.

2. Materials and methods

2.1. Chemicals

All reagents used in this study were HPLC grade. Deionized distilled water was used throughout the work (U.S. Filter, US Filter Corporation). Methanol, acetonitrile, chloroform, triethylamine (TEA) and trifluoroacetic acid (TFA) were obtained from Fisher (Fair Lawn, NJ, USA). Cell culture medium (Eagle's MEM) was purchased from Gibco (Gaithersbury, MD, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA) and was heat-inactivated at 56°C for 30 min before use.

2.2. Cell lines and cultures

RBL-2H3 cells (a rat origin mucosal mast cell line) was kindly provided by Dr. R. P. Siraganian (Laboratory of Microbiology and Immunology, NIH, Bethesda, MD, USA). RBL-2H3 mast cells were maintained in culture at 37°C and 5% CO_2–95% air as a monolayer in Eagle's MEM supplemented with Earle's balanced salts, 10 000 U/ml penicillin, 100 μg/ml streptomycin, 4 m*M* L-glutamine and 15% heat inactivated fetal calf serum. The cells were passaged twice a week and harvested by incubation with trypsin–EDTA for 5 min. Cell counts were performed by hemacytometer using tryptan blue exclusion as the criterion for viability.

2.3. Drugs

Highly pure (>99% by elemental analysis and HPLC) WHI-P131 and its internal standard WHI-P154 were synthesized, as previously described [6–8] (chemical structures shown in Fig. 1A). Stock solutions of WHI-P131 and WHI-P154 were prepared in methanol–water (50:50, v/v) at a concentration of 1 m*M*, and stored at −20°C. The stock solutions were then diluted further to yield appropriate working solutions for generation of calibration curve (see below).

2.4. HPLC determination of WHI-P131

2.4.1. Apparatus and chromatographic conditions

The HPLC system (Hewlett-Packard, Palo Alto,

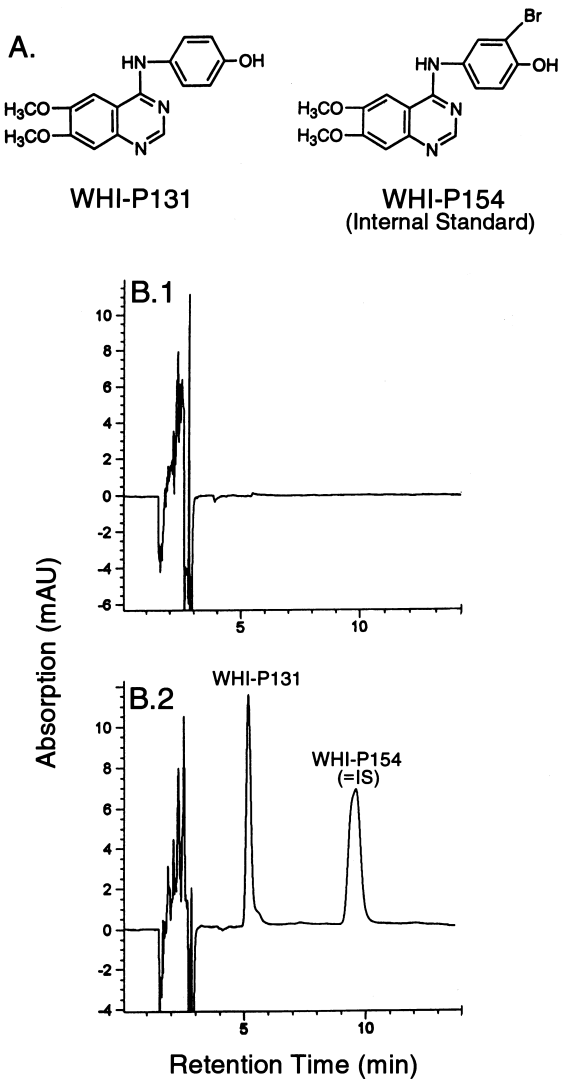

Fig. 1. (A) Chemical structures of WHI-P131 and its internal standard, WHI-P154 . Molecular mass of WHI-P131 was 297. Representative chromatograms from blank plasma (B.1) and from plasma sample 1 h after i.p. injection of 40 mg/kg WHI-P131 (B.2). I.S. = internal standard.

CA, USA) consisted of a HP series 1100 instrument equipped with a quaternary pump, an autosampler, an auto electronic degasser, an automatic thermostatic column compartment, a diode array detector and a computer with a CHEMSTATION software program for data analysis. A 250×4 mm Lichrospher 100, RP-18 (5 μm) analytical column and a 4×4 mm Lichrospher 100, RP-18 (5 μ*M*) guard column were obtained from Hewlett-Packard. Acetonitrile–water containing 0.1% of TFA and 0.1% TEA (28:72, v/v) was used as the mobile phase. The mobile phase was degassed automatically by the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow-rate of 1.0 ml/min at ambient temperature. The wavelength of detection was set at 340 nm for WHI-P131. Peak width, response time and slit were set at >0.03 min, 0.5 s and 8 nm, respectively.

2.4.2. Extraction procedures and extraction recovery.

For determination of WHI-P131 levels in 100-μl plasma samples, 10 μl of the internal standard WHI-P154 (at 50 μ*M*) was also added to the plasma. For extraction, 7 ml of chloroform was added to the plasma sample, and the mixture was vortexed thoroughly for 3 min. Following centrifugation (300 *g*, 5 min), the aqueous layer was frozen using acetone–dry ice and the organic phase was transferred into a clean test tube. The chloroform extracts were dried under a slow steady stream of nitrogen gas. The residue was reconstituted in 100 μl of methanol: water (9:1, v/v) and a 50-μl aliquot of this solution was injected for HPLC analysis. All extraction procedures were performed at room temperature.

For detection of WHI-P131 in cell lysates, 10 μl of the internal standard WHI-P154 (at 50 μ*M*) (=0.5 nmol) was added to 100 μl cell lysate and then the mixture was extracted with chloroform as described above. Replicate (n=5) plasma samples (100 μl/sample) were spiked with known amounts of WHI-P131 to yield a final concentration of 0.5 μ*M* and 10 μ*M* of WHI-P131, and replicate (n=5) lysates samples (100 μl/sample) were spiked with 1 nmol WHI-P131. The samples were extracted following the above described extraction procedures. The extraction recovery (ER) was calculated using the formula: ER% = {Peak Area $[\text{WHI-P131}]_{\text{extracted}}$/Peak Area $[\text{WHI-P131}]_{\text{unextracted}}$} × 100.

2.4.3. Calibration curve

A calibration curve was generated to confirm the linear relationship between the peak area ratio (peak area of WHI-P131 over the peak area of internal standard WHI-P154), and the concentration of WHI-P131 in the test samples. WHI-P131 was added to

plasma to yield final concentrations of 0.1, 0.25, 0.5, 1, 5, 10 and 20 μ*M*, and to cell lysates from $5 \cdot 10^6$ RBL-2H3 cells to yield the quantities of 0.01, 0.025, 0.1, 0.5, 1, 2 and 5 nmol. Subsequently, 10 μl of the internal standard WHI-P154 (50 μ*M*) (=0.5 nmol) was added to each sample. The plasma and cell lysate samples with known amounts of WHI-P131 and its internal standard were extracted as described above, and the standard curves were generated by plotting the peak area ratios (WHI-P131/WHI-P154) against the drug concentrations tested. Linear regression analysis of the standard curve was performed by using the CA-CRICKET GRAPH III computer program, Version 1.5 (Computer Association, Islandia, NY, USA). The unweighted linearity was confirmed using the INSTAT program V3.0 (GraphPad Software, San Diego, CA, USA).

2.4.4. Intra-assay and inter-assay accuracy and precision

To evaluate the intra-assay accuracy and precision, WHI-P131 and its internal standard WHI-P154 were added to drug-free plasma at concentrations of 0.75 and 7.5 μ*M*. These standard samples were prepared and analyzed within 24 h following addition of the internal standards. The concentrations were calculated using a standard curve. The ratio of the calculated concentration over the known concentration of WHI-P131 was used as the accuracy of the analytical method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined using six independent experiments. To evaluate the intra-assay and inter-assay accuracy and precision of the assay for measurement of WHI-P131 content in cells, 0.075 nmol and 0.75 nmol WHI-P131 were added to cell lysates from $5 \cdot 10^6$ RBL-2H3 cells, and these lysate samples were analyzed as described above. One-way analysis of variance (ANOVA) was performed using the INSTAT Program V3.0 to determine the statistical significance of differences between expected vs measured WHI-P131 levels in spiked samples.

2.5. Drug administration to mice and blood sampling

Female BALB/c mice purchased from Charles River (Wilmington, MA, USA) were housed in a controlled environment (12-h light/12-h dark photoperiod, 22±1°C, 60±10% relative humidity), which is fully accredited by the USDA (US Department of Agriculture). The mice were allowed free access to autoclaved pelleted food and tap water. Animal studies were approved by Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the guidelines found in the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

WHI-P131 was dissolved in DMSO and further diluted with alkalized PBS (pH ~9). The mice were injected intraperitoneally (i.p.) with a 40 mg/kg bolus dose of WHI-P131. Blood samples (~0.2 ml) were collected from the ocular plexus via retro-orbital venipuncture prior to drug administration and at 5, 15, 30 min and 1, 2, 4 and 8 h following i.p. injection. These blood samples were heparinized and centrifuged at 7000 *g* for 10 min in a microcentrifuge to obtain plasma. The plasma samples were stored at −20°C until analysis. Aliquots (100 μl) of plasma were used for extraction and HPLC assays.

2.6. Cellular uptake of WHI-P131 in RBL-2H3 cells

RBL-2H3 mast cells were grown to confluency in 25 cm^2 tissue culture flask. The confluent monolayers were treated with 33.6 μ*M* WHI-P131 or vehicle alone for 15, 30, 60 and 120 min at 37°C. After incubation, monolayers were washed three times with PIPES buffered saline containing 1 m*M* calcium chloride to remove excess of test compound. The monolayers were harvested by scraping and transferred to 15-ml capacity polypropylene tubes. The cells were lysed in 100 μl deionized water and saved at −70°C until processing.

2.7. Pharmacokinetic analysis

Data fitting and pharmacokinetic parameter calculations were carried out using the pharmacokinetic software, WINNONLIN program, version 2.0 (Scientific Consulting, Cary, NC, USA) [9]. An appropriate pharmacokinetic model was chosen to fit the plasma WHI-P131 concentration–time curves based on the lowest weighted squared residuals, lowest Schwartz criterion (SC), lowest Akaike's information criterion (AIC) value, lowest standard errors of the fitted

parameters, and dispersion of the residuals. The area under the plasma concentration time curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C/k, where C is the concentration of last sampling and k is the elimination rate constant. Apparent systemic clearance (CL) was determined by dividing the dose by the AUC. Cellular WHI-P131 pharmacokinetic profiles were analyzed by noncompartmental methods, where the AUC was estimated by the linear trapezoidal method to the last point.

3. Results and discussion

3.1. Sensitivity and accuracy of the HPLC detection method for WHI-P131 levels

Several combinations of acetonitrile, methanol and water (with 0.1% TFA and 0.1% TEA) were evaluated as possible mobile phases. It was determined that acetonitrile–water containing 0.1% of TFA and 0.1% TEA (28:72, v/v) is the most suitable mobile phase for separating WHI-P131 and its internal standard, WHI-P154. TEA has been demonstrated to be an important modifier for most of nitrogen-containing compounds [10–15]. Presence of TEA in the described mobile phase shortened the retention time and sharpened the peak for the compound WHI-P131. Under the described chromatographic conditions, the retention time for WHI-P131 was 5.4 min and the retention time for WHI-P154 was 9.5 min. At the retention time, WHI-P131 and its internal standard WHI-P154 eluted without an interference peak from the blank plasma (Fig. 1B.1. and 1B.2.) or cell lysate samples (data not shown). With the described extraction conditions, the recovery of WHI-P131 was 88.4% for plasma and 75.7% for the cell lysate samples (Table 1). Under the same extraction conditions, the extraction recovery of the internal standard was 78.3% for both plasma and cell lysates.

The standard curves obtained from extraction of plasma and cell lysate samples containing known amounts of WHI-P131 and its internal standard WHI-P154 were linear ($r>0.999$) over the concentration ranges tested (i.e. 0.1–20 μM in plasma and 0.01–5 nmol in cells) and could be described by the regression equations: $y=6.186x-0.002$ ($r>0.999$) for WHI-P131 in plasma, and $y=0.640x-0.012$ ($r>0.999$) for WHI-P131 in cell lysates, in which y is the WHI-P131 recovered in μM in plasma and nmol in cell lysate, and x is the peak area ratio (agent WHI-P131/WHI-P154). The linearity was statistically confirmed using the INSTAT Program V3.0. At a signal-to-noise ratio of ~2, the lowest limits of detection of WHI-P131 were 0.05 μM in plasma and 0.005 nmol in cells, respectively. The intra- and inter-assay coefficients of variance (C.V.) in plasma and in cell lysate samples for both WHI-P131 were <7%. The mean accuracy of this detection method was 98.6% for plasma samples and 99.7% for cell lysate samples (Table 2). There was no statistically significant difference by ANOVA analysis between expected and experimentally determined WHI-P131 levels in spiked samples.

3.2. Pharmacokinetics of WHI-P131 in mice

We next used the established HPLC method in a pilot study to determine the pharmacokinetics of WHI-P131 in BALB/c mice. Following i.p. administration of a non-toxic 40 mg/kg WHI-P131 to BALB/c mice, the plasma WHI-P131 concentration changes could be described by a two-compartment model (Fig. 2). The predicted maximum concentration (C_{max}) was 92.7 μM which is approximately

Table 1
Extraction recovery (mean±S.D.) of WHI-P131 from plasma and from cells ($n=5$)

Added	Peak area (mAU·s)		Recovery (%)	Average (%)
	Unextracted	Extracted		
Plasma				
0.5 μM	31.4±1.2	26.1±0.7	83.1±2.2	
10 μM	442.0±14.3	395.4±11.9	93.7±2.8	88.4
Cells				
1.0 nmol	402.8±57.9	304.8±23.9	75.7±6.0	75.7

Table 2
Intra- and inter-assay accuracy and precision of WHI-P131 in plasma and in cells[a]

	Added	Found	Accuracy (%)	C.V. (%)
Intra-assay ($n=6$)				
Plasma	0.75 μ*M*	0.747±0.028	99.6±3.7	3.8
	7.5 μ*M*	7.070±0.355	94.3±4.7	5.0
Cells	0.075 nmol	0.0756±0.003	100.8±4.2	4.2
	0.75 nmol	0.746±0.015	99.5±2.1	2.1
Inter-assay ($n=6$)				
Plasma	0.75 μ*M*	0.775±0.037	103.4±4.9	4.8
	7.5 μ*M*	7.292±0.459	97.2±6.1	6.3
Cells	0.075 nmol	0.0754±0.003	100.5±4.6	4.6
	0.75 nmol	0.734±0.013	97.9±1.8	1.8

[a] Data are expressed as mean±S.D; C.V., coefficient of variance.

1-log higher than the effective in vitro mast cell inhibitory concentrations of WHI-P131 [5]. WHI-P131 demonstrated rapid absorption with an absorption half-life ($t_{1/2\alpha}$) of 2.9 min, and the estimated time (t_{max}) to reach C_{max} was 8.3 min. WHI-P131 was cleared with an apparent systemic clearance rate of 2586 ml/h/kg and an elimination half-life of 107.6 min.

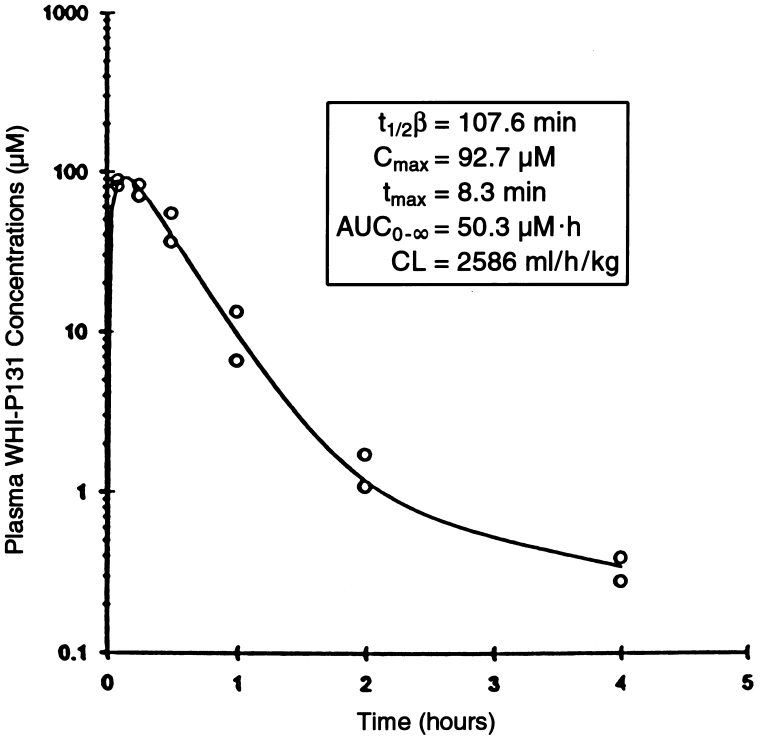

Fig. 2. Plasma concentration–time profiles in BALB/c mice following intraperitoneal administration of WHI-P131 (40 mg/kg; $n=2$). Data are expressed as means.

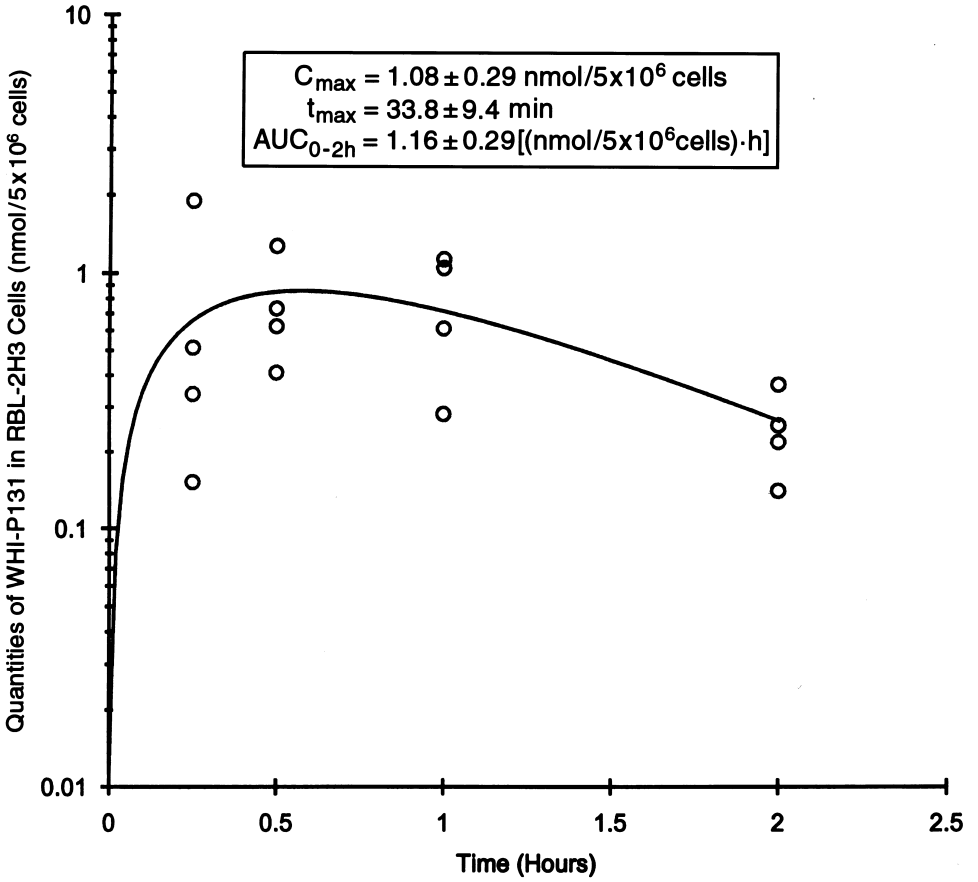

Fig. 3. Accumulation of WHI-P131 in RBL-2H3 cells following incubation with 33.6 μ*M* WHI-P131 ($n=4$). Data are expressed as mean±S.E.M.

3.3. Cellular uptake of WHI-P131 by RBL-2H3 mast cells

The cellular WHI-P131 concentration–time profiles of RBL-2H3 cells treated with 33.6 μ*M* WHI-P131 are shown in Fig. 3. The predicted maximum cellular WHI-P131 content was 1.08 nmol per 5 million cells (~0.2 fmols/cell or $5.1\cdot10^{-5}$ $M=51$ μ*M* concentration for an average cell volume of $4.2\cdot10^{-12}$ l) [15], which was about 1.5 fold higher than the drug concentration in the treatment medium (33.6 μ*M*). The $AUC_{0-2\ h}$ was 1.16 (nmol/$5\cdot10^6$ cells)·h (~55 μ*M*·h). The estimated time required to reach maximum cellular WHI-P131 concentration was 33.8 min.

In summary, we have developed a highly sensitive and accurate analytical HPLC method for the quantitative detection of the novel mast cell inhibitors WHI-P131 in plasma and cell lysate samples. The availability of this assay will now permit detailed pharmacodynamic and pharmacokinetic studies of WHI-P131.

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