ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 1057–1064

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Separation of the four stereoisomers of a potent inhibitor (L-694,458) of human leukocyte elastase and its determination in human plasma using achiral/chiral chromatography with column switching[1]

J. Zagrobelny [a], B.K. Matuszewski [a,*], W.F. Kline [a], S.H. Vincent [b]

[a] *Merck Research Laboratories, West Point, PA 19486, USA*
[b] *Merck Research Laboratories, Rahway, NJ 07065, USA*

Received 27 October 1997

Abstract

A stereoselective method based on high-performance liquid chromatography (HPLC) and ultraviolet detection at 235 nm for the separation of the four possible stereoisomers of compound **1** ((*S*-(*R**,*S**))-2-(4-((4-methylpiperazin-1-yl)carbonyl)phenoxy)-3,3-diethyl-*N*-(1-(3,4-(methylenedioxy)phenyl)butyl)-4-oxo-1-azetidinecarboxamide, L-694,458), a potent, selective, and orally active human leukocyte elastase (HLE) inhibitor, in human and monkey plasma has been developed. The molecule of **1** contains two chiral centers and is being developed as a single stereoisomer with the absolute configuration *S* and *R* in positions 'a' and 'b', respectively. Although the baseline separation of each of the two pairs of enantiomers (*SS*/*RR* and *SR*/*RS*) was achieved on a single chiral column (Chiralcel OD-H) using hexane–methyl-*t*-butyl ether (MTBE)–methanol 80/10/10, (v/v/v) as a mobile phase ($\alpha_{RS,SR} = 2.03$, $\alpha_{RR,SS} = 4.97$), only partial separation of *RS* from *RR* was observed under these conditions ($k'_{RS} = 3.32$, $k'_{RR} = 3.08$). Baseline separation of all four stereoisomers from each other and from endogenous plasma components required the initial chromatography of the two diastereomeric racemates (*SS*/*RR* and *SR*/*RS*) on the achiral silica column (50 × 4.6 mm, 5 μm), followed by column switching and further separation of the stereoisomers on a Chiralcel OD-H column (250 × 4.6 mm, 5 μm) using isopropanol (IPA)–hexane–diethylamine (DEA), 65/35/0.3, (v/v/v) on both columns as a mobile phase. The drug was extracted from basified (pH 11) plasma (1 ml) using liquid–liquid extraction with MTBE. After evaporation of the extract to dryness, the residue was reconstituted in the mobile phase (200 μl) and part of the extract (125 μl) was injected into the HPLC system. Using this method, it was demonstrated that after oral dosing of monkeys at 40 mg kg^{-1} with **1** the only stereoisomer detected in the post-dose plasma samples was the starting material **1**, and no inversion of the configuration at positions 'a' and 'b' of **1** had occurred in vivo. Based on this observation, a non-chiral assay for **1** in human plasma was also developed. The method was validated in the concentration range 10 – 500 ng ml^{-1} with the assay precision (expressed as the coefficient of variation, CV) better than 9% and assay accuracy in the range of 95–107% of the nominal concentrations at all concentrations

* Corresponding author. Tel.: +1 215 6526685; fax: +1 215 6524524; e-mail: bogdan_matuszewski@merck.com
[1] Presented at the 9th International Symposium on Chiral Discrimination, 27–30 October, 1997, Nagoya, Japan.

PII S0731-7085(98)00071-5

within the standard curve range. The total run time in the non-chiral assay was 12 min. The details of both chiral and non-chiral methods are provided.

Keywords: Potent inhibitor; Human leukocyte elastase; Achiral/chiral chromatography; Column switching; L-694,458

1. Introduction

Compound **1** ((*S*-(*R**,*S**))-2-(4-((4-methylpiperazin-1-yl)carbonyl)phenoxy)-3,3-diethyl-*N*-(1-(3,4-(methylenedioxy)phenyl)butyl)-4-oxo-1-azetidinecarboxamide (L-694,458), Fig. 1) was shown to be a remarkably potent human leukocyte elastase (HLE) inhibitor [1]. Among the β-lactam-based inhibitors reported, compound **1** is a selective, non-cytotoxic, and orally active inhibitor [2], and has been selected for further development [3]. Disposition of **1** in rats and rhesus monkeys has been recently reported [4].

Compound **1** contains two chiral centers (Fig. 1) and is being developed as a single stereoisomer with the absolute configuration *S* and *R* in positions 'a' and 'b', respectively. There were two major objectives of our studies. Initially, in order to assess the potential for an in vivo inversion of the configuration at one or both chiral centers of **1**, a method for the chiral separation of the four possible stereoisomers of **1** and their analyses in plasma without interference from endogenous plasma components was needed. In the second step, assuming that no in vivo inversion of the configuration was observed, the development of a fully validated and more rapid assay for determination of **1** in human plasma was necessary to support the anticipated clinical pharmacokinetic studies. The development of the chiral separation method based on achiral/chiral high pressure liquid chromatography (HPLC) with column switching and ultraviolet (UV) detection, and an achiral assay based on HPLC/UV with an on-line sample clean-up, are the subject of this paper.

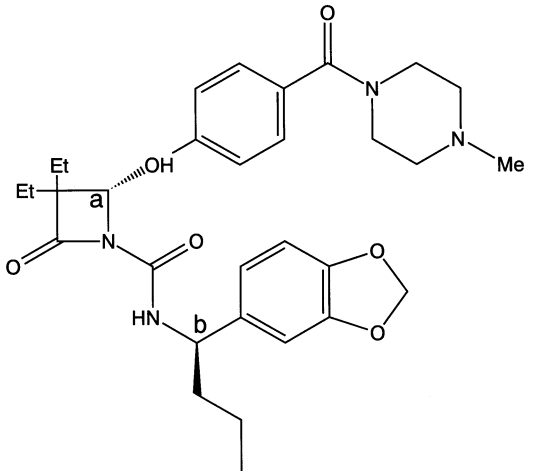

Fig. 1. Chemical structure of **1**.

Stereoselective assays follow two general approaches. One involves indirect enantiomeric separation after derivatization of the enantiomers with a chiral derivatizing reagent and formation of diastereomers. The other utilizes direct enantiomer separation through the use of chiral stationary phases (CSP's) or chiral modifiers in the mobile phase and non-chiral chromatography. Some recent examples from our laboratories using these two general approaches are listed in references [5–7] and [8,9], respectively. In the case of compound **1**, a direct chiral separation approach was utilized because of the lack of easily derivatizable functional groups in **1** for reaction with enantiomerically pure reagents. Instead, a method based on achiral/chiral chromatography of the underivatized stereoisomers of **1** was developed. In the case of the non-stereoselective assay, the relatively high absorption of the compound in the near ultraviolet region of the spectrum allowed development of an assay in human plasma with sensitivity in the low ng ml^{-1} range using normal-phase HPLC with UV detection. In rat and monkey plasma, **1** was determined using HPLC with tandem mass spectrometric detection [4] but the details of the assay methodology and validation data were not provided.

2. Experimental

2.1. Materials

Compound **1** (*SR*) and its three steroisomers with *RS*, *SS*, and *RR* stereochemistry were synthesized at Merck Research Laboratories (Rahway, NJ). All solvents were HPLC grade (Fisher, Fair Lawn, NJ). Heparnized human control plasma was obtained from Sera-Tec Biologicals (North Brunswick, NJ). Water was deionized using a Milli-Q reagent water system (Millipore, Milford, MA) resulting in 18 MΩ conductivity. Diethylamine (DEA) was obtained from Sigma (St. Louis, MO). All other chemicals were from EM Science (Gibbstown, NJ).

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of a series 410 LC pump (pump 1) (Perkin-Elmer, Norwalk, CT), a model 6000A isocratic pump (pump 2) (Waters, Milford, MA), a model WISP 715 autosampler (Waters) and a model 785A absorbance detector (Applied Biosystems, Ramsey, NJ). A six port switching valve (Valco, Houston, TX) was utilized to divert the eluent from column 1 to waste or onto column 2. The switching valve was controlled via time event programming on pump 1. The times at which the events on pump 1 were set to trigger the valve were determined prior to analysis by injecting a standard solution of **1** (in the non-chiral assay) or a mixture of the four steroisomers **1**, *RS*, *SS*, and *RR* (in the chiral method) and monitoring the retention times after elution from column 1. The timed events were then set to place the valve in position A at the beginning of the run, switch the valve to position B at 1.7 min (non-chiral assay) or 3.6 min (chiral method), and return the valve to position A at 2.5 or 6.5 min for non-chiral and chiral assays, respectively. The chromatographic data were acquired and analyzed with an automated laboratory system (PE/Nelson Access*Chrom V 1.7, Cupertino, CA). Unknown sample concentrations were calculated from the weighted linear regression of the standard line constructed by plotting peak areas of **1** versus drug concentration. All calculations were performed using PE Nelson Access*Chrom software. The absorption and fluorescence spectra were obtained using a model 8452A photodiode array spectrophotometer (Hewlett Packard, Palo Alto, CA) and a model F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), respectively.

2.2.1. Chiral method

The mobile phase for pumps 1 and 2 was the same and was composed of a 65/35/0.3 (v/v/v) mixture of isopropanol (IPA)–hexane–DEA delivered at a flow rate of 0.5 and 0.4 ml min^{-1}, respectively. Column 1 was a non-chiral Nucleosil

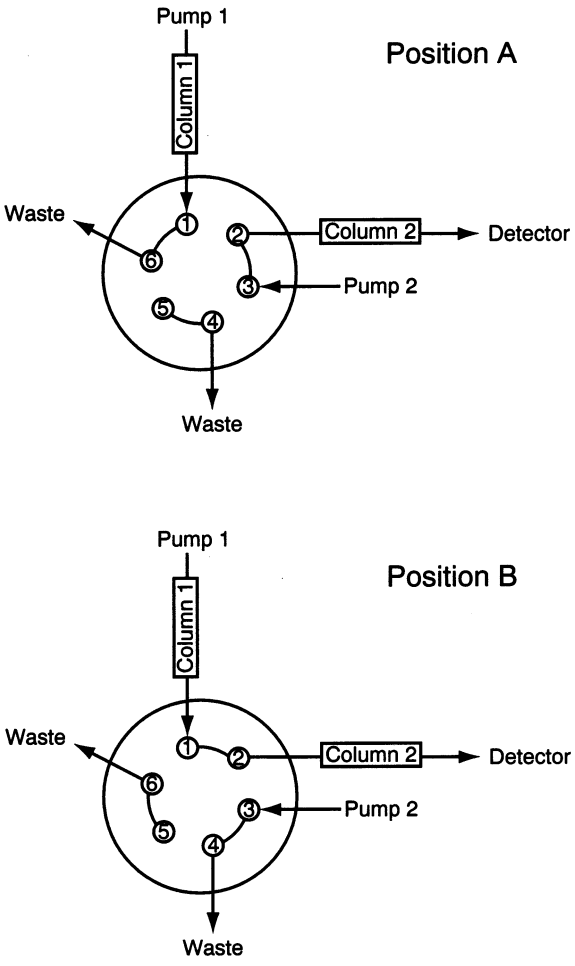

Fig. 2. HPLC column switching system used in the chiral and non-chiral HPLC assays.

silica column (50 × 4.6 mm, 5 μm), and column 2 was a Chiralcel OD-H (250 × 4.6 mm, 5 μm). The achiral column was purchased from Keystone Scientific (Bellefonte, PA) whereas column 2 was from Chiral Technologies (Exton, PA). For the analyses of monkey plasma extracts, in order to shorten the analysis time and sharpen the chromatographic peaks, the chiral column 2 was heated to 35°C. The mobile phase was filtered through a nylon filter (0.2 μm).

2.2.2. Non-chiral assay

The mobile phase for pump 1 was a 70/30/0.3/0.2 mixture of hexane–IPA–water–DEA (v/v/v/v), and for pump 2 a 65/35/0/5/0.2 mixture of the same chemicals. Column 1 was an amino (NH_2) Spherisorb column (50 × 4.6 mm, 5 μm), and column 2 was a Nucleosil silica (100 × 4.6 mm, 5 μm) both purchased from Keystone Scientific.

2.3. Sample preparation

The drug was extracted from basified plasma (pH 11) using liquid–liquid extraction with methyl-*t*-butyl ether (MTBE). A pH 11 carbonate–bicarbonate buffer was prepared by mixing 46 ml solution A containing 21.2 g sodium carbonate in 1000 ml water with 4 ml solution B prepared by dissolving 16.8 g sodium bicarbonate in 1000 ml water and diluting the mixture to 200 ml with water. This buffer solution (1 ml) was added to a 1 ml aliquot of plasma to which 100 μl ethanol was added. After vortexing and after the addition of 5 ml MTBE, the sample was shaken and centrifuged. The organic layer, after freezing the lower aqueous layer in a dry ice–acetone bath for 15 min, was transferred into a borosilicate culture tube. The organic layer was evaporated to dryness using a Turbo Vap evaporator (Zymark, Hopkinton, MA), the residue was reconstituted in 200 μl mobile phase, and 125 μl of the sample was injected into the HPLC system. In the chiral method the extraction procedure was the same as in the non-chiral method, but reconstitution of the residue after evaporation to dryness was in the mobile phase used in the chiral method. Standard plasma samples used for constructing a standard line were prepared in the same way as the study samples except that the control plasma (1 ml) was spiked with the respective standard solutions of **1** (100 μl) in ethanol before addition of the buffer and extraction with MTBE. The concentration of **1** in the standard solution in plasma was 10, 25, 50, 100, 250, and 500 ng ml^{-1}.

2.4. Precision, accuracy, specificity, and recovery of *1* from plasma

The precision of the non-chiral method was determined by the replicate analyses ($n = 5$) of human plasma containing **1** at all concentrations utilized for constructing the calibration curves. The accuracy of the method was expressed by: (mean observed concentration)/(spiked concentration) × 100. The recovery was determined by comparing the peak area of **1** extracted from the plasma to that of the standards injected directly. Assay specificity was assessed by analyzing blank control plasma from five different sources. No endogenous interference was observed.

2.5. Dosing of monkeys with *1*

Three rhesus monkeys weighing 4.5–7 kg were chaired without anesthesia and dosed orally with **1** at 40 mg kg^{-1} using a pediatric nasogastric tube. Heparnized blood was collected from the femoral vein using a previously implanted cannula and plasma was obtained by centrifugation at 4°C and stored at −20°C until analyzed. Compound **1** as a free base was prepared as a suspension in 0.5% methylcellulose for dosing.

3. Results

3.1. Chiral separation of steroisomers of *1*

Initially, a single-column configuration using a Chiralcel OD-H column with a variety of mobile-phase compositions was utilized to effect the separation of the steroisomers. The *SR*/*RS* and the *RR*/*SS* enantiomeric pairs were well separated using a mixture of hexane–MTBE–methanol (80/10/10, v/v/v) as the mobile phase at a flow rate of 1 ml min^{-1}. Retention times (RT) were 13.47,

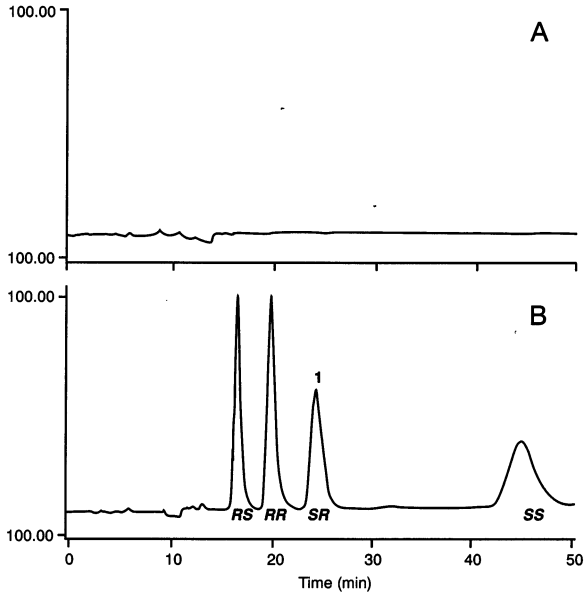

Fig. 3. Representative chromatogram showing the chiral separation of the four stereoisomers of **1**. (A) Human control plasma, (B) control plasma spiked with 2.5 μg ml^{-1} of each stereoisomer. The analytical column was kept at room temperature during analysis.

14.25, 25.55 and 53.86 min for *RR*, *RS*, *SR*(**1**), and the *SS* isomers, respectively, and the corresponding capacity factors (k') were 3.08, 3.32, 6.74, and 15.32, respectively. Under these conditions baseline separation of *RR* from *RS* was not observed. Decreasing the amount of methanol in the mobile phase allowed baseline separation of this diastereomeric pair, but the RT of the *SS* isomer increased to more than 90 min under these conditions. Separation of the diastereomers has been attempted using a variety of achiral columns and mobile phases but an efficient separation was not observed.

The baseline separation of all four steroisomers of **1** was achieved using a combination of achiral (silica) and chiral (Chiralcel OD-H) columns, as described in Section 2.

Representative chromatograms are shown in Fig. 3. Both chiral and achiral columns were kept at room temperature. A baseline separation required a relatively long analysis time of 50 min.

For analyses of post-dose plasma samples the analysis time was shortened to 40 min to sharpen the chromatographic peaks and decrease the limit of detection (LOD). In this case, the analytical column 2 was heated to 35°C. The chromatograms in Fig. 4 illustrate the pre-dose (Fig. 4A) monkey plasma, pre-dose monkey plasma spiked with 1500 ng ml^{-1} of each stereoisomer (Fig. 4B), and 6 h post-dose plasma samples of a monkey dosed orally with 40 mg kg^{-1} of **1**. The concentration of **1** in Fig. 4C corresponded to about 800 ng ml^{-1}. Under these conditions, the

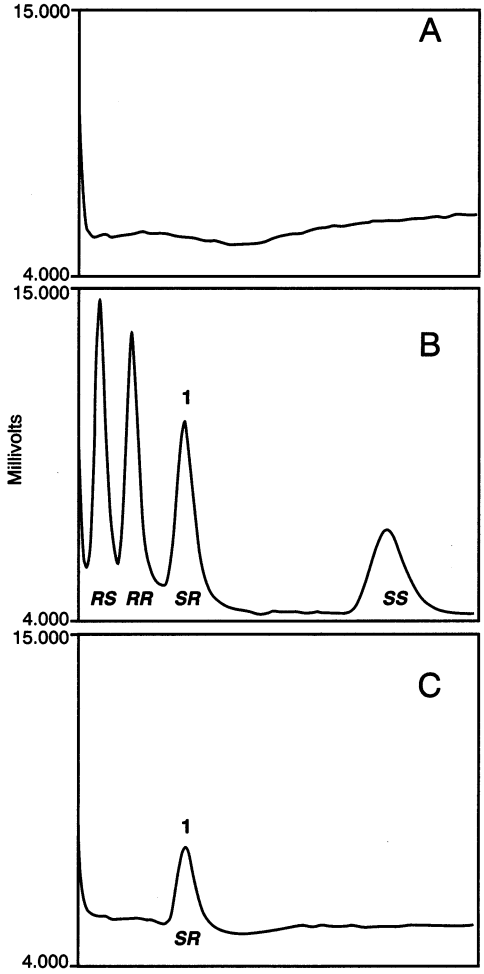

Fig. 4. Representative chromatograms in the 15–40 min region of the pre-dose monkey plasma (A), pre-dose monkey plasma spiked with 1500 ng ml^{-1} of each stereoisomers of **1** (B), and a 6 h post-dose plasma sample of a monkey after oral dose of 40 mg kg^{-1} of **1** (C). The analytical column was heated to 35°C during analysis.

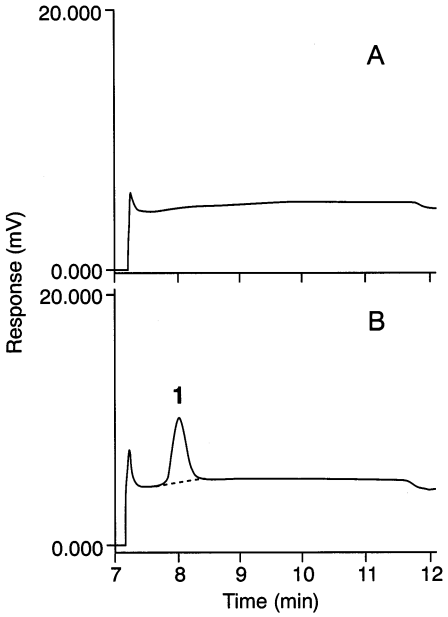

Fig. 5. Representative chromatograms of **1** extracted from human plasma and analyzed using the non-chiral assay. (A) Human control plasma, (B) human plasma spiked with 50 ng ml^{-1} of **1**. Only the elution region of the drug is shown.

estimated LOD of the steroisomers of **1** was about 50 ng ml^{-1} at a signal-to-noise ratio of 3:1 (expanded chromatograms are not shown). There was no indication of the presence of stereoisomers other than **1** in any of the post-dose plasma samples analyzed.

3.2. Non-chiral assay of *1* in plasma

The procedure for the extraction of **1** from plasma was the same as in the chiral method and was based on liquid–liquid extraction from basified (pH 11) plasma with MTBE (5 ml). Additional sample clean-up was necessary and was performed on-line using a column switching system (Fig. 2) described in Section 2. Under these conditions, a remarkably clean chromatograms of **1** from the plasma extracts were obtained (Fig. 5).

The assay was validated in control human plasma in the concentration range 10–500 ng ml^{-1}. The mean recovery of the drug from plasma was about 85% at all concentrations within the standard curve range.

The limit of quantification (LOQ) was 10 ng ml^{-1}. The LOQ was defined here as the lowest concentration of the standard line for which precision, expressed as the coefficient of variation (C.V., %) was better than 10%, and accuracy, defined as: (mean observed concentration)/(nominal spiked concentration) × 100, was within 15% at all points within the standard curve range (Table 1).

4. Discussion

4.1. Chiral analyses

The direct separation on CSP's was chosen here over an indirect method based on chiral derivatization due to the absence of an easily derivatizable functional group in the molecule of **1**. The baseline separation of the four steroisomers of **1** required utilization of both achiral and chiral chromatography and column switching. After separation of the diastereomeric mixture of **1** from endogenous plasma components on an achiral column, the isomers were 'heart-cut' and transferred onto the chiral column for final separation. Also, any coeluting endogenous plasma components that could interfere with the chiral analyses were mostly removed on the achiral column.

Using the achiral/chiral approach baseline separation of all four stereoisomers of **1** was achieved (Fig. 3). This separation allowed analyses of post-dose plasma samples for the presence the stereoisomers of **1** at a relatively high concentration of **1**. In addition, the LOD of all isomers was estimated at 50 ng ml^{-1} which was sufficient to assess qualitatively the potential for the in vivo inversion of the configuration at one or both chiral centers of **1**. The data obtained in this study indicated that the steroisomers *SS*, *RR*, and *RS* were not present at detectable concentrations in plasma after oral dosing with the single isomer *SR*(**1**) (Fig. 4), and no indication of an in vivo inversion of the configuration at either one or both chiral centers of **1** was observed in rhesus monkeys. Based on these results a non-stereoselective assay for the determination of **1** in human plasma by HPLC with UV detection was developed.

Table 1
Precision and accuracy data for the determination of **1** in human plasma

Concentration (ng ml^{-1})	Precision[a]	Mean found concentration[b] (ng ml^{-1})	Accuracy[c]
10	4.9	9.73	97.3
25	8.1	23.83	95.3
50	4.8	53.66	107.3
100	5.0	103.7	103.7
250	3.4	243.6	97.4
500	2.2	501.9	100.4

[a] Precision expressed as the coefficient of variation (C.V., %) of peak areas of **1** in five different lots of plasma.
[b] From linear regression analysis using all five replicate values at each concentration.
[c] Expressed as (mean calculated concentration)/(spiked concentration) × 100.

4.2. Development of a non-chiral assay

Initially, in order to establish the most sensitive method of detection of **1** using conventional methods, the absorption and fluorescence spectra of the molecule were obtained. The absorption spectrum in ethanol indicated the presence of three absorption maxima at 204, 236, and 284 nm with the molar absorption coefficients (ε) of 55 000, 17 000, and 4900 M^{-1} cm^{-1}, respectively. The compound was practically non-fluorescent following excitation at 235 nm. In addition, a series of photochemical experiments with **1** were performed to investigate a possible increase in assay sensitivity through the use of post-column irradiation and also to study the photochemical stability of **1** in standard solutions and in plasma. These experiments showed that photodecomposition after irradiation at 254 nm and the formation of fluorescent or highly absorbing compounds was not observed. Based on all these preliminary evaluations, an HPLC assay with UV detection at 236 nm was developed. It is our practice to develop a quantitative method for the determination of a drug based on a conventional detection method rather than HPLC with tandem mass spectrometric (MS/MS) detection when the sensitivity of a conventional method and the speed of analysis may be sufficient to support clinical studies. Only in cases where conventional methods are not efficient and/or sensitive enough to support studies with relatively low doses of the drug, the HPLC/MS/MS approach is evaluated. Contrary to the common belief, the development of the HPLC/MS/MS assays requires extensive method validation including the studies of the matrix effect, studies of assay specificity in the presence of metabolites, and the assessment of 'cross-talk' effect [10,11]. In many cases, the sensitivity of conventional methods and speed of analysis are comparable or better than methods based on HPLC/MS/MS [12,13]. Therefore, in order to support the anticipated first clinical study of **1**, the method based on the conventional HPLC/UV approach rather than HPLC/MS/MS was developed.

After liquid–liquid extraction of the drug from basified plasma with MTBE, an additional sample clean up was required on line using column switching technique. The endogenous plasma components were separated from **1** under normal-phase conditions on a short (50 × 4.6 mm) amino (NH_2) column and were diverted to waste. The peak of interest was 'heart-cut' into the analytical column where final separation of **1** from other endogenous plasma impurities was achieved. Remarkably clean chromatograms (Fig. 5) of **1** in different sources of human plasma were obtained. Using this method, the assay with the total analysis time of 12 min was validated in the concentration range 10–500 ng ml^{-1}.

In conclusion, the method for the chiral separation of four stereoisomers of the novel HLE inhibitor **1** was developed. Using this method it was demonstrated that in vivo inversion of the configuration at either one or both chiral centers of **1** did not occur after dosing rhesus monkeys with **1**. In addition, a non-chiral method based on

HPLC with UV detection for determination of **1** in human plasma with the LOQ of 10 ng ml^{-1} was established.

References

[1] R.J. Cvetovich, M. Chartrain, F.W. Hartner Jr., C. Roberge, J.S. Amato, E.J.J. Grabowski, J. Org. Chem. 61 (1996) 6575–6580.

[2] P.E. Finke, C.P Dorn, Jr, A.L. Kissinger et al., Orally active intracellular inhibitors of human leukocyte elastase (HLE), 22nd Natl. Meet. of the American Chemical Society, Chicago, 1995, MEDI 083.

[3] L-694,458 has been licensed to DuPont Merck Pharmaceutical Company (DMP-777).

[4] S.H. Vincent, S.K. Painter, D. Luffer-Atlas, et al., Drug Metab. Dispos. 25 (1997) 932–939.

[5] B.K. Matuszewski, M.L. Constanzer, G.A. Hessey II, W.F. Bayne, Anal. Chem. 62 (1990) 1308–1315.

[6] B.K. Matuszewski, M.L. Constanzer, Chirality 4 (1992) 515–519.

[7] B.K. Matuszewski, M.L. Constanzer, M. Kiganda, Pharm. Res. 11 (1994) 449–454.

[8] B.K. Matuszewski, M.L. Constanzer, G.A. Hessey II, W.F. Bayne, J. Chromatogr. 526 (1990) 461–473.

[9] J. Zagrobelny, B.K. Matuszewski, Enantiomer 2 (1997) 37–43.

[10] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. (in press).

[11] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.

[12] B.K. Matuszewski, Pharmaceutical and Biomedical Analysis 8th Int. Symp., Orlando, FL, 4–8 May, 1997, abstract no. M/P-A9.

[13] J. Stewart, M. Silveru, W. Bachman, T. Venkateschwaran, D. Delinky, B.K. Matuszewski, Methodological surveys in bioanalysis of drugs, R. Soc. Chem. (in press).