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High-throughput, semi-automated determination of a cyclooxygenase II inhibitor in human plasma and urine using solid-phase extraction in the 96-well format and high-performance liquid chromatography with post-column photochemical derivatization-fluorescence detection

C.Z. Matthews, E.J. Woolf*, L. Lin, W. Fang, J. Hsieh, S. Ha, R. Simpson, B.K. Matuszewski

Merck Research Laboratories, Department of Drug Metabolism, West Point, PA 19486, USA

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Abstract

Compound **I**, 5-chloro-3-(4-methanesulfonylphenyl)-6′-methyl-[2,3′]bipyridinyl, has been found to be a specific inhibitor of the enzyme cyclooxygenase II (COX II). The anti-inflammatory properties of this compound are currently being investigated. HPLC assays for the determination of this analyte in human plasma and human urine have been developed. Isolation of **I** and the internal standard (**II**) was achieved by solid-phase extraction (SPE) in the 96-well format. A C_8 SPE plate was used for the extraction of the drug from human plasma (recovery >90%) while a mixed-mode (C_8/Cation) SPE plate was used to isolate the analytes from human urine (recovery approximately 71%). The analyte and internal standard were chromatographed on a Keystone Scientific Prism-RP® guard column (20×4.6 mm) connected to a Prism-RP® analytical column (150×4.6 mm), using a mobile phase consisting of 45% acetonitrile in 10 m*M* acetate buffer (pH=4); the analytes eluted at retention times of 5.2 and 6.9 min for **I** and **II**, respectively. Compounds **I** and **II** were found to form highly fluorescent products after exposure to UV light (254 nm). Thus, the analytes were detected by fluorescence (λ_{ex}=260 nm, λ_{em}=375 nm) following post-column photochemical derivatization. Eight point calibration curves over the concentration range of 5–500 ng/ml for human plasma and human urine yielded a linear response (R^2>0.99) when a $1/y$ weighted linear regression model was employed. Based on the replicate analyses (n=5) of spiked standards, the within-day precision for both assays was better than 7% C.V. at all points on the calibration curve; within-day accuracy was within 5% of nominal at all standard concentrations. The between-run precision and accuracy of the assays, as calculated from the results of the analysis of quality control samples, was better than 8% C.V. and within 8% of nominal. **I** was found to be stable in human plasma and urine for at least 8 and 2 months, respectively. In addition, the human plasma assay was semi-automated in order to improve sample throughput by utilizing a Packard liquid handling system and a Tom-Tec Quadra 96 SPE system. The precision and accuracy of the semi-automated procedure were comparable to the manual procedure. Over 5000 clinical samples have been analyzed successfully using these methods.

*Corresponding author. Fax: +1-215-652-4524.

E-mail address: woolf@merck.com (E.J. Woolf).

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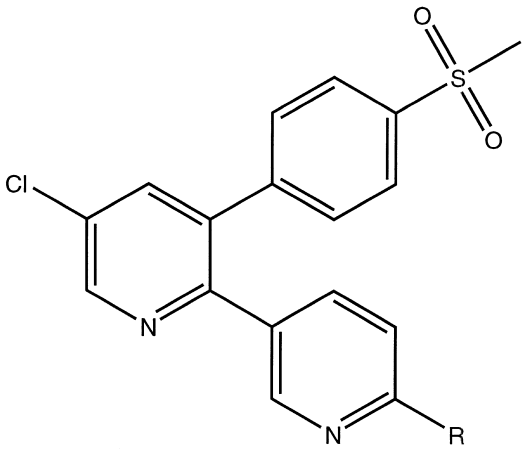
1. Introduction

Prostaglandin (PG) synthesis in man has been found to be catalyzed by at least two forms of the enzyme cyclooxygenase; cyclooxygenase-I (COX-I) and cyclooxygenase-II (COX-II) [1,2]. COX-I is constitutively expressed and enzymatically active in a variety of tissues including the stomach, intestine, kidneys and platelets [1]. COX-II is cytokine-inducible and is expressed in inflammatory cells [1]. The identification of multiple isoforms of COX enzymes has led to the hypothesis that COX-II is primarily responsible for PGs produced during inflammation, while COX-I is involved in the PGs synthesis that is required for normal homeostasis [1]. Currently-available nonsteroidal anti-inflammatory drugs (NSAIDs) have been characterized as dual COX-I and COX-II inhibitors [2]. Toxicities, such as gastrointestinal bleeding, that commonly occur when NSAIDs are used to treat arthritis and other inflammatory disorders have recently been hypothesized to be caused by the inhibition of COX-I [2]. Therefore, a COX-II specific inhibitor may have the potential to suppress the response to inflammation without causing the toxicity associated with the inhibition of COX-I derived PGs.



I: R=CH_3 **II**: R=CH_2CH_3

Fig. 1. Chemical structures of compound **I** and internal standard, **II**.

Compound **I** (Fig. 1), 5-chloro-3-(4-methanesulfonylphenyl)-6′-methyl-[2,3′]bipyridinyl, has been found to be a cyclooxygenase II specific inhibitor. Its in-vivo anti-inflammatory properties are currently being investigated in clinical studies. In order to support clinical pharmacokinetic studies, HPLC assays for the determination of **I** in human plasma and urine were required. Assays for the determination of **I** in plasma and urine that utilize solid-phase extraction in the 96-well format followed by HPLC with post-column photochemical derivatization and fluorescence detection are the subject of this publication.

2. Experimental

2.1. Materials

Compound **I** and internal standard, **II** (Fig. 1) (purities >99%) were provided by Merck-Frosst (Montreal, Canada). Acetonitrile (ACN), methanol and methylene chloride (Omnisolve HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Ammonia, 2.0 *M* solution in methanol, was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). All other reagents were ACS grade from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified with a Milli-Q system and had a resistivity of 18.2 Ω at the outlet. Drug free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). Control urine was provided by volunteers in the Department of Drug Metabolism of Merck Research Labs.

Empore™ 96-well disk plates (C_8 and C_8/cation-exchange) were purchased from 3*M* (St. Paul, MN, USA). Polypropylene 96 deep well plates (2.0 ml) were obtained from Matrix Technologies Corporation (Lowell, MA, USA). ELISA plates were from Corning-Costar (Cambridge, MA, USA). Racked collection tubes in a 96-well plate format (1.2 ml) were obtained from Marsh Biochemical Products (Rochester, NY, USA). The 96-well filter plate (25–

30 μm polypropylene upper pre-filter and 0.45-μm nylon lower filter) was from Whatman (Clifton, NJ, USA).

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump, a WISP 715 automatic injector (Waters Assoc., Milford, MA, USA), a AURA Industries (Staten Island, NY, USA) photochemical reactor consisting of a 254-nm UV lamp mounted with a 5.0-m, 0.3-mm ID reaction coil and a Perkin-Elmer model LC 240 fluorescence detector (Norwalk, CT, USA). The photochemical reactor was installed between the analytical column and the fluorescence detector. The detector output was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 941 analog-to-digital interface.

UV absorption and fluorescence spectra were obtained using a diode array spectrophotometer (HP 8452, Hewlett-Packard, Palo Alto, CA, USA) and an Hitachi (Danbury, CT,USA) Model F-4500 spectrofluorometer, respectively.

Off-line irradiations of samples were performed using a ''merry-go-round'' photochemical reactor (Rayonet, Hamden, CT, USA) equipped with four 254-nm mercury lamps.

The HPLC–UV–fluorescence system for the photochemical studies consisted of a HP (Hewlett-Packard, Palo Alto, CA, USA) series 1100 pump, HP series 1100 automatic injector, a HP series 1100 UV diode array spectrophotometer (UV–DAD) and a Perkin-Elmer LC 240 fluorescence detector (Norwalk, CT, USA).

An API 3000 triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer atmospheric pressure chemical ionization interface was used to obtain mass spectra of the photolysis products of **I** and **II**. The mass spectrometer was operated in the positive-ionization mode. The heated nebulizer temperature was set at 400°C.

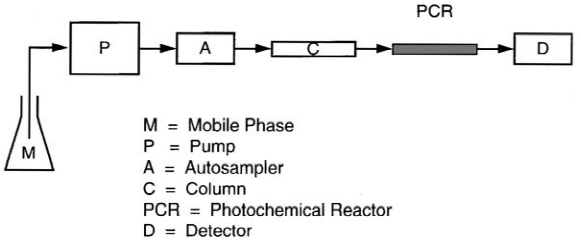


Fig. 2. A schematic of the HPLC and post-column photochemical reactor system.

A Matrix Impact[2] eight-channel expandable electronic pipet was used for manual sample manipulations, while a Packard MultiProbe® 204DT liquid handling system (Downers Grover, IL, USA) and a TomTec Quadra 96 SPE workstation (Model 320, Hamden, CT, USA) were used to automate the liquid transfer procedures. The QIAvac® vacuum manifold for solid-phase extraction (SPE) and the Sigma® 4 K15 96-well format centrifuge were obtained from QIAGEN (Chatsworth, CA, USA).

2.3. Chromatographic conditions

The mobile phase, (45/55 v/v acetonitrile/10 m*M*, pH 4 acetate buffer) was prepared by adding 1.2 ml of acetic acid to 1.1 l of purified water and adjusting the solution pH to 4 with sodium hydroxide prior to the addition of 900 ml ACN. The mobile phase was filtered through a nylon filter (0.45 μm) prior to use. The flow-rate was 1.2 ml/min through a Keystone Prism® RP guard column (20×4.6 mm, 5 micron, Keystone Scientific, Bellefonte, PA, USA) connected to a Keystone Prism® RP analytical column (150×4.6 mm, 5 micron, Keystone Scientific). The column was operated at ambient temperature (approximately 22°C). Injection volume was 35 μl for both the human plasma and urine assays.

2.4. Preparation of standards

A 20 μg/ml stock solution of **I** was prepared by weighing 1.0 mg of reference material into a 50-ml red volumetric flask, dissolving the compound in 25 ml of ACN and filling the flask to volume with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of 20 μg/ml solution to 50 ml with ACN–water (1:1,v/v). Working standards of 10, 8, 4 and 2 μg/ml were prepared by dilution of 20 μg/ml stock solution with ACN–water (1:1, v/v). Working standards of 1, 0.4, 0.2 and 0.1 μg/ml were prepared

by dilution of 2.0 μg/ml stock solution with ACN–water (1:1, v/v).

Standard plasma or urine samples at concentrations of 5, 10, 20, 50, 100, 200, 400 and 500 ng/ml were prepared by spiking 25 μl of each working standard to 0.5 ml of drug-free plasma or urine. These standards were used to analyze plasma or urine samples containing **I** over the range of 5 to 500 ng/ml. Use of standard curves containing fewer than eight points was not evaluated. Working standards solutions were found to be stable for at least 6 months when stored at room temperature and protected from light. The stability of working standard solutions stored at reduced temperatures was not tested.

Quality control (QC) samples containing **I** at concentrations of 375 ng/ml (High QC) and 15 ng/ml (Low QC) were prepared by diluting 1-ml aliquots of solutions of **I** in 50/50 v/v % acetonitrile–water at concentrations of 37.5 and 1.5 μg/ml to 100 ml with control plasma or urine. The QC samples were then stored in 1-ml aliquots at −20°C.

2.5. Extraction of human plasma

Aliquots (0.5 ml) of clinical samples were pipetted into 13×85 mm polypropylene tubes (Sarstedt, Newton, NC, USA). A 25-μl aliquot of ACN–water (50/50, v/v, %) was added to each of the samples to make their volume equal to those of the standards. A 25-μl volume of internal standard (Compound **II**) solution, (0.8 μg/ml in 50/50 ACN–water, v/v, %) was added to the samples and standards and the tube contents were vortex mixed. One milliliter of phosphate buffer (0.1 *M*, pH 7) was added and the resulting solution was vortex mixed. A 96-well disk SPE plate (C_8, standard density) was conditioned by passing 0.3 ml of methanol followed by 0.6 ml of water through each of the wells. Low negative pressure was applied (<2 in Hg) during plate conditioning to prevent drying of the wells. Aliquots (0.6 ml) of the buffered plasma solutions were transferred into the conditioned Empore 96-well disk SPE plate using a Matrix Impact^2 eight-channel expandable electronic pipette. The solutions were drawn through the plate wells using negative pressure (10–15 in Hg). One milliliter of 0.1 *M* pH 7 phosphate buffer was passed through each of the extraction wells. The plate then was positioned on the top of an ELISA plate and the extraction plate/ELISA plate assembly was centrifuged for 5 min at 1500 rpm (514 *g*) to remove residual phosphate buffer. The extraction plate was placed on top of a rack of 96 collection tubes (1.2 ml) that contained 300 μl of 10 m*M* acetate buffer (pH 4). ACN (200 μl) was added to each of the extraction wells. The plate–collection tube assembly was centrifuged for 5 min at 1500 rpm (514 *g*) to elute the analytes. The resulting solutions in the collection tubes were mixed and transferred onto a filter plate placed on the top of a new rack of collection tubes (1.2 ml). The assembly was centrifuged for 5 min at 3500 rpm (2800 *g*) in order to remove particulate matter from the samples. The filtered solutions were transferred into autosampler vials prior to injection to the HPLC system.

2.6. Extraction of human urine

Aliquots (0.5 ml) of clinical samples were pipetted into 13×85 mm polypropylene tubes (Sarstedt, Newton, NC, USA). A 25-μl aliquot of ACN–water (50/50, v/v, %) was added to each of the samples to make their volume equal to those of the standards. Internal standard solution (25 μl of 0.8 μg/ml in 50/50 ACN–water (v/v, %) was added to the standards and samples and the contents of the tubes were vortex mixed. A 1.0-ml volume of 0.15 *M* of phosphate buffer (pH 2) was added to acidify the samples and standards. A 96-well disk SPE plate (MPC, C_8/Cation-exchange, standard density) was conditioned by drawing 0.3 ml of methanol followed by 0.6 ml of water through each of the extraction wells. Low negative pressure was applied (<2 in Hg) during plate conditioning to prevent drying of the wells. Aliquots (1.0 ml) of the acidified urine samples and standards were transferred to the preconditioned 96-well SPE plate using a Matrix Impact^2 eight-channel expandable electronic pipette. The samples and standards were drawn through the extraction well using negative pressure (10–15 in Hg). The extraction wells were washed by sequentially passing 1.0 ml of water, 1.0 ml of acetic acid and 2 ml of methanol through them using high negative pressure (10–15 in Hg). The plate was placed on top of a rack of collection tubes (1.2 ml).

Two 500-μl aliquots of 98/2 (v/v, %) methylene chloride–2 *M* ammonia in methanol were used to elute the analytes. The elution solvent was passed through the extraction wells using centrifugation at 1000 rpm (228 *g*) for 5 min. The eluents were transferred into 12×75 mm glass culture tubes and evaporated with nitrogen using a Turbo Vap LV evaporator set at 50°C for 10 min. The residue was reconstituted initially in 200 μl of ACN, to which 300 μl of 10 m*M* acetate buffer (pH 4) was added. The reconstituted samples were transferred into autosampler vials prior to injection into the HPLC system.

2.7. Automation of liquid transfer steps

A Packard MultiProbe 204 DT liquid handling system (Downers Grover, IL, USA) equipped with four fixed tip probes, was used to transfer the diluted plasma samples from the polypropylene test tubes to a deep well 96 position plate. A 1.2-ml volume of tip-dip solution (ACN–water, 50/50, v/v, %) was used to wash the probes between each transfer. The deep well plate then was placed onto the platform of a TomTec Quadra 96 SPE workstation (Model 320, Hamden, CT, USA). The SPE plate conditioning, sample loading, SPE plate washing, elution solvent addition, sample dilution, sample mixing and sample transfer steps that are described above in the manual sample preparation procedure were carried out by TomTec Quadra 96 SPE workstation. This workstation simultaneously processed all 96 wells in the plate in a single step. Centrifugation was used, as in the manual procedures, to remove residual wash solvent, perform analyte elution and sample filtration.

3. Results and discussion

3.1. Spectroscopic characterization of analytes

To establish the best conditions for the sensitive detection of **I**, a series of spectroscopic and photochemical studies were performed. The ultraviolet (UV) spectrum of **I** dissolved in ACN–phosphate buffer (50/50, v/v, %) showed maxima at 238 and 280 nm. The position and molar extinction coefficients of these bands were not significantly affected by the pH of the solvent. **I** was found not to exhibit significant fluorescence.

Acidic solutions of **I** exposed to UV light (254 nm) were found to exhibit significant fluorescence, which was believed to result from the photocyclization of the compound (Fig. 3).

To confirm this hypothesis, additional experiments to study the photochemistry of **I** were conducted. A photochemical reactor consisting of a 5-m PTFE reaction coil capable of withstanding high pressure (greater than 1000 psi) and a 254-nm lamp was installed in an HPLC system between the injector and an HPLC column (Hypersil, Quest 50×3 mm, 5 μm, HyPURITY®-C_{18}). A mobile phase consisting of 35/65 (v/v, %) acetonitrile–5 m*M* ammonium acetate buffer (pH 4) at a flow-rate of 0.6 ml/min was utilized for these experiments. UV photodiode array (wavelength range from 190 to 400 nm) and fluorescence detectors (EX: 260 nm, EM: 375 nm) were used to detect the separated photolysis products. When **I** was injected onto the system with the reactor lamp off, its retention time was 3.3 min. When **I** was injected onto the system with the reactor lamp on, three photolysis products were detected at retention times of 4.7, 7.0 and 19.6 min; no unchanged **I** was detected under these conditions. The products eluting at 4.7 and 19.6 min were fluores-

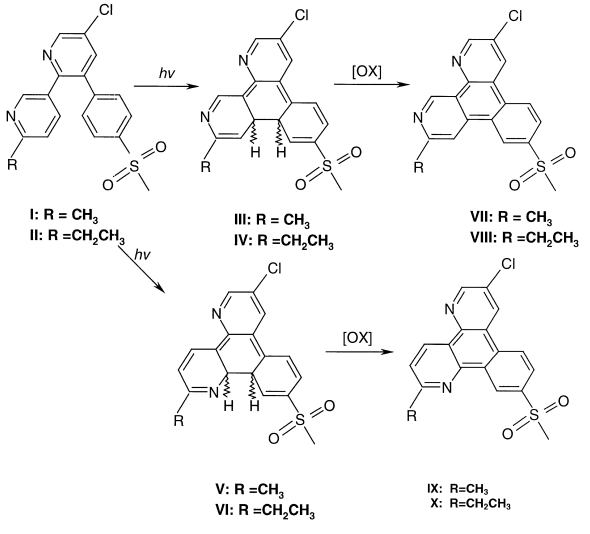


Fig. 3. Photocyclization of **I** and **II**.

cent, the product eluting at 7.0 min was not fluorescent. This data is consistent with the photocyclization scheme shown in Fig. 3. The photolysis products that eluted at retention times of 4.7 and 19.6 min may be either **VII** or **IX** (Fig. 3) as these conjugated compounds would be expected to be fluorescent. The large difference in retention time of these products may be due to differences in pK_a of the two species. The product that eluted at 7.0 min may correspond to **III** and/or **V**; as these products are not fully conjugated, they would not be expected to be fluorescent.

HPLC with tandem mass spectrometric detection was utilized to provide additional structural information regarding the photolysis products. The photolysis products that eluted at 4.7 and 19.6 min were found to have protonated molecular ions at m/z 357. The photolysis product that eluted at 7.0 min had a protonated molecular ion at m/z 359. This data is consistent with the scheme shown in Fig. 3.

Similar HPLC–UV–fluorescence and LC–MS–MS results were obtained when the photochemistry of the internal standard was studied, indicating that it under went reactions analogous to those described for **I.**

Based on these results, fluorescence detection following post-column photochemical derivatization was chosen as the method for detecting **I** following HPLC separation. The application of this detection method made a detection limit in the low ng/ml range possible. A similar method has recently been employed for the determination of rofecoxib in human plasma [3].

3.2. Chromatographic system development

Utilization of post-column photochemical derivatization–fluorescence detection required the development of chromatographic conditions that made use of an acidic mobile phase. Under acidic conditions, however, the pyridine nitrogens of **I** are protonated, thus **I** was found to elute near the solvent front from conventional reversed-phase columns unless very weak mobile phases having an organic content of less than 20% were utilized. Experience has shown that endogenous components from biofluid samples are more likely to cause interference problems with analyte quantitation when such weak mobile phases are employed.

In contrast to traditional reversed-phase columns, a column packed with Prism® RP material (Keystone Scientific) enabled the analytes to be separated using a moderately strong mobile phase containing 45% acetonitrile. This stationary phase is claimed by the manufacturer to have polar groups incorporated into the bonded phase, which results in increased retention for ionic analytes. The separation on this column could be optimized through slight changes in mobile phase pH, thus confirming the importance of the interaction between the ionized pyridine group and the stationary phase. Based on these results, the column packed with Prism® RP material was chosen to be used for the analysis of **I** in the biofluid samples.

*3.3. Development of procedures to extract **I** from biofluids*

Development of suitable procedures to extract **I** and **II** from biofluid samples focused on the use of solid-phase extraction (SPE). This approach was chosen because we desired to take advantage of 96-well SPE technology [4–9] in order to ensure maximum sample throughput. Extraction plates containing Empore® C_8 and C_{18} disks as well as plates containing Waters Oasis® packing were investigated for the preparation of plasma samples. Best results in term of recovery and fewest endogenous peaks were obtained using an Empore® C_8 disk plate to extract plasma samples. Processing the wash and elution steps via centrifugation as opposed to vacuum was found to improve assay consistency and eliminate cross sample contamination.

A major advantage of this extraction procedure was the ability to elute the extraction disks with a relatively small volume of solvent. The elution solvent could be diluted and injected directly into the HPLC without the need for an evaporation–reconstitution step. However, during the injection of these samples, a gradual increase in system back pressure and deterioration in the analyte peak shape was observed. Filtration of the samples through a 96-well filter plate was found to eliminate the pressure increase and peak shape deterioration.

Attempts to use the plasma extraction procedure to process human urine samples failed due to the presence of endogenous peaks in the resulting chro-

matograms. Urine samples were thus processed using an SPE plate containing mixed cation-exchange–reversed-phase extraction disk (Empore® Cation/C_8, MPC). Under acidic conditions, compound **I** was retained on the MPC plate through an ion-exchange mechanism. The plate could then be washed with 100% methanol in order to elute non-ionic endogenous species from the plate. The analytes were then eluted from the plate using a mixture (98/2,v/v, %) of methylene chloride–2 *M* ammonia in methanol.

At least 1 ml of elution solvent was required to obtain satisfactory recoveries. In contrast with the plasma assay, these extracts needed to be evaporated and reconstituted prior to injection.

3.4. Assay automation

The initial assay development and validation work was performed manually using an adjustable eight-channel pipette. Following this work, manual assay steps were automated using a combination of a Packard Multi-Probe® liquid-handling system and TomTec Quadra 96 SPE workstation.

The Packard Multi-Probe® liquid-handling system was used to transfer samples from regular individual test tubes into a 96 deep well plate. The probes used for samples transfer were washed with 1.2 ml of a solution of 50/50 acetonitrile–water in order to eliminate cross sample contamination.

The TomTec Quadra 96 SPE workstation was used to perform the plate conditioning and sample load steps of SPE procedure. In addition, the system was used to load the wash and elution solvent onto the SPE plate, prior to the centrifugation steps.

During automation of the assays using TomTec 96 SPE Quadra workstation we found that it was important to adjust the height of tips so as to avoid sample splash, which would have the potential of causing cross-sample contamination.

3.5. Assay selectivity and specificity

Fig. 4 shows chromatograms of extracted control plasma, a plasma standard containing **I** (10 ng/ml, t_r=5.2 min) and **II** (40 ng/ml, t_r=6.9 min) and a plasma sample taken from a subject 48 h after receiving a 25 mg oral dose of **I**. Fig. 5 shows chromatograms of extracted control urine, a urine

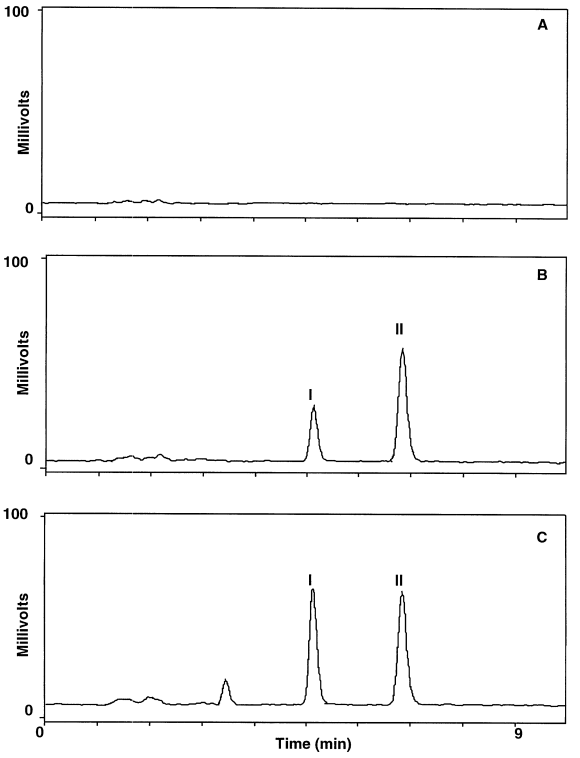


Fig. 4. Representative chromatograms of plasma samples. (A) Control human plasma, (B) Plasma spiked with 10 ng/ml **I** and internal standard **II** (40 ng/ml), (C) Plasma sample from a human subject at 48 h after oral administration of 25 mg **I**. The concentration of **I** is equivalent to 45.9 ng/ml.

standard containing **I** (10 ng/ml) and **II** (40 ng/ml) and a urine sample collected between 0 and 4 h following the administration of 500 mg oral dose of **I**. The 500 mg oral dose was the only dose level for which urine samples were analyzed. A comparison of Fig. 3A with Fig. 3B and Fig. 4A with Fig. 4B illustrates that no endogenous peaks co-elute with either the analyte or internal standard. The selectivity of the assays was further confirmed by the fact that pre-dose plasma and urine samples from subjects involved in clinical trials were free of interfering peaks.

The specificity of the assay is illustrated by the fact that metabolites of **I** were found to elute at significantly earlier retention times (t_r<4 min, Figs. 4C and 5C) and did not interfere with the quantitation of parent compound.

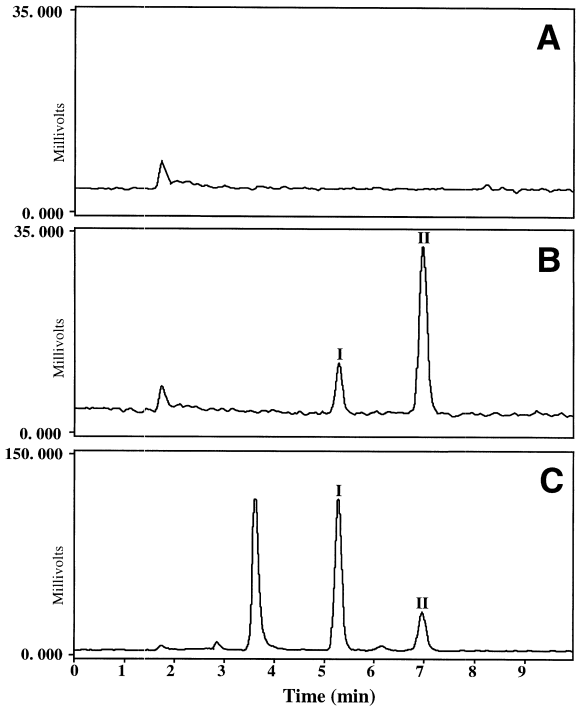


Fig. 5. Representative chromatograms of urine samples. (A) Control human urine, (B) Urine spiked with 10 ng/ml **I** and internal standard **II** (40 ng/ml), (C) 0–4 h urine collection from a human subject obtained after oral administration of 500 mg **I**. Sample diluted 1:10 prior to the addition of internal standard **II** at a concentration of 40 ng/ml. The concentration of **I** is equivalent to 1549 ng/ml.

3.6. Assay linearity

Weighted (weighting factor=$1/y$, where y=peak height ratio) least-squares regression calibration curves, constructed by plotting the peak height ratio of **I**/**II** vs. nominal standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration ranges of the plasma and urine assays. The mean (n=7) slope of the calibration curve was 0.0358 with a coefficient of variation of 5.8%. The use of the weighted least-squares regression resulted in less than a 7% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equation.

3.7. Extraction recovery

The recovery of the extraction procedures was determined by comparing the responses (peak area) of the working standards of **I** and **II** injected directly into the HPLC system with those of extracted plasma and urine standards. The results (Table 1) indicate that the recovery of **I** from human plasma was greater than 93% at all concentrations tested. The recovery of **I** from human urine averaged 71%. Recovery of internal standard (**II**) from human plasma was greater than 97%, while for urine it was 73.5% at a concentration of 40 ng/ml.

3.8. Assay precision and accuracy

Replicate (n=5 at each concentration) standards at concentrations of 5, 10, 20, 50, 100, 200, 400 and 500 ng/ml in plasma or urine prepared from the working solutions were analyzed to assess the within-day variability of the assays. The mean assayed concentrations as well as the mean accuracy and relative standard deviations (RSDs) of the analyses, obtained using the manual and semi-automated assays, are shown in Table 2 for human plasma and human urine. The mean RSDs for the manual and semi-automated methods were 1.2% and 2.2%, respectively. The difference in precision between the manual and semi-automated procedures was not considered scientifically meaningful.

3.9. Quality control samples

Quality control samples (QCs), containing concentrations of 15 and 375 ng/ml of **I** in plasma and

Table 1
Extraction recovery of **I** from human plasma and human urine

Concentration (ng/ml)	Mean (n=3) recovery (%)[a]	
	Plasma	Urine
5	108.2 (5.0)	66.9 (8.0)
50	98.2 (5.2)	77.8 (2.9)
500	97.7 (7.5)	67.7 (3.0)

[a] Values in parentheses are RSDs.

Table 2
Intraday precision and accuracy of the assays as assessed by the replicate ($n=5$) analysis of standards

Nominal standard concentration (ng/ml)	Plasma						Urine		
	Manual			Semi-automated			Mean ($n=5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	RSD (%)
	Mean ($n=5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	RSD (%)	Mean ($n=5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	RSD (%)			
5	5.4	108.4	1.6	5.0	100.5	3.6	5.2	103.7	6.2
10	10.1	101.3	1.8	10.1	101.2	4.6	9.8	98.2	2.4
20	19.4	97.2	1.1	19.7	98.4	1.7	19.8	99.0	2.4
50	48.0	96.1	0.9	50.3	100.5	0.5	50.5	101.0	1.1
100	97.2	97.2	0.9	99.9	99.9	2.0	98.7	98.7	1.9
200	195.3	97.7	0.3	199.9	100.0	1.1	200.6	100.3	1.2
400	405.4	101.4	1.3	395.7	98.9	2.0	394.5	98.6	1.0
500	504.6	100.9	1.4	504.8	101.0	2.3	506.2	101.2	1.5

[a] Calculated as (mean analyzed concentration/nominal concentration)×100.

urine were prepared and frozen (−20°C) in 1-ml volumes. It was found that quality control human plasma and urine samples were stable through three freeze–thaw cycles. The quality control samples were analyzed each day along with human clinical samples from clinical studies to assess the inter-day variability of the assay. Representative plasma quality control sample data acquired over a 1-month period during the analysis of clinical samples is shown in Table 3. Results from the analysis of human urine quality control samples are also shown in Table 3. Based on long-term analysis of quality control samples (data not shown), **I** was found to be stable in human plasma and urine for at least 8 and 2 months, respectively.

4. Conclusion

High throughput semi-automated assays have been developed for determination of **I** in human plasma and urine. The Prism® RP column enabled the analytes to be separated under conditions that were optimal for post-column photochemical derivatization and eliminated the need for ion-pair reagents. Application of post-column photochemical derivati-

Table 3
Inter-day variability of the assays for determination of **I** as assessed by RSDs of low and high quality control (Q.C.) samples

Assay	Nominal Q.C. concentration (ng/ml)	Mean analyzed	RSD (%)
Plasma Assay	15	15.2	2.0[a]
	375	371.8	4.2[a]
Urine Assay	15	13.9	7.8[b]
	375	358.6	6.3[b]

[a] $n=11$ runs over a 1-month period.
[b] $n=4$ runs over a 1-week period.

zation technique made it possible to quantitate **I** at low ng/ml concentrations in plasma and urine. The application of 96-well SPE technology and automation workstations greatly improved the assay sample through put, at least by 2-fold, compared with conventional SPE using discrete columns. The assays have been found to be precise, accurate and suitable for the analysis of plasma and urine samples collected during clinical studies.

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