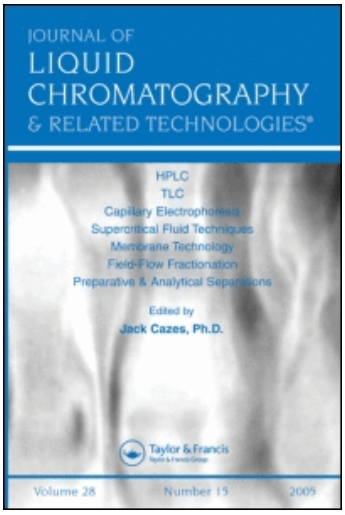

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SAMPLE PREPARATION VIA SOLID PHASE EXTRACTION IN THE 96-WELL FORMAT FOR HPLC/UV DETECTION-BASED BIOFLUID ASSAYS. APPLICATION TO THE DETERMINATION OF A NOVEL CYCLOOXYGENASE II INHIBITOR IN HUMAN PLASMA AND URINE

R. S. Mazenko[a]; A. Skarbek[a]; E. J. Woolf[a]; R. C. Simpson[a]; B. K. Matuszewski[a]
[a] Department of Drug Metabolism, Merck Research Laboratories, West Point, PA, U.S.A.

Online publication date: 31 October 2001

J. LIQ. CHROM. & REL. TECHNOL., 24(17), 2601–2614 (2001)

SAMPLE PREPARATION VIA SOLID PHASE EXTRACTION IN THE 96-WELL FORMAT FOR HPLC/UV DETECTION-BASED BIOFLUID ASSAYS. APPLICATION TO THE DETERMINATION OF A NOVEL CYCLOOXYGENASE II INHIBITOR IN HUMAN PLASMA AND URINE

R. S. Mazenko, A. Skarbek, E. J. Woolf,* R. C. Simpson, and B. K. Matuszewski

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

ABSTRACT

Methods using solid phase extraction in the 96-well format, together with HPLC and UV detection, are described for the determination of low ng/mL concentrations of 2-(3,5-difluorophenyl)-3-(4-methanesulfonylphenyl)-cyclo-pent-2-enone, a cyclooxygenase II inhibitor, in human plasma and urine. The plasma assay utilizes a packed-bed SPE plate, while the urine assay employs a 96 well plate containing membrane-based extraction disks. Both matrices are diluted with 17% acetonitrile in water prior to application to the extraction plates.

Following sample application, the plates are washed with acetonitrile/water mixtures to remove endogenous components prior

* Corresponding author. E-mail: woolf@merck.com

to elution of the analytes with 100% acetonitrile. The urine extracts are diluted with water and injected directly into the HPLC system. The plasma extracts, however, require evaporation and reconstitution prior to injection. An HPLC/column switching system is employed to separate the analyte and an internal standard from co-extracted endogenous species.

The compounds are detected based on their UV absorbance at 288 nm. The 96-well SPE assays were validated over concentration ranges of 5 to 500 ng/mL for plasma (1 mL sample) and 10 to 1000 ng/mL for urine (0.5 mL sample). Replicate analyses (n=5) of spiked plasma and urine standards yielded linear responses with coefficients of variation below 10% and accuracy within 6% of nominal concentrations.

INTRODUCTION

Solid phase extraction (SPE) in the 96-well format has, in recent years, become a method of choice in many laboratories to prepare biological fluid samples for analysis.(1) Numerous examples of assays utilizing this technology have appeared in the literature.(2-5) The published methods give the impression, however, that this sample preparation methodology is useful primarily to prepare samples for analysis via HPLC in combination with mass spectrometric (MS) detection; only a small percentage of the published methods utilize other detection methodologies.(6-10) Furthermore, all methods that have been published to date that use UV detection in combination with 96-well SPE, have limits of quantitation that are greater than 100 ng/mL.

In contrast to the literature, we have applied 96-well SPE to numerous assays utilizing both UV and fluorescence based detection methods. An assay with low ng/mL limits of quantitation for the determination of the cycloxygenase II inhibitor, 2-(3,5-difluorophenyl)-3-(4-methanesulfonyl-phenyl)-cyclopent-2-enone (Compound I, Figure 1) in human plasma and urine, using 96 well SPE together with HPLC, column switching, and UV detection is presented here to illustrate the applicability of 96-well SPE to highly sensitive, non-MS based assays.

EXPERIMENTAL

Materials

Compounds **I** and internal standards, **II** (plasma assay), and **III** (urine assay) were obtained from the Medicinal Chemistry Department of Merck

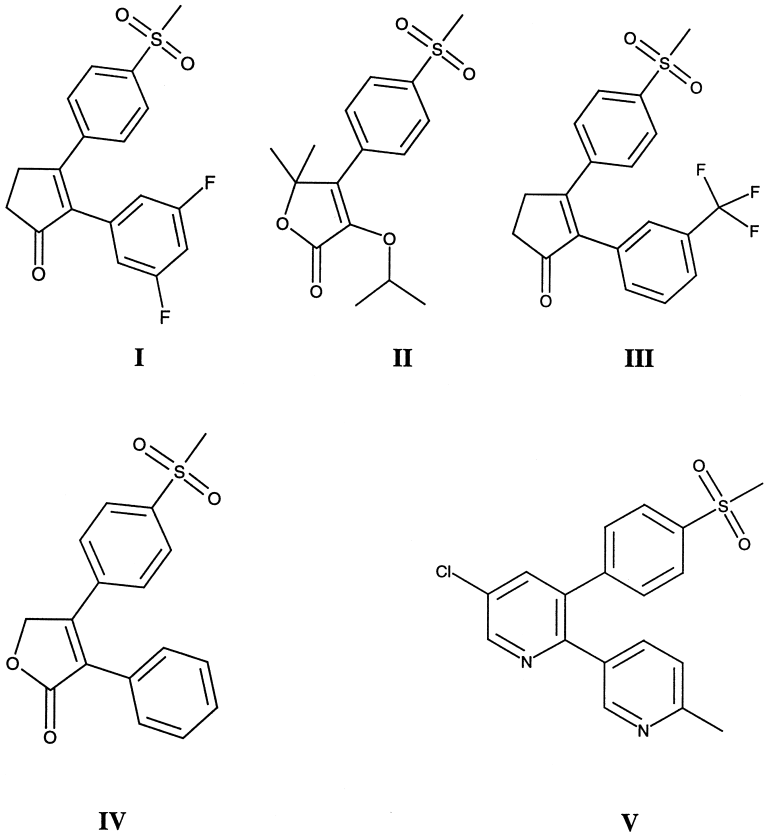

Figure 1. Chemical structures of Compounds **I–V.**

Frosst Canada, Inc. (Kirkland, Quebec, Canada). Each of the compounds was reported to be greater than 98% pure. All other chemicals were of research grade and were purchased from EM Science (Gibbstown, NJ, USA) or Fisher Scientific (Fair Lawn, NJ, USA). Drug free heparinized human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). Drug free control urine was obtained from healthy donors within the Department of Drug Metabolism of Merck Research Laboratories (West Point, PA, USA).

OASIS® 96-well solid phase extraction plates were purchased from Waters Associates (Milford, MA, USA). Empore™ Standard Density (SD) C8 96-well solid phase extraction plates were purchased from 3M Corporation (St. Paul, MN, USA).

Instrumentation

The HPLC system consisted of two Perkin-Elmer (Norwalk, CT, USA) model 410 pumps, a WISP 717 + autosampler (Waters Assoc., Milford, MA, USA), an electrically activated, 2-position, 10-port switching valve (Valco Instruments, Houston, TX, USA) and a Perkin-Elmer/Applied Biosystems 785A UV/VIS detector. The detector output was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 900 series interface. The switching valve was controlled via contact closures from the data system interface. A block diagram of the HPLC system is shown in Figure 2.

UV spectra were obtained using a Hewlett Packard (Palo Alto, CA, USA) 8452A diode array spectrophotometer. A Hitachi (San Jose, CA, USA) F-4500 fluorescence spectrophotometer was used to obtain fluorescence spectra of the analytes.

Chromatographic Conditions

Plasma Analysis

The mobile phase for both pumps consisted of a mixture of 34% acetonitrile in water. Both pumps were set to deliver the mobile phase at a flow rate of 1.2 mL/min. Column 1 was a BDS Hypersil Phenyl column (50 X 4.6 mm, 5 μm; Keystone Scientific, Bellefonte, PA, USA). Column 2 was a 50 x 4.6 mm column packed with Zorbax XDB C8 material (5 μm; MAC-MOD Analytical, Chadds Ford, PA, USA). Samples were injected (100 μL) while the valve was in Position A (Figure 2). The valve switched from Position A to Position B (Figure 2) at 2.8 minutes following injection. Following the transfer of the analytes from column 1 to column 2, the valve was switched back to its original position at 3.7 minutes following injection. The total run time for each sample was 12 minutes. The analytes were detected based on their UV absorbance at 288 nm.

Urine Analysis

The mobile phase for Pump 1 consisted of a mixture of 35% acetonitrile in water, while a mobile phase composed of 40% acetonitrile in water, was delivered by Pump 2. Flow rates of 1.0 and 1.2 mL/min were used for Pumps 1 and 2, respectively. Column 1 was a 50 x 4.6 mm Hypersil BDS phenyl column (5 μm, Keystone Scientific). Column 2 (150 x 4.6 mm) was packed with Prism RP

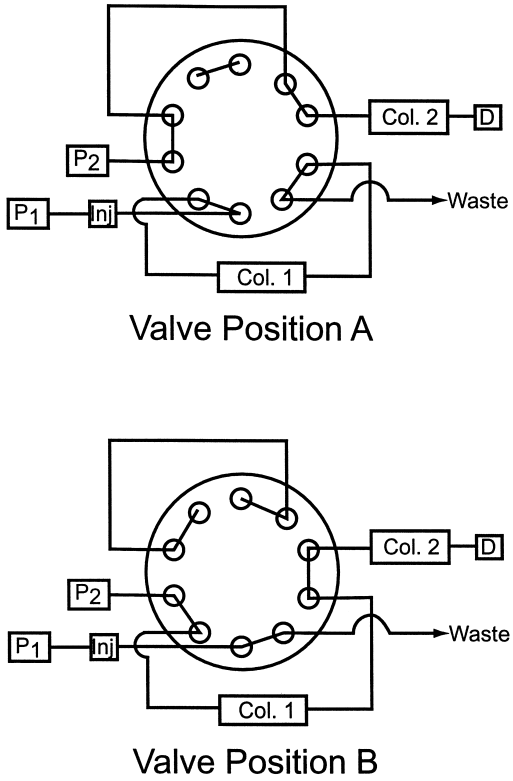

Figure 2. Block diagram of HPLC column switching system**.**

material (5 μm, Keystone Scientific). Samples were injected (50 μL) while the valve was in Position A (Figure 2); interfering endogenous compounds were directed to waste, while the analyte and internal standard were retained on column 1. After 3.15 minutes, the valve was switched to Position B; Column 1 was switched in-line with Column 2, and the mobile phase from Pump 2 was delivered to the coupled columns for elution and subsequent UV detection (288 nm) of the analyte and internal standard.

Preparation of Standards

A 1 mg/mL stock solution of **I** was prepared by dissolving the reference standard material in acetonitrile. Additional stock solutions of **I** at concentrations

of 100, 10, and 1 μg/mL were prepared by serial dilution of the 1 mg/mL solution with acetonitrile. Working standards at concentrations of 20, 15, 10, and 5 μg/mL were prepared by dilution of the 100 μg/mL stock solution. Working standards of 2, 1, and 0.5 μg/mL were prepared by dilution of the 10 μg/mL stock solution. Working standards of 0.2 and 0.1 μg/mL were prepared by dilution of the 1 μg/mL stock solution.

Plasma standards were prepared by adding 50 μL of each of the 10, 5, 2, 1, 0.5, 0.2, and 0.1 μg/mL working standards of **I** to 1 mL of drug-free plasma contained within a disposable polypropylene tube (13 x 85 mm). The resulting standards were used to quantitate plasma samples ranging in concentration from 5 to 500 ng/mL.

Urine standards were prepared by adding 25 μL of each of the 20, 15, 10, 5, 2, 1, 0.5, and 0.2 μg/mL working standards of **I** to 0.5 mL of drug-free urine. The resulting standards were used to quantitate urine samples ranging in concentration from 10 to 1000 ng/mL.

Working standard solutions were found to be stable at room temperature for at least 2 weeks when stored in light protected volumetric flasks.

Plasma Extraction Procedure

One mL aliquots of plasma samples were pipetted into disposable polypropylene tubes (13 x 85 mm). A 50 μL aliquot of acetonitrile was added to each of the samples. To the tubes containing the samples and standards (prepared as described previously), was added a 50 μL aliquot of a 1 μg/mL acetonitrile solution of **II** as internal standard. The contents of the tubes were vigorously vortexed for 30 seconds. A 1 mL aliquot of 17 % acetonitrile in water was added to the tubes, followed by 30 seconds of further vigorous vortexing. The tubes were then centrifuged at 2050 x g for 10 minutes at 15°C.

The samples and standards were extracted using a 96 well OASIS® SPE plate. The SPE plate was first conditioned by aspirating 1 mL of methanol through the wells, followed by 1 mL of water; low vacuum pressure (< 5 inches Hg) was used for the conditioning steps. A 1.8 mL aliquot of each of the samples to be extracted was transferred to a well on the SPE plate. The plasma was aspirated through the extraction bed using moderate vacuum pressure (10-12 inches Hg). After aspirating 2 mL of 20 % acetonitrile in water through each well, the analyte and internal standard were eluted by aspiration of 0.50 mL of 100 % acetonitrile. Elutions were carried out under low vacuum pressure into deep well (2 mL well volume) polypropylene collection plates. The eluents were evaporated under a stream of heated (40°C) nitrogen using a 96-well evaporator. The extracts were each reconstituted in 250 μL of 25% acetonitrile in water. To reduce backpressure buildup on the HPLC system, the reconstituted samples were filtered

using a 96 well filter plate (0.45 μm nylon filter with 20 μm pre-filter, Applied Separations, Allentown, PA). The samples were transferred to autosampler vials, which were subsequently capped and made ready for injection into the HPLC system for analysis.

Urine Extraction Procedure

Aliquots (0.5 mL) of urine samples were pipetted into disposable polypropylene tubes (13 x 85 mm). A 25 μL aliquot of acetonitrile was added to each of the tubes. To the tubes containing the samples and the standards (prepared as described previously), was added a 25 μL aliquot of a 1 μg/mL acetonitrile solution of **III** as internal standard. The resulting solution was vigorously vortexed for 30 seconds. A 0.50 mL aliquot of 17% acetonitrile in water was added to each of the tubes, followed by an additional 30 seconds of vigorous vortexing.

The samples and standards were then extracted using a 96 well Empore® Standard Density C8 SPE plate. The SPE plate was first conditioned by aspirating 0.5 mL of methanol through the wells, followed by 0.5 mL of water; low vacuum pressure (< 5 inches Hg) was used for the conditioning steps. A 0.9 mL aliquot of each of the samples to be extracted was transferred to a well on the SPE plate. The diluted urine was aspirated through the extraction bed using moderate vacuum pressure (10-12 inches Hg). After aspirating 1 mL of 15% acetonitrile in water through each well, the analyte and internal standard were eluted with 100 μL of acetonitrile into a polypropylene collection plate (individual well volume = 1 mL). A 200 μL aliquot of water was added to each well, such that the solvent content of the extracted samples approximated that of the mobile phase. The samples were transferred to autosampler vials, which were subsequently capped and made ready for injection into the HPLC system for analysis.

RESULTS AND DISCUSSION

Spectroscopic Characterization of the Analyte

The spectral characteristics of **I** were examined in order to establish the best detection method for HPLC assays to support human clinical studies. The UV spectrum of **I** in acetonitrile:buffer (50:50, v/v%) showed an absorption maximum at 288 nm (ε = 21000 $M^{-1}cm^{-1}$). The position and intensity of this absorption band were not affected by changes in pH. Compound **I** was not found to exhibit significant native fluorescence.

Rofecoxib (**IV**) and etoricoxib (**V**) are cyclooxygenase 2 inhibitors that are structurally similiar to **I**.(11-13) From a photochemical standpoint, the major dif-

ference between these compounds is that **I** possesses an α,β unsaturated ketone moiety, whereas this group is replaced by either an α,β unsaturated lactone group or a 2,3,5 substituted pyridine ring in **IV** and **V**, respectively. Compounds **IV** and **V** have been been found to form highly fluorescent species on exposure to UV light, hence, we have previously described methods for their determination in human plasma using postcolumn photochemical derivatization, followed by fluorescence detection.(10,14) The fluorescent products of **IV** and **V** resulted from their photocyclization, followed by oxidation to form phenanthrene-like species.(10,14) The lowest excited singlet state of these compounds were apparently π–π* in nature, which is known to deactivate efficiently to the ground state through a radiative (ie., fluorescent) pathway.

In order to evaluate whether **I** behaved similarly, solutions of the compound were irradiated in a "merry-go-round" reactor equipped with four 254 nm mercury lamps. No change in the UV spectra of the solutions were observed following brief (30 second) irradiations. UV spectral changes were observed, however, following an irradiation for an extended period (15 minutes), indicating that **I** most likely undergoes photochemical reactions, similar to those described previously for **IV** and **V**, albeit at a slower rate. Although UV spectral changes were observed following extended irradiation, these solutions were not found to be significantly fluorescent. Assuming that **I** formed a photocyclization product, the lack of its fluorescence may indicate that the lowest excited singlet state of the product is n-π* in nature, due to the presence of the α,β unsaturated ketone moiety in the molecule. For such molecules, the radiative pathway of deactivation of the lowest excited singlet state, is insignificant in comparison with other nonradiative processes. Studies of the photochemistry of **I** and **III** and of the photophysical processes of the products of their photolysis, will be the subject of more detailed studies. Based on the results of these experiments, assays utilizing UV detection at 288 nm were developed for the determination of **I** in plasma and urine.

Development of the Extraction Procedure

Solid phase extraction in the 96 well format, due to its high throughput nature, is currently the method of choice in our laboratories to prepare biological fluid samples for analysis. Thus, we desired to take advantage of this technique to prepare plasma and urine samples for the analysis of **I**.

The desire to achieve a limit of quantitation of 5 ng/mL for the plasma assay, coupled with the need to use UV detection, necessitated that a full 1 mL of plasma be extracted. This requirement limited the type of extraction plate that could be utilized to those containing packed beds; attempts to pass this volume of plasma through Empore® membrane based extraction plates resulted in well plug-

ging. Plates containing both silica and polymeric based sorbents were evaluated for the extraction of plasma samples. Best results in terms of analyte recovery and sample cleanliness were obtained using plates containing Oasis® polymeric sorbent (30 mg). The presence of endogenous interferences in the sample extract was significantly reduced by pre-diluting the sample with an acetonitrile/water mixture (17/83 v/v%), and washing the plate with a 2 mL aliquot of a 20% solution of acetonitrile in water following sample application. Analyte recovery was maximized by using a 500 μL aliquot of acetonitrile to elute **I** and internal standard (**II**) from the plate. In order to obtain an additional degree of sample preconcentration, the elution solvent was evaporated to dryness and the extracts were reconstituted in a reduced volume (250 μL) of solvent prior to injection into the HPLC system.

In contrast with the plasma assay, the target limit of quantitation for the urine assay was 10 ng/mL. The less demanding requirements of the urine assay could be met through the extraction of a sample size of 0.5 mL, thus permitting the use of Empore® membrane based extraction plates, as this sample volume did not cause the wells of this type of plate to plug. The major advantage of using the membrane based plate for the urine assay was the ability to elute the analytes with only a 100 μL aliquot of acetonitrile; thus, urine extracts were diluted with water to approximate the solvent strength of the mobile phase and injected directly into the HPLC system, without the need for evaporation and reconstitution steps.

Chromatographic System Development

Although the plasma and urine extraction procedures were optimized with respect to wash steps to eliminate the majority of interfering endogenous species present in the samples, low levels of interferences were found to be present in the sample extracts. The interferences could not be chromatographically resolved from the analytes through either mobile phase or column manipulations.

An observation was made that the pattern of the endogenous peaks appeared different on a phenyl column, as compared to a C_{18} column. The peaks that interfered with the detection of the analytes on the phenyl column apparently were different than those that interfered on the C_{18} column. A column switching system was, thus, employed to take advantage of the different selectivities of the columns and, thus, resolve the interferences from the peaks of the analytes of interest.

Assay Selectivity

Figure 3 shows chromatograms of extracted drug-free plasma and a plasma sample containing 5 ng/mL **I** and 50 ng/mL **II** (internal standard). Figure 4

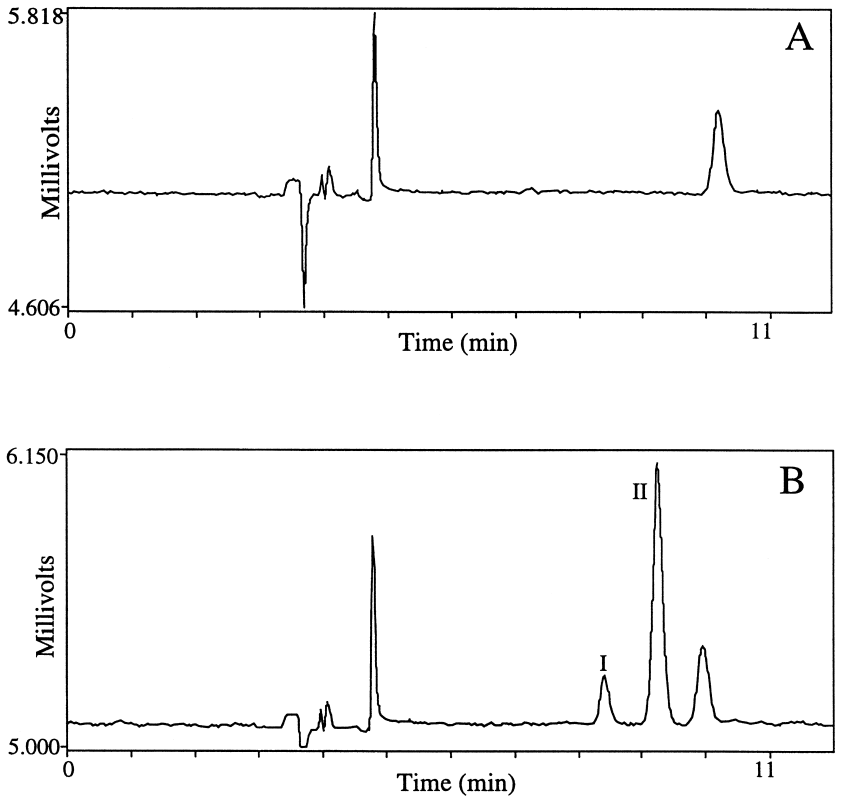

Figure 3. Representative chromatograms of human plasma. A) chromtogram of control plasma, B) chromatogram of a sample containing 5 ng/mL **I** and 50 ng/mL **II**.

shows chromatograms of extracted drug-free urine and a urine sample containing 10 ng/mL **I** and 50 ng/mL **III** (internal standard). A comparison of the chromatograms of the drug-free extracts to those containing **I** at the quantitation limit of the assays, along with internal standard, show no interfering peaks under the conditions of the assays.

Linearity

Weighted (weighting factor = *1/y* , where *y* = peak height) least-squares regression calibration curves, constructed by plotting the standard concentration

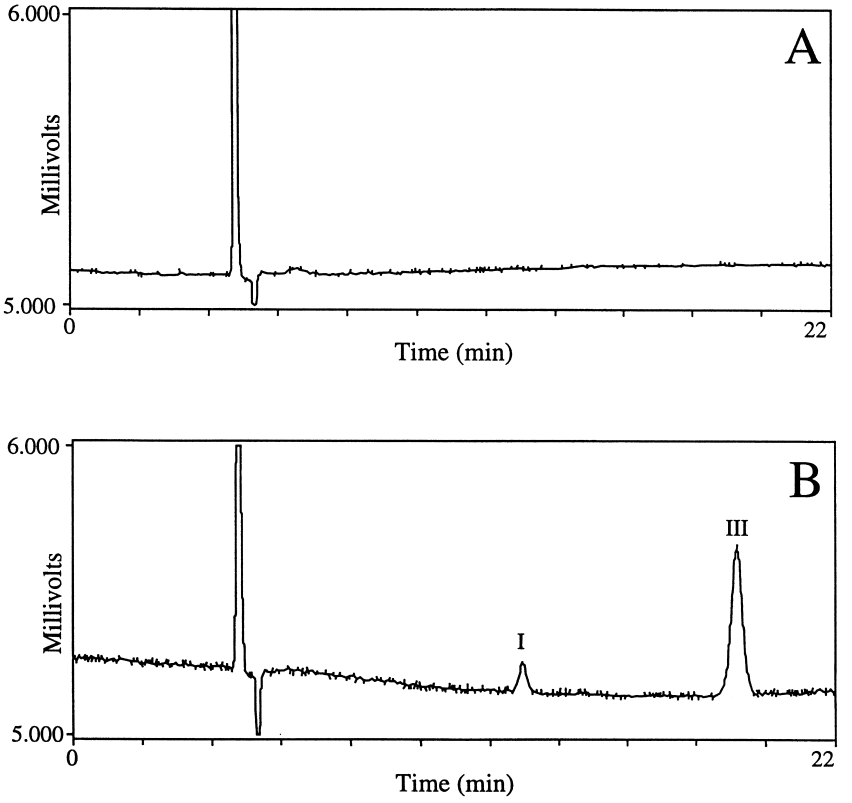

Figure 4. Representative chromatograms of human urine. A) chromatogram of control urine, B) chromatogram of a sample containing 10 ng/mL **I** and 50 ng/mL **III**.

of **I** versus the peak height ratio of analyte to internal standard, yielded coefficients of regression typically greater than 0.999 over concentrations of 5-500 ng/mL, and 10-1000 ng/mL for the plasma and urine standard curves, respectively.

The use of a weighted least-squares regression analysis generally resulted in less than a 10% deviation between the nominal standard concentration and the experimentally determined standard concentration calculated from the regression equation.

Extraction Recovery

The recovery of the extraction method was determined by comparing the mean responses of **I** dissolved in mobile phase and injected directly into the HPLC system, with those of extracted plasma or urine standard replicates (n=5) at each concentration on the standard curves. Each replicate, at a given concentration, was prepared in control plasma or urine from a different donor. The mean recovery of **I** from plasma over the concentration range of 5-500 ng/mL was 97.7% (± 2.6 %). The mean recovery of **I** from urine over the concentration range of 10-1000 ng/mL was 102.2% (± 3.4%). The mean recoveries of **II** and **III** at their working concentrations were 94.9% (± 1.7 %), and 100.6 (± 3.4%) for **II** in plasma and **III** in urine, respectively.

Assay Precision and Accuracy

Replicate standards (n=5) were analyzed to assess the within-day variability of the plasma and urine assays. The mean accuracy of the assayed concentration, as well as the coefficient of variation (C.V.) of the replicates for plasma and urine, are shown in Tables 1 and 2, respectively.

Sample Stability

Samples containing **I** in plasma were prepared at concentrations of 13.5 and 375 ng/mL and frozen at –20°C. The samples were analyzed after 1, 2, and 3

Table 1. Within-Day Accuracy and Precision Data for the Analysis of I in Plasma

Nominal Concentration (ng/mL)	Determined Concentration (ng/mL)	Precision (% C.V.)[a]	Accuracy (%)[b]
5	5.2	4.5	104.3
10	9.9	2.0	99.5
25	25.0	1.2	100.1
50	48.4	0.8	96.7
100	100.1	0.4	100.1
250	246.9	1.1	98.8
500	504.3	0.8	100.9

[a]Calculated as: [(standard deviation)/(mean determined value)] x 100.
[b]Calculated as: [(mean determined concentration)/(nominal concentration)] x 100.

Table 2. Within-Day Accuracy and Precision Data for the Analysis of I in Urine

Nominal Concentration I (ng/mL)	Determined Concentration I (ng/mL)	Precision (% C.V.)[a]	Accuracy (%)[b]
10	9.5	7.6	94.6
25	25.7	2.4	103.0
50	50.8	2.5	101.6
100	100.3	2.6	100.3
250	254.1	2.3	101.6
500	496.7	3.3	99.3
750	763.1	2.9	101.8
1000	986.6	3.6	98.7

[a]Calculated as: [(standard deviation)/(mean determined value)] x 100.
[b]Calculated as: [(mean determined concentration)/(nominal concentration)] x 100.

freeze-thaw cycles, in order to determine the stability of the analyte towards repetitive freezing and thawing. Following a single freeze-thaw cycle, the analyzed concentrations of these samples were found to be within 5% of their nominal concentrations. Additional freeze-thaw cycles were not found to affect the results of the analysis, indicating that **I** in plasma is stable toward repetitive sample freezing and thawing. Urine samples prepared to contain I at concentrations of 35 and 800 ng/mL were similarly evaluated. Concentrations of **I** in urine were found to be within 5% of nominal values and were not affected by additional freeze-thaw cycles.

CONCLUSIONS

Solid phase extraction in the 96-well format, together with HPLC with UV detection, has been successfully utilized for the determination of low ng/mL concentrations of **I** in human plasma and urine. The methods have been found to be precise, accurate, and suitable for the analysis of plasma and urine sample collected during human clinical studies

REFERENCES

1. Wells, D.A. LC-GC **1999**, *17*, 600-610.
2. Hempenius, J.; Steenvoorden, R.J.J.M.; Lagerwerf, F.M.; Wieling, J.; Jonkman, J.H.G. J. Pharm. Biomed. Anal. **1999**, *20*, 889-898.

3. Rule, G.; Henion, J. J. Amer. Soc. Mass Spec. **1999**, *10*, 1322-1327.
4. Cenacchi, V.; Baratte, S.; Cicioni, P.; Frigerio, E.; Long, J.; James, C. J. Pharm. Biomed. Anal. **2000**, *22*, 451-460.
5. Rose, M.J.; Merschman, S.A.; Eisenhandler, R.; Woolf, E.J.; Yeh, K.C.; Lin, L.; Fang, W.; Hsieh, J.; Braun, M.P.; Gatto, G.J.; Matuszewski, B.K.; J. Pharm. Biomed. Anal. **2000**, *24*, 291-305.
6. Plumb, R.S.; Gray, R.D.M.; Jones, C.M. J. Chromatogr. B. **1997**, *694*, 123-133.
7. Harrison, A.C.; Walker, D.K. J. Pharm. Biomed. Anal. **1998**, *16*, 777-783.
8. Cheng, Y.F.; Neue, U.D.; Bean, L. J. Chromatogr. A.**1998**, *828*, 273-281.
9. Hempenius, J.; Wieling, J.; Brakenhoff, J.P.G.; Maris, F.A.; Jonkman, J.H.G. J. Chromatogr. B. **1998**, *714*, 361-368.
10. Matthews, C.Z.; Woolf, E.J.; Lin, L.; Fang, W.; Hsieh, J.; Ha, S.; Simpson, R.; Matuszewski, B.K. J. Chromatogr. B. **2001**, *751*, 237-246.
11. Prasit, P.; Wang, Z.; Brideau, C.; Chan, C.-C.; Charleson, S.; Cromlish, W.; Ethier, D.; Evans, J.F.; Ford-Hutchinson, A.W.; Gauthier, J.Y.; Gordon, R.; Guay, J.; Gresser, M.; Kargman, S.; Kennedy, B.; Leblanc, Y.; Leger, S.; Mancini, J.; O'Neill, G.P.; Ouellet, M.; Percival, M.D.; Perrier, H.; Riendeau, D.; Rodger, I.; Tagari, P.; Therien, M.; Vickers, P.; Wong, E.; Xu, L.-J.; Young, R.N.; Zamboni, R.; Boyce, S.; Rupniak, N.; Forrest, M.; Visco, D.; Patrick, D. Bioorganic Med. Chem. Lett. **1999**, *9*, 1773-1778.
12. Friesen, R.; Brideau, C.; Chan, C.; Charleson, S.; Deschenes, D.; Dube, D.; Ethier, D.; Fortin, R.; Gauthier, J.; Girard, Y.; Gordon, R.; Greig, G.; Riendeau, D.; Savoie, C.; Wang, Z.; Wong, E.; Visco, D.; Xu, L.; Young, R. Bioorganic Med. Chem. Lett. **1998**, *8*, 2777-2782.
13. Riendeau, D.; Percival, M.D.; Brideau, C.; Charleson, S.; Dube, D.; Ethier, D.; Falgueyret, J.-P.; Friesen, R.W.; Gordon, R.; Greig, G.; Guay, J.; Mancini, J.; Ouellet, M.; Wong, E.; Xu, L.; Boyce, S.; Visco, D.; Girard, Y.; Prasit, P.; Zamboni, R.; Rodger, I.W.; Gresser, M.; Ford-Hutchinson, A.W.; Young, R.N.; Chan, C.-C. J. Pharmacol. Expt. Therapeutics **2001**, *296*, 558-566.
14. Woolf, E.; Fu, I.; Matuszewski, B.K. J. Chromatogr. B. **1999**, *730*, 221-227.

Received April 20, 2001 Manuscript 5573
Accepted May 22, 2001