ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1601–1608

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

An automated sample preparation and high performance liquid chromatographic method for the determination of MK-591, a novel leukotriene biosynthesis inhibitor, in human plasma

Charles Lin*, John Y.-K. Hsieh, Bogdan K. Matuszewski, Michael R. Dobrinska

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

Received for review 31 March 1995; revised manuscript received 18 December 1995

Abstract

A high performance liquid chromatography assay utilizing an automated sample preparation procedure for the determination of a novel leukotriene biosynthesis inhibitor, (MK-591), in human plasma has been developed. After aliquoting plasma samples and adding internal standard manually, the BenchMate™ Workstation executed protein precipitation and solid-phase extraction. Following evaporation to dryness, the residue was reconstituted and chromatographed isocratically on a cyano-phase analytical column. MK-591 and the internal standard were separated from each other and from endogenous plasma substances and detected with an absorbance detector. The assay has been validated in the concentration range 10–1000 ng ml^{-1} and has the sensitivity and specificity necessary to quantify plasma concentrations from several clinical studies.

Keywords: Automated sample preparation; BenchMate™ Workstation; HPLC; Leukotriene biosynthesis inhibitor; MK-591; plasma assay

1. Introduction

3-[1-(4-chlorobenzyl)-3-(*t*-butylthio)-5-(quinolin-2-yl methoxy)-indol-2-yl]-2,2-dimethylpropanoic acid (**I**, Fig. 1) is a potent leukotriene biosynthesis inhibitor that binds to the 5-lipoxygenase-activating protein [1–3], and thereby inhibits the translocation and activation of 5-lipoxygenase [4–6]. Inhibition of the biological effects of leukotrienes may provide therapeutic benefits in a number of hypersensitivity and inflammatory disorders. The therapeutic efficacy of compound (**I**) for treatment of asthma and inflammatory bowel disease has been investigated.

To support the efficacy and biopharmaceutical trials, a sensitive, specific and highly efficient assay for (**I**) was desirable. In addition, automation of at least some of the steps of sample preparation was considered highly advantageous. The use of laboratory automated workstations is a viable alternative to manual sample preparation [7,8]. It saves a significant amount of the analyst's time

* Corresponding author. Tel.: (+1) 215-652-4515.

PII S0731-7085(96)01737-2

[9–11], and allows continuous sample preparation over 24 h.

BenchMate™ Workstations are a family of automation devices which perform well-defined laboratory operations such as sample aliquoting, reagent and internal standard addition, vortex-mixing, weighing, membrane filtration, solid-phase extraction, and high performance liquid chromatography (HPLC) auto-injection. The robotic arm is used to access test tubes, and different stations are used to perform the above functions. The workstation is controlled by a diskette containing the method and operating software, written for an IBM-compatible personal computer. One of the primary uses of this instrumentation in bioanalytical chemistry has been solid-phase extraction and biological sample preparation of drugs prior to chromatography. In this work the use of the BenchMate™ Workstation has been expanded to perform both protein precipitation and solid-phase extraction of drugs from biological fluids for HPLC analyses. The detailed description of the method for the determination of MK-591 in human plasma in the concentration range 10–1000 ng ml^{-1} using the BenchMate™ Workstation and assessment of long term assay performance is the subject of this paper.

2. Experimental

2.2. Chemicals and reagents

Compound (**I**) (Fig. 1) and its iso-(4-*t*-butylthiobenzyl) analogue (**II**, Fig. 1), used as an internal standard, were synthesized at the Merck Frosst Center for Therapeutic Research (Pointe Claire-Dorval, Canada). These compounds were supplied as the sodium salts, and all concentrations and doses are expressed in terms of the free acids. Heparinized human control plasma was obtained from Biological Specialty (Lansdale, PA).

Acetonitrile and methanol were both HPLC grade and were purchased from Fisher Scientific (Springfield, NJ). Trifluoroacetic acid was purchased from Sigma (St. Louis, MO). Water was purified using a Milli-Q-filtered system (Millipore, Milford, MA).

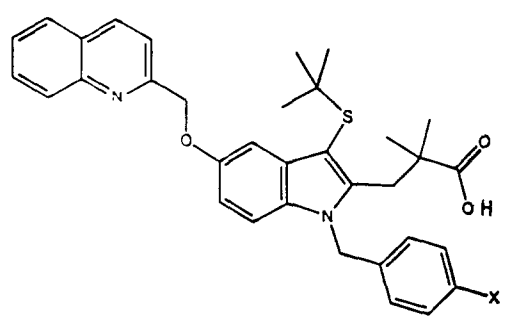
Solid-phase extraction (SPE) C_{18} cartridges (3 ml, 500 mg) originated from J. T. Baker (Phillipsburg, NJ). A Maxi Mix II vortex mixer from Thermolyne (Dubuque, IA) was also used.

2.2. Instrumentation

A Zymark BenchMate™ Workstation (Hopkinton, MA) was used for automated sample preparation and SPE. A Zymark TurboVap™ (Hopkinton, MA) was utilized for evaporating the methanol eluent to dryness at 45°C. The HPLC system included a Perkin-Elmer Series 250 pump and ISS-100 autosampler with 50 μl sample loop (Norwalk, CT). A CPS-Hypersil-2 analytical column (10 cm × 4.6 mm, 5 μm) protected with a cyano guard column (1 cm × 2 mm, 5 μm) and a column inlet-filter, all purchased from Keystone (Bellefonte, PA), were utilized. The absorbance spectra were recorded using a photodiode array spectrophotometer model 8542A (Hewlett-Packard, Palo Alto, CA). As an HPLC detector, An Applied Biosystems 783A programmable absorbance detector (Ramsey, NJ) was employed.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of 0.1% trifluoroacetic acid and acetonitrile (55:45, v/v, pre-mixed, pH 2.1) and was delivered isocratically at a flow rate of 1.6 ml min^{-1}. The UV detector was set at 290 nm with a sensitivity of 0.001 AUFS. A PE-Nelson ACCESS*CHROM



I, X=Cl
II, X=SC(CH$_3$)$_3$

Fig. 1. Chemical structures of (**I**) and internal standard (**II**)

laboratory data system was used for signal collection, peak integration, data acquisition and quantification.

2.4. Preparation of standard solutions and quality control samples

Stock solutions of (**I**) and (**II**) were prepared in methanol at a concentration of 0.5 mg ml^{-1}, and all subsequent dilutions were made using acetonitrile. Working solutions of (**I**) were prepared at concentrations of 20, 10, 4, 2, 1, 0.5 and 0.2 $\mu g\ ml^{-1}$ and the internal standard was prepard at a concentration of 10 $\mu g\ ml^{-1}$. An additional stock standard, independent of those used for preparing working standards, was used to make quality control (QC) samples. QC samples were prepared by pooling control plasma and spiking with the separately prepared stock standard solution of (**I**). The low quality control (LQC) sample was prepared by diluting the high quality control (HQC) sample with control plasma in a ratio of 1:24 (v/v). The QC samples were divided into 1.25 ml aliquots in glass vials and stored at −15°C until they were analyzed.

A seven-point calibration curve was run daily by spiking 1 ml of control plasma with 50 μl of (**II**) working solution (10 $\mu g\ ml^{-1}$). In addition, the appropriate working standard and two sets of HQC and LQC plasma samples were analyzed immediately after the analyses of standard line samples and later after the analyses of the subjects' plasma samples.

2.5. Sample preparation and analysis

The subjects' plasma samples were thawed to room temperature and vortexed vigorously for 5 s on a vortex mixer. 50–1000 μl of subject plasma diluted with 950–0 μl of control make-up plasma and 50 μl of (II) (10 $\mu g\ ml^{-1}$) was pipetted into a disposable culture glass tube and placed in the Benchmate™ Workstation for further sample preparation. The procedure used for the extraction of (**I**) from plasma is outlined in Fig. 2.

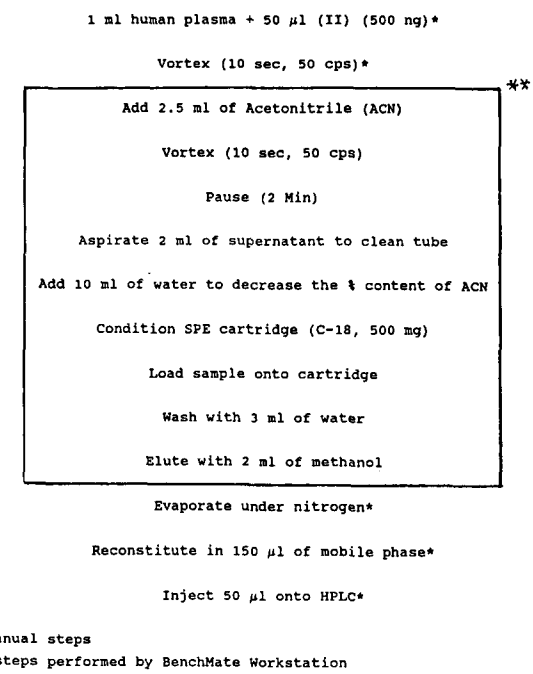


Fig. 2. Sample preparation procedure using the BenchMate™ Workstation

2.6. Precision, linearity, accuracy, sensitivity, and specificity

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma containing (**I**) at all concentrations utilized for constructing the calibration curves. The linearity of each standard was confirmed by plotting the peak-height ratio of (**I**) to (**II**) vs. concentration of (**I**). The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted $(1/y)$ linear regression of the standard line. The standard curve was prepared and assayed daily with quality control and unknown samples. The accuracy of the method was expressed as: (mean observed concentration)/(spiked concentration) × 100%. The limit of quantification (LOQ), defined as the lowest concentration on the standard line for which acceptable accuracy ($\pm$ 10% of the nominal values) and precision (expressed as the relative standard deviation, RSD $\leq$ 10%) were obtained, was 10 ng ml^{-1} of plasma. Assay specificity was assessed by

Table 1
Validation of BenchMate Workstation

Nominal volume	Volume found (ml)		Precision[a] (%)	Accuracy[b] (%)
	Mean[c]	SD[d]		
Dispense 2.5 ml of Acetonitrile	2.42	0.0026	0.11	96.8
Transfer 2.0 ml of supernatant	1.94	0.0042	0.22	97.0
Add 6.0 ml of water	5.82	0.0009	0.02	97.0
Add 4.0 ml of water	3.88	0.0011	0.03	97.0
Load 6.0 ml of supernatant	5.81	0.0043	0.07	96.8
Load 6.0 ml of supernatant	5.79	0.0042	0.07	96.5
Elute 2.0 ml of methanol	1.87	0.0054	0.29	93.5

[a] RSD ($n = 5$)
[b] Expressed as [(mean observed volume/set volume) × 100].
[c] $n = 5$.
[d] Standard deviation.

running blank control and patients pre-dose plasma samples.

2.7. Data acquisition and analysis

Peak heights for (**I**) and (**II**), used as an internal standard, were measured and automatically processed using a PE-Nelson ACCESS*CHROM laboratory data system. Concentrations of (**I**) in subject plasma samples were calculated from the daily standard curve obtained by least-squares linear regression of peak-height ratios of (**I**) to (**II**) vs. concentraion. When a calcualted amount exceeded the standard curve range, the sample was diluted with control plasma and re-analyzed.

3. Results

3.1. BenchMate™ Workstation suitability tests and system productivity

Before using the BenchMate™ Workstation, the accuracy and precision of the sample and solvent transfers were determined and the results are shown in Table 1.

Samples were prepared in batches when manual preparation was performed. However, each sample was processed through serial steps using the workstation. Therefore the system prepared 50 samples in a 16 h period.

3.2. Method validation

Typical chromatograms of control human plasma and plasma spiked with (**I**) and (**II**) (25 ng ml^{-1} and 500 ng ml^{-1} respectively) are shown in Fig. 3. The total analysis time was 12 min. The specificity of the assay was demonstrated by the lack of endogenous interference observed at the retention times of these compounds in any of the control human plasma or pre-dosed plasma samples from subjects participating in clinical trials.

Plots of the peak-height ratios of (**I**) to (**II**) vs. drug concentrations were linear over the concentraton range 10–1000 ng ml^{-1} in plasma. Unknown sample concentrations were calculated from the equation $Y = mX + b$, as determined by the weighted $(1/Y)$ linear regression of the standard line. The typical equation describing the standard line was $Y = 0.00283X \pm 0.00571$. An average correlation coefficient of determination (γ^2) of 0.9999 was obtained.

The precision of the described procedures was assessed by calculating the intra-day variation for each point on the standard lines. Intra-day RSDs for the calibration standards and QC samples were all less than 10% (Table 2). The inter-day accuracy and precision data are presented in Table 3.

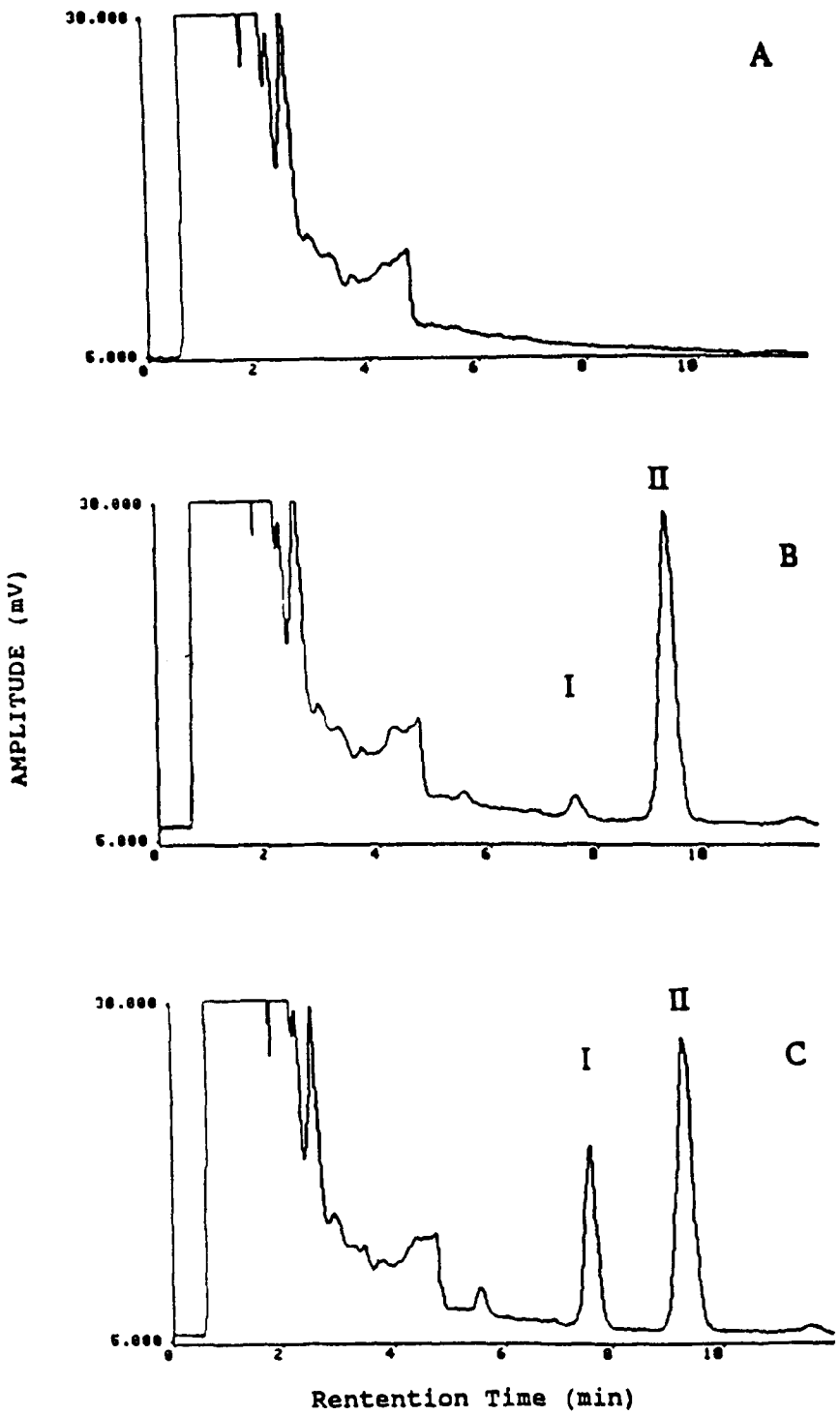


Fig. 3. Representative chromatogram of (I) and internal standard (II) in human plasma (1 ml): (A) control plasma; (B) control plasma (1 ml) spiked with 25 ng of (I) and 500 ng of (II); (C) subject's plasma (1 ml) after oral dosing with 250 mg of (I), collected at 4 h post-dose, and spiked with 500 ng of (II); concentration of (I) equivalent to 227 ng ml^{-1}.

Table 2
Intra-day precision and accuracy of determination of (I) in plasma

Nominal concentration (ng ml^{-1})	Concentration found (ng ml)$^{-1}$		Precision[a] (%)	Accuracy[b] (%)
	Mean[c]	SD[c,d]		
Standard				
10	9.5	0.75	7.89	95.0
25	23.9	0.35	1.46	95.6
50	50.3	0.88	1.75	100.6
100	99.8	1.53	1.53	99.8
200	205.5	2.29	1.11	102.8
500	501.6	5.18	1.03	100.3
1000	992.5	18.68	1.88	99.3
QC[c]				
32.0	31.7	0.71	2.24	99.1
800	800.1	8.75	1.09	100.0

[a] RSD ($n = 5$).
[b] Expressed as [(mean observed concentration/spiked concentration) × 100].
[c] $n = 5$, five replicate samples analyzed on the same day.
[d] Standard deviation.

The accuracy of the method was established by preparing QC samples at low and high concentrations on the standard line. These samples were frozen, stored with clinical samples and assayed daily together with the subjects' plasma samples. Samples with concentrations above the linear calibration range were diluted with control plasma and re-assayed. The accuracy data for QC samples are also summarized in Tables 2 and 3.

3.3. Recovery and stability

Extraction efficiencies from plasma were determined by comparing the peak heights of the analyte extracted from plasma to the peak heights of directly injected standards of (I). Across the range of the calibration curve, the recoveries of (I) ranged from 90% to 95%.

The data in Table 3 also indicates that (I) was stable in plasma during storage at −20°C for at least 6 months.

3.4. Application

The applicability of the method was demonstrated by analyzing plasma samples from nine clinical studies. Those studied included normal volunteers [6], asthmatic patients, and ulcerative colitis patients.

As an example, from a single study, the mean plasma concentration ($n = 8$ for all doses) vs. time profiles of (I) following single oral doses of 25, 50, 125, 250, and 500 mg of (I) under fasting conditions are shown in Fig. 4.

4. Discussion

The UV absorption spectrum of (I) in methanol indicated the presence of two absorption bands with maxima at around 230 and 290 nm and molar absorption coefficients (ε) of 190 000 and 43 000 M^{-1} cm^{-1} respectively. The high UV absorption of (I) in the accessible UV region (290 nm), allowed the development of a sensitive assay in biological fluids in the low nanograms per milliliter range. Since no detectable endogenous interferences were found at the retention times of (I) and (II) at 290 nm, UV detection at this wavelength was chosen for the assay.

Both (I) and (II) were found to be thermally stable. Due to the presence of an absorption band

Table 3
Inter-day precision and accuracy of determination of (I) in plasma

Nominal concentraton (ng ml^{-1})	Concentration found (ng ml^{-1})		Precision[a] (%)	Accuracy[b] (%)
	Mean[c]	SD[c,d]		
Standard				
10	9.9	0.6	6.06	99.0
25	24.6	0.8	3.25	98.4
50	50.7	1.4	2.76	101.4
100	100.2	2.0	2.00	100.2
200	203.6	5.4	2.65	101.8
500	500.1	10.4	2.08	100.0
1000	996.5	12.4	1.24	99.7
QC[e]				
32.0	34.6	2.5	7.14	108.1
800	817.5	5.5	0.67	102.2

[a] RSD ($n = 13$).
[b] Expressed as [(mean observed concentration/spiked concentration) × 100].
[c] $n = 13$, mean values calculated from standard curves constructed with daily study samples.
[d] Standard deviation.
[e] Three QC samples at each concentration were analyzed daily with the study samples on 13 different days and over a period of 6 months.

extending beyond 300 nm, and in order to prevent photolysis of the compounds, all steps during the sample preparation procedure were performed under yellow light.

Several different approaches to the isolation of (I) from plasma have been studied. Direct liquid–liquid extraction at different pH using solvents ranging in polarity from hexane to ethyl acetate produced extracts not only containing several major interferences but with low and variable recoveries. Therefore, isolation of (I) from plasma was attempted using SPE. When applied from water or plasma, (I) was retained to varying degrees on a variety of SPE cartridges, including Si, C-8, and C-18, but the recoveries were irreproducible and were dependent on loading speeds and types of cartridges. Therefore, in order to improve recovery, plasma samples were treated with acetonitrile to denature plasma proteins prior to extraction. The careful control of sample loading speed and flow rate of solvents during cartridge elution was found to be critical in achieving high recovery and reproducibility. These steps were not only difficult to control manually but were also time-consuming and tedious. The BenchMate™ Workstation is an automated instrument designed to eliminate routine laboratory sample preparation tasks such as dilutions, reagent and buffer additions, vortex mixing, SPE, and HPLC auto-injection. The precise and constant control of the syringe speed allowed accurate flow control through all the liquid transfer steps. The loading and eluting speeds were set at 0.05 and 0.10 ml s^{-1} respec-

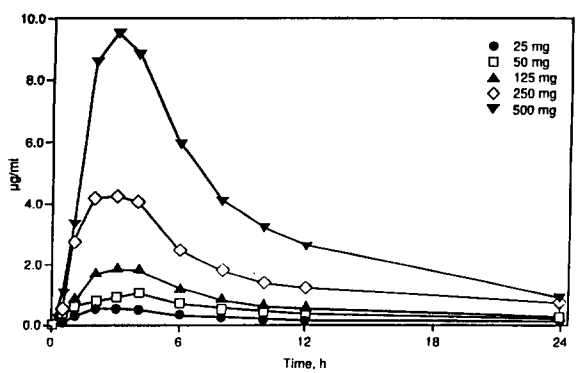


Fig. 4. Mean plasma concentration–time profiles of eight fasting subjects for each dosing range after single oral doses of (I) of 25, 50, 125, 250, and 500 mg

tively. The use of an automated system for sample clean-up prior to HPLC analysis provided a method that was both robust and efficient. Automation of the protein precipitation step with acetonitrile followed by SPE is an efficient way to assay highly protein-bound compounds. In this assay, plasma samples were aliquoted and an internal standard (**II**) was added manually. The samples were then loaded on the BenchMate™ Workstation, acetonitrile was dispensed to precipitate plasma proteins, the supernatant was transferred to a clean tube and, after addition of water, the mixed solution was loaded onto a C_{18} solid-phase cartridge where (**I**) and (**II**) were retained. After washing the cartridge with water, both compounds were eluted with methanol, the methanol eluent was evaporated to dryness, and the residue was dissolved in the mobile phase. (**I**) and (**II**) were separated from each other and from endogenous components in plasma on a HPLC cyano analytical column and the eluent was monitored using a UV detector (at a wavelength of 290 nm). The applicability and ruggedness of the assay were confirmed by analyzing more than 3500 plasma samples over a 3 year period in support of various human pharmacokinetic studies.

In conclusion, an automated HPLC method utilizing the Zymark BenchMate™ Workstation has been developed, validated, and successfully implemented for quantifying (**I**) in human plasma. In addition to SPE, the BenchMate™ Workstation was shown to be capable of performing an additional task of protein precipiatation before SPE, extending its capability in bioanalysis.

Acknowledgements

The authors thank Dr. B. Friedman for directing and monitoring the clinical programs from which the plasma samples were obtained for analyses.

References

[1] B. Samuelsson, Science, 220 (1983) 568.
[2] L. Menard, M. Laviolette and P. Borgeat, Can. J. Physiol. Pharmacol., 70 (1992) 808.
[3] C. Brideau, C. Chan and S. Charleson, Can. J. Physiol. Pharmacol., 70 (1992) 799.
[4] B. Samuelsson, S. E. Dahlen, J. A. Lindgren, C. A. Rouzer and C. N. Sherhan, Science, 237 (1987) 1171.
[5] D. K. Miller, J. W. Gillard, P. J. Vicker, S. Sandowski, C, Leveille, J. R. Mancini, P. Charleson, R. A. F. Dixon, A. W. Ford-Hutchinson, R. Fortin, J. Y. Gauthier, R. C. Rodkey, R. Rosen, C. Rouzer, I. S. Sigal, C. D. Strater and J. F. Evans, Nature, 343 (1990)278.
[6] M. Depre, B. Friedman, A. Van Hecken, I. De Lepeleire, W. Tanaka, A. Dallob, S. Shingo, A. Porras, C. Lin and P. DeSchepper, Clin. Pharmacol. Ther., 56 (1994) 22.
[7] J. Hsieh, C. Lin, B. Matuszewski and M. Dobrinska, J. Pharm. Biomed. Anal., 12 (1994) 1573.
[8] J. Hsieh, C. Lin and B. Matuszewski, J. Chromatogr. B, 661 (1994) 307.
[9] A. Bruns, H. Waldhoff, A. Wilsch-Irrgang and W. Winkle, J. Chromatogr., 583 (1992) 183.
[10] A. R. Newmann, Anal. Chem., 62 (1990) 29.
[11] T. L. Isenhour, S. E. Eckert and J. C. Marshall, Anal. Chem., 61 (1989) 805.