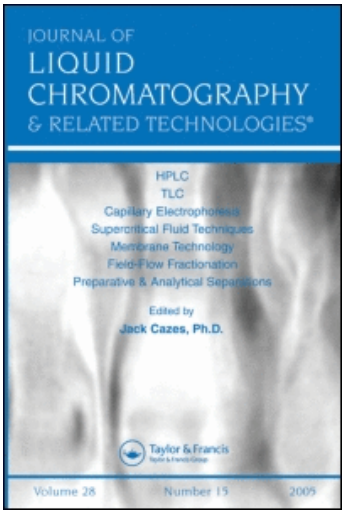


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The Analysis of Ro 24-4736 in Human Plasma by Multidimensional Reversed Phase Microbore HPLC/UV

T. E. Mulligan[a]; R. W. Blain[a]; N. F. Oldfield[a]; B. A. Mico[a]
[a] Department of Drug Metabolism and Pharmacokinetics, Hoffmann-La Roche, Inc., Nutley, New Jersey

To cite this Article Mulligan, T. E. , Blain, R. W. , Oldfield, N. F. and Mico, B. A.(1994) 'The Analysis of Ro 24-4736 in Human Plasma by Multidimensional Reversed Phase Microbore HPLC/UV', Journal of Liquid Chromatography & Related Technologies, 17: 1, 133 — 150
To link to this Article: DOI: 10.1080/10826079408013440
URL: http://dx.doi.org/10.1080/10826079408013440

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(1), 133-150 (1994)

THE ANALYSIS OF RO 24-4736 IN HUMAN PLASMA BY MULTIDIMENSIONAL REVERSED PHASE MICROBORE HPLC/UV

THOMAS E. MULLIGAN*, ROGER W. BLAIN,
NEIL F. OLDFIELD, AND BRUCE A. MICO
Department of Drug Metabolism and Pharmacokinetics
Hoffmann-La Roche, Inc.
340 Kingsland Street
Nutley, New Jersey 07110

ABSTRACT

A highly sensitive HPLC method for Ro 24-4736, 5-[3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f]=[1,2,4]triazolo[4,3-a][1,4]diazepin-2-y-1]-2-propynyl]=phenanthrydin-6(5H)-one, in human plasma was developed employing multidimensional reversed phase microbore HPLC. First dimension chromatography with a (3 cm x 2 mm i.d.) base deactivated C8 stationary phase column followed by second dimension chromatography on a diisopropyl octyl silane bonded phase HPLC column (15 cm x 1.0 mm i.d.) was used. Detection was by UV (239 nm, $\epsilon \approx 61{,}000$). Differences in the stationary bonding chemistry and pore size provide remarkable selectivity, yielding an assay method limited mainly by the inherent detector sensitivity. Differences in analyte retention caused by differences in the stationary phases allowed use of a weak mobile phase for the first dimension, focusing the analyte on the head of the second column. Use of smaller i.d. second dimension columns (2.0 mm and 1.0 mm) further concentrated the analyte and decreased the limit of quantitation to 0.05 ng/ml. Plasma concentration versus time profiles of Ro 24-4736 following oral doses in man were obtained using this method. The use of

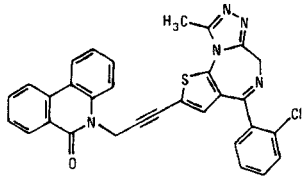
multidimensional chromatography with two reversed phase columns of different selectivity may provide a general technique for exploiting the advantages of microbore chromatography.

INTRODUCTION

Ro 24-4736 (I) (Figure 1) is a highly potent platelet aggregating factor receptor antagonist. A multidimensional reversed phase HPLC assay procedure with UV detection for the quantitation of I in human plasma using Ro 24-3729 (II) (Figure 1) as the internal standard has been developed.

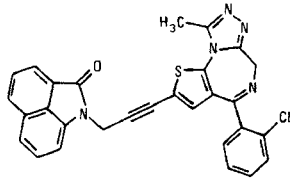
A very sensitive HPLC method for I in human plasma was needed to support drug development studies. As is so often the case, single column HPLC detection was limited not by inherent detector sensitivity, but rather by co-elution of endogenous components. A multidimensional HPLC method was developed (1,2), employing sample cleanup by liquid-liquid extraction (3-9) into hexane/methylene chloride. First dimension chromatography included a short base deactivated C8 HPLC column (3 cm x 2 mm i.d.), then second dimension chromatography on an RX-C8 microbore HPLC column (15 cm x 1 mm i.d.). Direct transfer, using a switching valve, was used as a means of transferring I and II from the first dimension column to the second dimension column. The effect of stationary phase chain length in the first dimension, mobile phase composition (1,2), column temperature, and the use of

I



Ro 24-4736

II



Ro 24-3729

Figure 1. Molecular Structures for Ro 24-4736 (I) and Ro 24-3729 (II).

conventional bore, narrowbore, and microbore columns in the second dimension were investigated. With UV detection at 239 nm, the limit of detection was 1 ng/ml using a 4.6 mm i.d. column, 0.1 ng/ml using a 2.0 mm i.d. column, and 0.05 ng/ml using a 1.0 mm column.

EXPERIMENTAL

Chemicals and Materials

Ro 24-4736 (I) was obtained from the Quality Control Department and Ro 24-3729 (II) was obtained from the Medicinal Chemistry Department, Hoffmann-La Roche, Inc., Nutley, NJ 07110 (U.S.A.). All solvents were HPLC grade

and obtained from Fisher Scientific, Fairlawn, NJ 07410. Ammonium acetate, ACS grade, was obtained from J.T. Baker Inc., Phillipsburg, NJ 08865. Distilled water was purified with a Milli-Q UF Plus water purification unit, Millipore Corp., Bedford, MA 01730. Human plasma was obtained from Rockland Inc., Gilbertsville, PA 19525.

Instrumentation

A multidimensional liquid chromatography system is used for this assay (Figure 2). The HPLC instrument consisted of a WISP 712 autosampler, Waters Div., Millipore Corp., Milford, MA 01757; two Beckman model 126 gradient HPLC pumps, Beckman Instruments, San Ramon, CA 94583; two SpectraFocus UV detectors, Spectra-Physics, San Jose, CA 95134; a Chrompak HPLC column oven, Chrompak Inc., Raritan, NJ 08869; and a Rheodyne model 7163-031 (120V AC) solenoid valve kit, Rheodyne Inc., Cotati, CA 94928. Data collection was carried out by a P.E. Nelson 3000 series chromatography software with a model 960 Intelligent Interface, Perkin-Elmer Systems Inc., Cupertino, CA 95014. A relay module consisting of a 5 volt power supply and two single pole double throw relays allowed contact closures on the A/D module to control the solenoid valves, and thereby change the position of the switching valve during the chromatographic run.

It is essential that the first dimension column be thermostated in a column oven to allow accurate

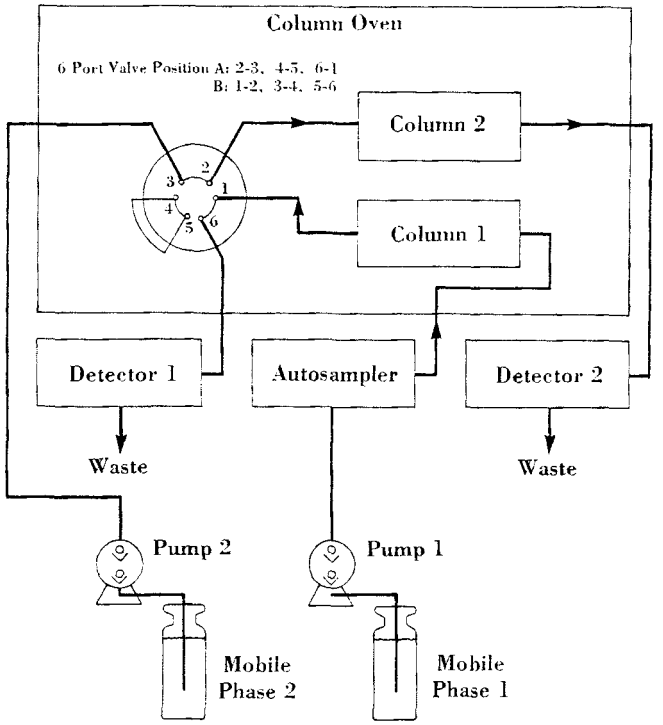


Figure 2. Schematic Representation of the Multidimensional HPLC System.

collection of the peaks of interest within the time window set by the analyst. The column switching valve and the second dimension column are also contained in the column oven.

The switching valve is activated using compressed air controlled through dual Rheodyne 120V AC air solenoids switched on and off by contact closures on the Nelson Analytical chromatography data system. Timed event commands in the chromatography data system method

control the contact closures. A switching valve can often be ordered as a built in feature on integrated instruments and controlled through the instrument data system.

Chromatographic Procedure and Conditions

The microbore chromatographic system employed a narrowbore reverse phase column (column 1), C8-BD, 3 cm x 2 mm i.d., ES Industries, Berlin, NJ 08009, and a microbore column (column 2), Zorbax RX-C8, packing from MacMod Inc., Chadds Ford, PA 19317, 15 cm x 1 mm, column packed by ES Industries.

The conventional bore assay used a 4 mm x 5 cm C8-BD column in the first dimension and a 4.6 mm x 15 cm RX-C8 column in the second dimension. The narrowbore assay utilized a 3 mm x 3 cm C8-BD column in the first dimension and a 2.1 mm x 15 cm RX-C8 column in the second dimension.

The mobile phase for the first dimension column of the microbore system was acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (40:60 v/v) (mobile phase 1) with a flow rate of 0.2 ml/min.; for the second dimension column, the mobile phase was acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (60:40 v/v) (mobile phase 2) with a flow rate of 0.05 ml/min. Flow rates for the narrow bore and conventional bore systems were scaled up based on the ratio of the square of radii for the

respective columns of the respective systems, relative to the column radii and flow rates of the microbore system. The HPLC columns were thermostated at 42 °C. Both detectors were set for UV detection at 239 nm.

With the six port valve in position A (Figure 2), the sample was injected onto the C8-BD column (column 1). Just before the analytes started to elute from column 1, the valve was timed to switch to position B. This allowed the direct transfer of the eluent from column 1 to column 2, the microbore reverse phase column, where the analytes were further separated from endogenous components. As soon as the analytes were completely transferred to column 2, the valve was switched back to position A. A synthetic mixture containing 20 ng of **I** and **II** in reconstitution solvent was injected before each set of samples to determine correct valve timing, and the data system timed events file controlling the relays was modified accordingly. Valve timing was set so as to minimize collection of the first dimension eluent before and after the elution of the peaks of interest.

Sample Preparation

Calibration standards were prepared in duplicate by adding 1.0 ml aliquots of heparinized human plasma, 50 µl aliquots of **I** spiking solutions and 50 µl of **II** (internal standard) working solution (in acetonitrile) to 16 x 100 mm disposable culture tubes. The tubes were then vortex

mixed. Standards contained 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/ml of **I** and 20 ng/ml of **II**. For experimental samples, 50 μl of **II** (internal standard) working solution and 50 μl of acetonitrile were added.

Sample Extraction

To each sample, 5.0 ml of hexane/methylene chloride (9:1 v/v) were added. The samples were vortex mixed vigorously for 15 minutes. They were then centrifuged for 5 minutes @ 1500 RPM @ 10 °C. The organic layer from each sample was transferred to a 16 x 100 mm disposable culture tube. The samples were then evaporated to dryness under a stream of nitrogen in a 40 °C water bath. Each sample was reconstituted with 50 μl of acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (25:75 v/v). These reconstituted samples were vortex mixed for 2 minutes on a multitube vortex mixer and transferred to autosampler vials with limited volume inserts, ready for injection.

Sample Analysis and Calculation

One each of the duplicate set of calibration standards was injected at the beginning and the end of each sample analysis run, bracketing the quality assurance standards, control blanks and experimental samples. From the peak height ratios of **I** to **II** (internal standard), a linear calibration curve was constructed. The slope and the intercept of the

calibration curve were calculated by using weighted ($1/y^2$) linear regression. Concentrations of **I** were calculated from the peak height ratio of samples with the use of the parameters obtained above.

Sample Stability

The stability of **I** in human plasma was evaluated at room temperature over four time periods. (**I**) was spiked into drug free human plasma, which had been collected in the presence of sodium heparin anticoagulant. Replicates of six samples were left on the benchtop for periods of 24, 6, 3, and 0 hours. They were then all analyzed simultaneously.

The stability of **I** in human plasma through three cycles of daily freezing and thawing was investigated. (**I**) was spiked into drug free human plasma which had been collected in the presence of sodium heparin anticoagulant. Each day, triplicate samples stored at -20 °C were removed and allowed to thaw and equilibrate at room temperature. Aliquots were then analyzed and the samples refrozen. The procedure was repeated on the second day. The control samples were only thawed once for analysis.

Recovery

Absolute recovery of **I** from human plasma was determined by comparing the mean of the peak heights from a set of six replicate extracted samples to a set of six

replicate unextracted samples. The extracted samples were prepared by spiking I into human plasma at a level of 4 ng/ml and extracting as described above. The unextracted samples were made by spiking I into extracted control blanks prior to reconstitution.

RESULTS AND DISCUSSION

Method Development

The initial, single dimensional chromatographic system failed to separate I from endogenous components. Co-elution of endogenous components with the analyte limited sensitivity to 25 ng/ml. With multidimensional chromatography, differences in stationary phase bonding chemistry and pore size provide remarkable selectivity, yielding an assay method limited mainly by the inherent detector sensitivity.

Variations of sample preparation and HPLC conditions were examined. Two sample preparations were investigated. An acetonitrile protein precipitation used in initial method development, which failed to provide adequate sample cleanup and resulted in practically no first dimension column life, was changed to the presently accepted liquid-liquid extraction. A multidimensional HPLC system was required to separate I from co-eluting endogenous peaks. The original multidimensional assay had a lower limit of quantitation of 1.0 ng/ml using

conventional bore columns in the first and second dimension. A more sensitive method was needed to measure plasma levels of **I** following low doses to support drug development studies. Multidimensional narrowbore and microbore HPLC/UV assays were developed with a lower limit of quantitation of 0.1 ng/ml and 0.05 ng/ml, respectively.

In each case, the analyte was preconcentrated on the head of the second column, since the first dimension mobile phase had a much lower organic content than the second dimension mobile phase. This resulted in peak compression, and therefore, increased sensitivity. Since the compressed peak gave very good resolution, it was possible to further enhance sensitivity through improved signal to noise by using a relatively long flow cell (6 mm, 9 μl) with a wide bore (1.4 mm).

Figure 3 shows first dimension chromtograms, matrix free synthetic mixture of **I** and **II**, and a sample human plasma injection. Figure 4 shows chromatograms of both spiked and experimental samples. A typical run time for plasma samples was 20 minutes. Typical retention times for **I** and **II** on column 2 were 12.9 and 11.5 minutes, respectively. Control blanks showed no interfering peaks from endogenous components of the plasma matrix, and the analyte peak at the low concentration limit was easily detectable. Baseline resolution of **I** and **II** at the high

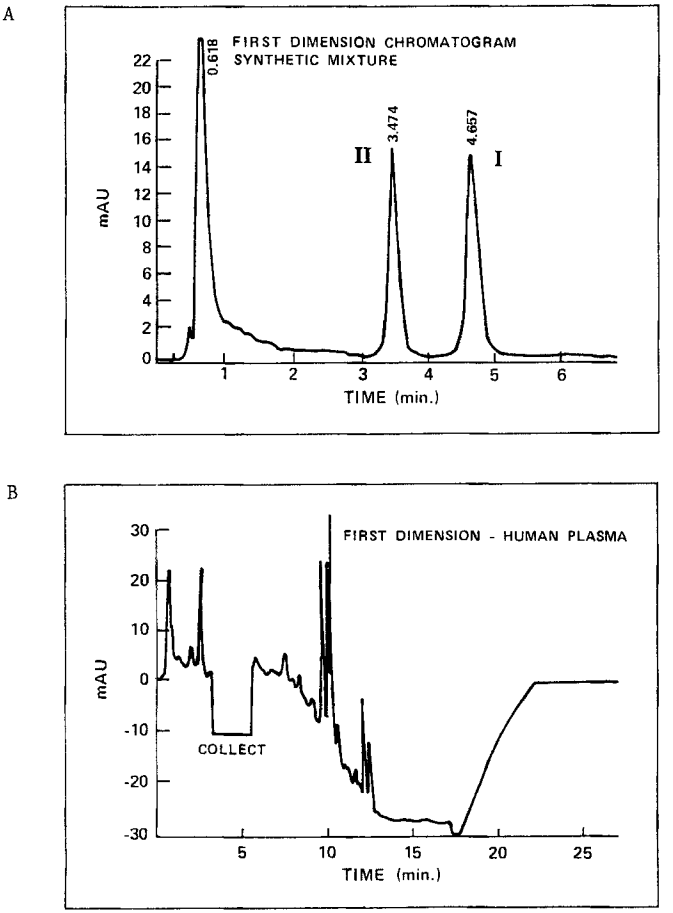


Figure 3. First Dimension Chromatograms: A) Synthetic Mixture of **I** and **II**; B) Human Plasma Injection Showing Collection of Analyte and Internal Standard into Sample Loop.

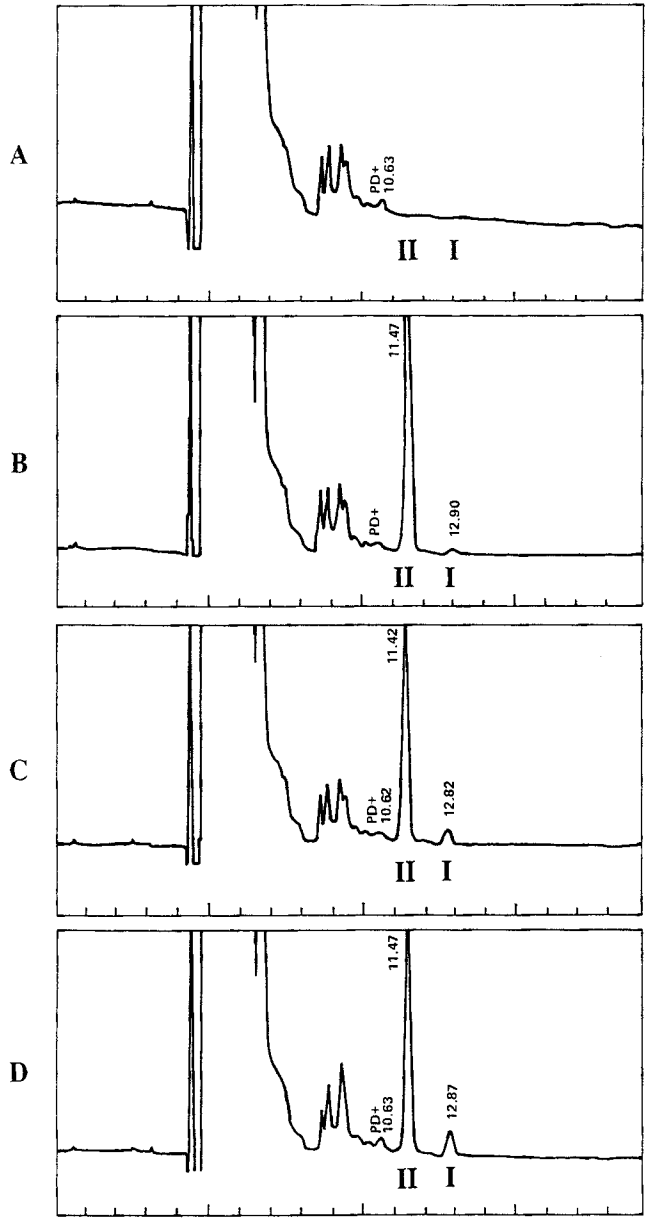


Figure 4. Typical Second Dimension Chromatograms of both Spiked and Experimental Samples: A) Human Plasma Blank; B) 0.1 ng/ml of **I** and 20 ng/ml of **II** in Human Plasma; C) 3.0 ng/ml of **I** and 20 ng/ml of **II**; D) Low Dose Experimental Sample.

concentration limit was still obtained. Operation of the column oven at 42 °C provided a stable temperature environment and high column efficiency through improved mass transfer.

Linearity

The response factor (analyte peak height ÷ [internal standard peak height x analyte concentration]) differed by less than 5% from the mean response factor for all calibration levels, indicating good linearity for the method.

Stability

The objective was to determine if I was stable under conditions in which plasma samples may be subject to during normal sample preparation. Analysis of plasma which had been allowed to stand for 0, 3, 6, and 24 hours at room temperature yielded mean ± S.D. values of 7.87 ng/ml ± 0.39 (n=6), 7.78 ng/ml ± 0.04 (n=6), 7.87 ng/ml ± 0.07 (n=6), and 7.72 ng/ml ± 0.07 (n=6), respectively. The mean concentration of I did not appear to change for as long as 24 hours under benchtop conditions. The percent difference from time zero to 24 hours is only 1.8%.

The stability of I to three cycles of daily freezing and thawing was intended to reproduce the conditions which plasma samples may be subject to if reanalysis was required. The difference between the first and third

cycle was -4.3%. (I) is stable in human plasma after three freeze/thaw cycles.

Recovery

Recovery of I was found to be acceptable at 79.3%. The recovery of II was not determined.

Precision

The inter-assay precision was estimated by determining the mean and the percent relative standard deviation for the values obtained for the high and low quality assurance samples on three separate days. The %R.S.D. for the low (0.35 ng/ml) quality assurance sample was 4.60%, while the %R.S.D. for the high (3.0 ng/ml) quality assurance sample was 8.15%. The overall %R.S.D. (average of %R.S.D.'s for the high and low standards) was 6.38%, indicating excellent day to day reproducibility.

Reliability

No problems in reliability were observed related to the increased complexity of the multidimensional system. Changing the precolumn filter element after every third tray was required to keep back pressure low on the first dimension system. No problems were encountered with the column switching valve. The first dimension column must be replaced when resolution between I and II is being reduced due to severe peak tailing. The first dimension column life is estimated to be approximately 250 injections. First dimension column life can be further

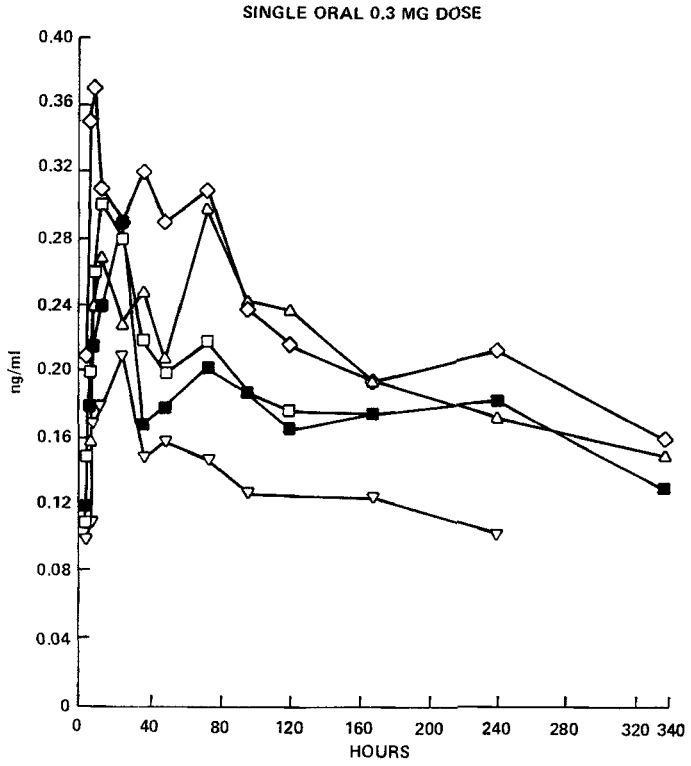


Figure 5. Plasma (I) Concentration vs. Time Profile: 0.3 mg Dose Given to Five Subjects.

extended by employing gradient elution after I and II have eluted from the column. This also prevents late eluting endogenous components from co-chromatographing with the analyte or internal standard in subsequent chromatographic runs.

Application to Experimental Samples

The plasma concentration versus time curves for 5 subjects given a 0.3 mg oral dose of compound I are shown in Figure 5.

CONCLUSIONS

A sensitive and reliable multidimensional HPLC/UV analytical method has been developed for the determination of **I** in human plasma. Liquid-liquid extraction of **I** and an internal standard, **II**, into hexane/methylene chloride (9:1 v/v) serves to separate the analytes from the bulk of the endogenous plasma components. The sample is injected onto the first dimension column containing a C8-BD packing. The portion of the eluent from this column containing **I** and **II** are transferred by switching a Rheodyne 7010P pneumatically actuated six port valve onto the second dimension reverse phase column, which separates **I** and **II** from endogenous components co-eluting with them on the first column. UV detection, using a wavelength of 239 nm, provides excellent sensitivity and selectivity. The high sensitivity of the method allows quantitation of **I** to 0.05 ng/ml using a 1.0 mm i.d. column; 0.1 ng/ml using a 2.0 mm i.d. column; and 1.0 ng/ml using a 4.6 mm column.

REFERENCES

[1] M.J. Koenigbauer and R.E. Majors, LC-GC. **8** (1990) 510.

[2] K.A. Ramsteiner, J. Chromatogr. **456** (1988) 3.

[3] H. Irth, R. Tocklu, K. Welten, G.J. DeJong, U.A. Th. Brinkman, and R.W. Frei, J. Chromatogr. **491** (1989) 321.

[4] A. Farjam, G.J. DeJong, R.W. Frei, U.A. Th. Brinkman, W. Haasnoot, A.R.M. Hamers, R. Schilt, and F.A. Huf, J. Chromatogr. **452** (1988) 419.

[5] J.V. Posluszny and R. Weinburger, Anal. Chem. **60** (1988) 1953.

[6] K. Matsumoto, S. Kanba, H. Kubo, G. Yagi, H. Iri, and H. Yuki, Clin. Chem. **35** (1989) 453.

[7] L.E. Mathes, G. Muschik, L. Demby, P. Polas, D. Weiss Mellini, H.J. Issaq, and R. Sams, J. Chromatogr. **432** (1988) 346.

[8] C.G. Kindberg, C.M. Riley, J.F. Stobaugh, and M. Slavik, J. Chromatogr. **473** (1989) 431.

[9] A. Wahlagen, L.-E. Edholm, C.E.M. Heeremans, R.A.M. Van Der Hoeven, W.M.A. Niessen, U.R. Tjaden, and J. Van Der Greef, J. Chromatogr. **474** (1989) 257.

Received: April 19, 1993
Accepted: May 19, 1993