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Quantitation of an orally available thrombin inhibitor in rat, monkey and human plasma and in human urine by high-performance liquid chromatography and fluorescent post-column derivatization of arginine

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Abstract

An assay for the quantification of plasma and urine levels of CVS 1123, an orally bioavailable thrombin inhibitor, and its desmethyl form, CVS 738, was developed to support clinical and toxicology studies. This assay uses solid-phase extraction, reversed-phase HPLC separation, and post-column fluorescent derivatization with ninhydrin. An internal standard is added to correct for recovery. In aqueous solution, the arginine aldehyde structures of CVS 1123 and CVS 738 exist in multiple forms which can be separated under standard reversed-phase HPLC conditions. HPLC conditions were optimized to give rapid interconversion of the forms on the separation time scale, and consequently a single chromatographic peak. Extraction conditions were modified for quantitative extraction of drug compounds from large volumes of human plasma. The assay was shown to be accurate and precise, with a quantification limit of 17 ng CVS 1123/ml human plasma.

Keywords: Derivatization, LC; Pharmaceutical analysis; Thrombin inhibitors

1. Introduction

The serine protease thrombin is a valuable target for the development of new drug therapies. CVS 1123 (Fig. 1) is a synthetic direct inhibitor of thrombin [1,16] which has been demonstrated to be both effective in animal models of thrombosis [2,3] and orally bioavailable in cynomolgus monkeys and in dogs [4]. In order to establish the pharmacokinetic parameters of CVS 1123, it was necessary to measure plasma levels of this compound and of its possible metabolite CVS 738 (Fig. 1).

The development of an assay for the direct analysis of low levels of CVS 1123 in plasma presented two major challenges. First, sensitive detection of CVS 1123 and CVS 738 proved difficult because the molecules are arginine aldehydes. This class of molecules exists in at least three equilibrium structures in aqueous solution [5,6] (Fig. 2). The major forms that are present are an aldehyde hydrate and two amino cyclols. These forms are baseline resolved in an acetonitrile–water–trifluoroacetic acid gradient HPLC C_{18} separation (Fig. 3). Different mobile phase pH, extraction conditions and other conditions affect the relative abundance ratio of the three different forms. Quantification of such a mixture of peaks would be very difficult. Another difficulty in the analysis of CVS 1123 is that there is

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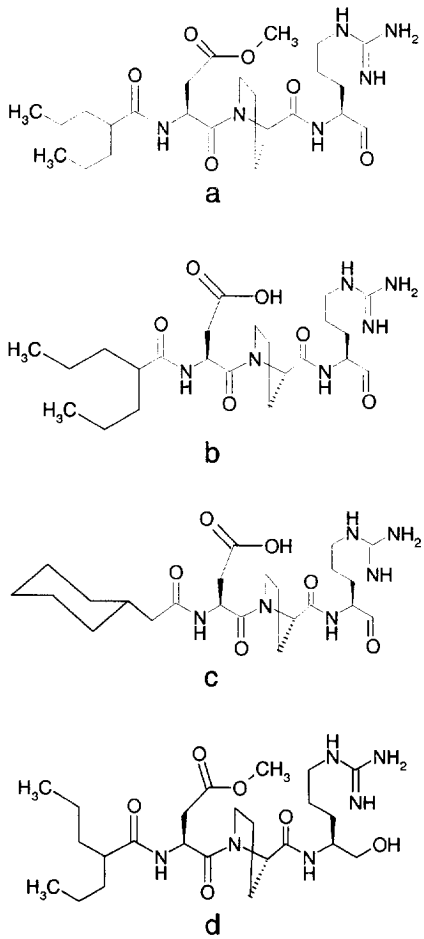

Fig. 1. Structures of compounds: a=CVS 1123; b=CVS 738; c=CVS 737; d=CVS 1235. The first three compounds are pictured in their free aldehyde form.

no chromophore which would allow for sensitive absorbance measurement of the compound in HPLC systems. No free primary amine group is available for derivatization, and pre column derivatization of small quantities of a free aldehyde can be difficult.

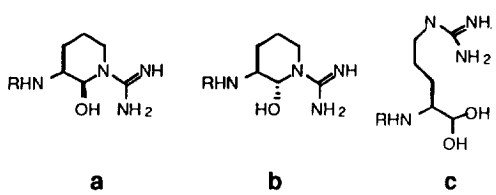

Fig. 2. Equilibrium forms of arginine aldehyde compounds. (a) and (b) are cyclols and (c) is the hydrate form.

This paper describes the assays which have been developed for determination of CVS 1123 and CVS 738 in rat, cynomolgus monkey and human plasma, and in human urine. The assays make use of post-column derivatization for sensitive and selective determination of the compounds, and mobile phase conditions which give a single chromatographic peak for CVS 1123. These assays were used to determine CVS 1123 and CVS 738 concentrations in plasma for toxicology studies in rat and cynomolgus monkey, and in plasma and urine for a safety assessment study in human volunteers.

2. Experimental

2.1. Materials

CVS 1123 (Fig. 1) used as the standard was chemically synthesized [1] either at Corvas International or at UCB-Bioproducts S.A. (Braine-l'Alleud, Belgium) under contract from Corvas International. CVS 738 (the desmethyl form of CVS 1123, Fig. 1) was synthesized at Corvas International. CVS 737 and CVS 1235 (Fig. 1) were synthesized at Corvas International and were used as the internal standards. All standards had purity greater than 98%. Water used for all buffers, mobile phases and solutions was deionized and then purified using a Nanopure water system (Barnstead, Dubuque, IA, USA). A 1 *M* stock solution of phosphate buffer for mobile phase was prepared by adding 67 ml of 85% phosphoric acid to 0.85 l of water, adjusting the pH to 6.0 by addition of a 45% KOH solution, allowing the solution cool to room temperature and then adjusting to a final volume of 1.0 l by addition of water. A 0.125% ninhydrin solution was prepared by adding 2.5 g of ninhydrin to 2 l of water. Methanol and acetonitrile were HPLC grade. All other chemicals were reagent grade or better.

Empore C_{18} and Empore S.D.B. extraction disk columns were obtained from 3M (Minneapolis, MN, USA). The 300 mg Bond Elut C_{18} columns were obtained from Varian Sample Preparation (Harbor City, CA, USA).

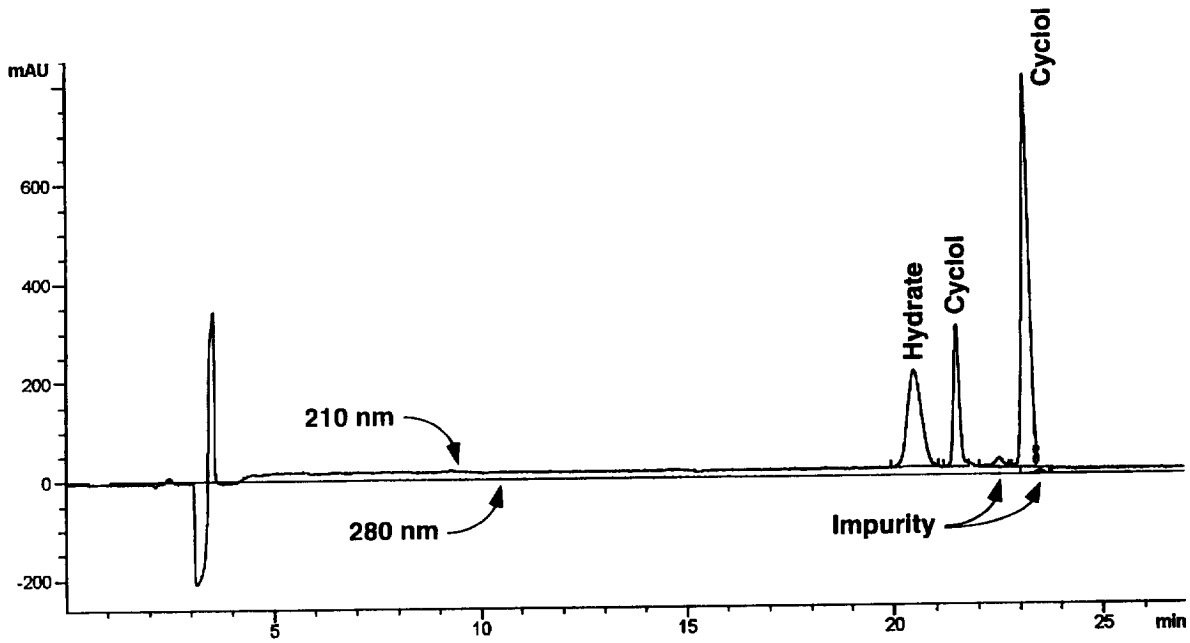

Fig. 3. HPLC separation of CVS 1123 in an acetonitrile–water–trifluoroacetic acid mobile phase. Column is a 25 cm×4.6 mm diameter Kromasil C_{18} column. Separation is from 10% acetonitrile–0.1% TFA to 35% acetonitrile–0.1% TFA in water–0.1% TFA in 20 min. Detection is by absorbance at 210 and 280 nm. The first peak is from the hydrate form, and the next two large peaks are from the cyclol forms. The peak at 22.5 min is from racemization at the arginine of CVS 1123. The peak which absorbs at 280 nm arises from dehydration of CVS 1123.

2.2. Extraction procedures

2.2.1. Rat and cynomolgus monkey plasma samples

The plasma (unknown samples, blank plasma for standards and quality control samples) was thawed and gently vortexed. The blank plasma was centrifuged at 18 000 *g* for 2 min to pellet aggregates if needed. Samples were up to 0.1 ml of rat or cynomolgus monkey plasma, and were put into 1.5 ml micro centrifuge tubes. If less than 0.1 ml of plasma was used, blank plasma was added to supplement the plasma volume to 0.1 ml. 6 μl of 1 *M* NaF was immediately added to the sample tube to give a final concentration of about 10 m*M* NaF. 10 μl of 10 μg CVS 737/ml water and 10 μl of 10 μg CVS 1235/ml water were added to each tube as internal standards. 500 μl of 0.1 *M* potassium phosphate, pH 7.0 was then added to each tube. The tubes were vortexed to mix. The Empore 4 mm C_{18} extraction disk cartridges were conditioned by adding 50 μl of methanol to wet the membrane, then by adding 0.5 ml of water. Centrifugation was used to force all solvents through the extraction cartridge, taking care not to dry out the column between solvent additions. The diluted plasma samples were applied to the conditioned extraction cartridges, one sample to each extraction cartridge. Samples were centrifuged through the extraction cartridge. Each extraction cartridge was subsequently washed with two applications of 0.5 ml of water. The relatively polar compound, CVS 738, and its internal standard, CVS 737, were selectively eluted by application of 0.15 ml of acetonitrile–water (50:50). CVS 1123 and its internal standard, CVS 1235, were eluted using 0.15 ml of methanol–trifluoroacetic acid (99.8:0.2) into a separate centrifuge tube. The eluates were dried under vacuum, and reconstituted using 0.1 ml of acetonitrile–water–trifluoroacetic acid (10:89.9:0.1). The first and second eluates were chromatographed separately. 90 μl of the reconstituted sample were injected onto the HPLC-post-column derivatization system for each chromatographic run.

2.2.2. Clinical plasma samples

The plasma (unknown samples, blank plasma for standards and quality control samples) was thawed and gently vortexed. The plasma (up to a volume of 1 ml) was put into 2 ml micro centrifuge tubes. If less than 1 ml of plasma was used, blank plasma was added to supplement the plasma volume to 1 ml. 10 ml of 10 μg CVS 1235/ml water was added to each standard and sample as an internal standard for both CVS 738 and for CVS 1123. 750 μl of 100 m*M* sodium citrate (pH 3) was added to each sample and standard. The diluted samples were then vortexed gently to mix, and then centrifuged at 18 000 *g* in a microcentrifuge for 10 min to remove any precipitate which could clog the extraction cartridges. As much sample as possible (over 90%) was loaded onto the extraction cartridge without disturbing the pellet of solid at the bottom of the centrifuge tube. The Empore 7 mm SDB extraction disk cartridges were conditioned with 300 μl MeOH and then with 2 ml water. Following cartridge conditioning, the samples were immediately applied to the cartridge and forced through by centrifugation. The cartridges were then washed with 2 ml of water. The CVS 1123, CVS 738, and internal standard were then eluted with 0.3 ml of 0.2% trifluoroacetic acid in methanol. 30 μl of 0.45 *M* NH_4OH was then added to the eluate to neutralize the solution and the samples were then dried under vacuum. The samples were reconstituted in 0.1 ml of a methanol–acetonitrile–0.05 *M* phosphate, pH 6 solution (31:10:59). 90 μl of this solution was injected onto the HPLC-post-column derivatization system for each chromatographic run.

2.2.3. Clinical urine samples

The urine (unknown samples, blank pooled urine for standards and quality control samples) was thawed, gently vortexed and then centrifuged to pellet solids if needed. The urine was put into 2 ml micro centrifuge tubes with snap tops. If the sample volume assayed was less than 1 ml, pooled blank human urine was added to the urine samples to bring the urine volume to 1 ml. 10 μl of 100 μg CVS 1235/ml water was added to each standard and sample as an internal standard for both CVS 738 and for CVS 1123. 750 μl of 100 m*M* sodium citrate, 6 *M* guanidine, pH 3, was added to each sample, and then vortexed gently to mix. The urine solutions were centrifuged at 18 000 *g* in a microcentrifuge for 10 min to separate out any precipitate which could clog the extraction cartridges. As much sample as possible (over 90%) was loaded onto the extraction cartridge without disturbing the pellet of solid at the bottom of the centrifuge tube. The Bond Elut 300 mg C_{18} solid-phase extraction cartridges were conditioned with 1 ml MeOH followed by 2 ml water. The samples were immediately applied to the column and were forced through the column by centrifugation. The extraction cartridges were then washed with 2 ml of water. The CVS 1123, CVS 738, and internal standard were eluted with 1 ml of 0.2% trifluoroacetic acid in methanol. 150 μl of 0.45 *M* NH_4OH was added to each eluate and the samples were then dried under vacuum. The samples were reconstituted in 0.1 ml of 31% methanol–acetonitrile–0.05 *M* phosphate, pH 6 (31:10:59). 90 μl of this solution was injected onto the HPLC-post-column derivatization system.

2.3. HPLC procedures

2.3.1. Rat and monkey toxicology samples

The analysis for the toxicology study samples depended on sequential extraction of CVS 738 and its internal standard, CVS 737, followed by elution of CVS 1123 and its internal standard, CVS 1235. The CVS 737–CVS 738 elution was analyzed on a separate HPLC system than the CVS 1123–CVS 1235 elution. The CVS 737–CVS 738 samples were separated using a methanol–0.05 *M* potassium phosphate, pH 6.0 (40:60) buffer mobile phase. The CVS 1123–CVS 1235 samples were separated using a methanol–acetonitrile–0.05 *M* potassium phosphate buffer, pH 6.0 (25:8:67) mobile phase. The HPLC system (Fig. 4) consisted of an HPLC pump, autosampler and fluorescence detector (Hitachi, San Jose, CA, USA) and a post column derivatization system (Pickering, Mountain View, CA, USA). The mobile phase for each separation was delivered at a flow-rate of 0.3 ml/min through a Symmetry 15 cm×2.1 mm diameter C_8 HPLC column (Waters, Milford, MA, USA) maintained at 60°C. The column was protected by an in-line filter and a 2 cm×2 mm self packed pellicular C_{18} guard column (Upchurch, Oak Harbor, WA, USA). The column effluent entered into the postcolumn derivatization system where it was

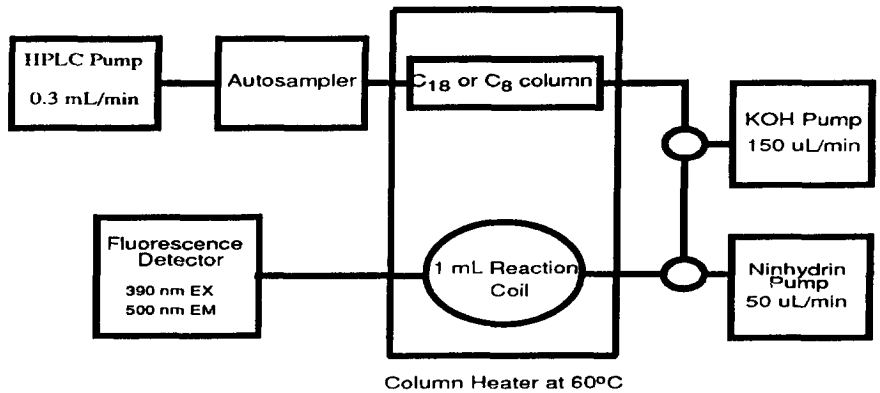

Fig. 4. Block diagram of HPLC system for determination of arginine aldehydes from physiological fluids.

successively mixed with a 0.15 ml/min stream of 0.4 *M* KOH and a 0.05 ml/min stream of 0.125% (w/v) ninhydrin. The liquid passed into a 1 ml reactor coil at 60°C. The samples were detected by a fluorescence detector with excitation at 390 nm, and emission monitored at 500 nm.

2.3.2. Human plasma and urine samples

These chromatographic separations used a single internal standard, CVS 1235, for both CVS 1123 and for CVS 738. We were able to use a single extraction step and a single HPLC separation for these samples. The mobile phase was MeOH–ACN–0.05 *M* potassium phosphate, pH 6 (31:10:59) flowing at a flow-rate of 0.3 ml/min through a Brownlee 22 cm×2.1 mm RP-18 HPLC column (ABI division of Perkin Elmer, Foster City, CA, USA) at 60°C which was protected by an in-line filter and a 2 cm×2 mm self packed pellicular C_{18} guard column (Upchurch). The post-column derivatization system was the same as that used for the rat and monkey samples.

2.3.3. Standard curves

Standard solutions of CVS 1123 and CVS 738 at concentrations of 10.0 μg/ml water were spiked into the plasma to form the calibration curves. The concentrations in plasma extend from the quantification limit (35 ng/0.1 ml plasma for rat and monkey plasma, 17 ng/ml for human plasma) up to the maximum of the curve (1.5 μg/0.1 ml for rat and cynomolgus monkey plasma, 1.5 μg/ml for human plasma). Volumes for the rat and monkey plasma standard curves were 0.1 ml and were 1.0 ml for the human plasma and human urine standard curves. The concentrations in human urine extend from the quantification limit (84 ng/ml urine for CVS 1123, 360 ng/ml urine for CVS 738) up to the maximum of the curve (1.26 μg/ml urine for CVS 1123, 2.88 μg/ml urine for CVS 738). Eight standard points at eight different concentrations were used for the curves in rat and cynomolgus monkey plasma and eight standard points at four different concentrations were used for the curves in human plasma and human urine. The curve was fitted by linear regression of the ratio of the standard peak area to the internal standard peak area versus the amount of drug spiked in plasma. This linear regression equation was used to calculate the concentrations of CVS 1123 and CVS 738 in unknown samples and in quality control samples. The standard curve linear regression was unweighted for the plasma samples and weighted with a $1/x$ value for the urine samples. A new set of standards was extracted and chromatographed for each set of samples.

3. Results and discussion

3.1. Selection of chromatography conditions

Peptides are typically separated on reversed-phase columns using aqueous mobile phases with an ion pairing agent and an organic modifier. The most commonly used ion pairing agent is trifluoroacetic acid, and acetonitrile is the most commonly used organic modifier. The first challenge in the develop-

ment of a quantitative HPLC method for CVS 1123 and for CVS 738 was that arginine aldehydes can exist in three separate forms which separate into three peaks when using reversed-phase chromatography with acetonitrile–water–TFA mobile phases [5,6]. Quantification of such a compound by constructing standard curves using three different chromatography peaks would be difficult. Potential matrix interferences would be more likely to coelute with one of the peaks. In experiments at Corvas it has been observed that preparation conditions, temperature and pH affect the relative amounts of the three different forms. Our solution to this problem was to use HPLC conditions which favor more rapid interconversion of the three forms. We hypothesize that as the compound travels down the HPLC column, the three forms interconvert so that each molecule spends a roughly equivalent amount of time in each form as the other molecules. Through NMR studies published in the literature [15], we found that the interconversion rate increases with higher pH, and our chromatographic studies have shown that higher temperatures speed the interconversion rate. Fig. 5 illustrates the effect of temperature on the peak shape of CVS 1123 in a methanol–0.05 *M* phosphate buffer, pH 6. In order to get the best peak shape, we heat the HPLC column to 60°C, the maximum recommended temperature by column manufacturers. We investigated different mobile phase buffers, including phosphate, ammonium acetate, ammonium formate and Tris buffer at pH units of 6–8. We found the best results, in terms of peak efficiency, were obtained with 0.05 *M* potassium phosphate buffer, pH 6. The use of these conditions gave a single peak for CVS 1123 and for CVS 738. A drawback to the use of this mobile phase is that the silica based column does not last very long (usually about 200 injections). Researchers have shown that neutral pH phosphate buffers at high temperatures (above 40°C) will rapidly degrade silica based columns [7]. We have tried using columns in which the silica is more highly end capped (which should protect the stationary phase better) to improve column lifetime, but the resolution between the internal standard and CVS 1123 suffered. We also tried using a polymeric based column, but this was not satisfactory for column efficiency. Unfortunately, the peaks using our HPLC system are broader than one would expect when using an optimized HPLC assay. This lack of peak efficiency is due to the finite amount of time that the arginine aldehyde spends in each form (the interconversion rate is too slow). Fig. 6 shows a typical chromatogram of CVS 738, CVS 1235 and CVS 1123 extracted from human plasma.

An additional problem with the use of HPLC at 60°C for the separation of samples extracted from physiological fluids is that the pre-column filters in the HPLC system showed a tendency to clog unless great care is taken in sample handling. Our solution to this problem was to reconstitute the samples in mobile phase to prevent solubility differences once the sample is injected, and to centrifuge the reconstituted sample in a microcentrifuge at 18 000 *g* for 10–15 min. This centrifugation step pellets any undissolved material. Care must be taken when transferring the reconstituted sample to the HPLC injection vial to avoid disturbing any pellet on the bottom of the centrifuge vial.

3.2. Post-column derivatization

Ninhydrin reacts with guanidine groups under basic conditions to form a fluorescent product [8–10]. Techniques for derivatization of guanidine containing compounds using ninhydrin were developed as HPLC post-column derivatization methods by workers in Japan [11] and at Smith Kline and French Laboratories [12,13]. The post-column derivatization method was optimized for 0.2 cm diameter columns by the group at Smith Kline and French Laboratories, and was used essentially unchanged in our laboratory. This method allowed us to measure the plasma levels of a large number of guanidine containing compounds. This general applicability to guanidine containing compounds allowed us to screen other compounds structurally related to CVS 1123 for pharmacokinetic parameters and facilitated the selection of a compound for clinical studies.

3.3. Extraction procedures

Our objective in developing this assay was to quantitate both CVS 1123 and CVS 738 in plasma samples from rats, cynomolgus monkeys, and humans and in urine samples from humans. Preliminary extraction studies using C_{18} extraction

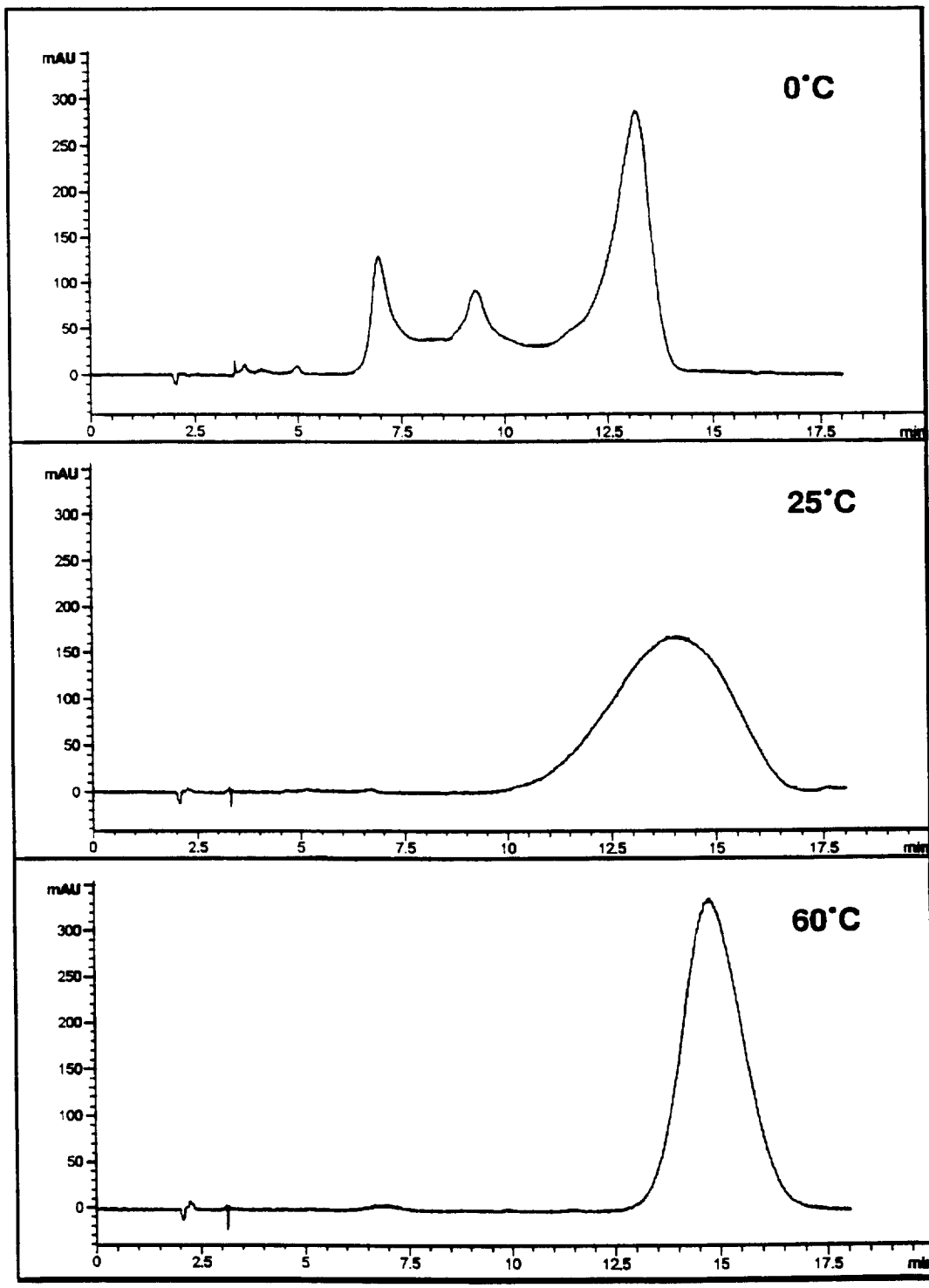

Fig. 5. Effect of temperature variation on CVS 1123 chromatography. The mobile phase is acetonitrile–0.05 *M* potassium phosphate, pH 6.0 (27:73). Column is a Brownlee RP-18 100×2.1 mm. UV detection at 210 nm.

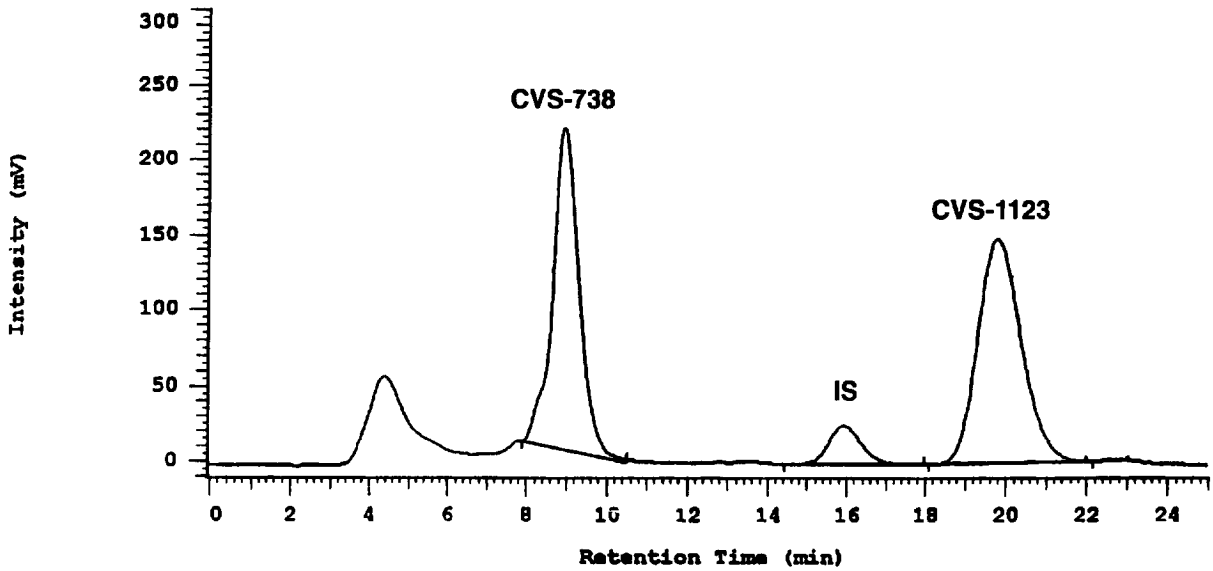

Fig. 6. Separation of CVS 738, Internal Standard (IS), and CVS 1123 extracted from human plasma.

cartridges demonstrated that CVS 738 is more polar than CVS 1123. CVS 738 was not quantitatively retained on the extraction cartridge with a rinsing solution of greater than 10% methanol or acetonitrile, while CVS 1123 could be quantitatively retained using rinses stronger than 50% methanol–water (50:50) solutions. CVS 1123 could also be quantitatively retained on the C_{18} extraction cartridge using a 100% acetonitrile wash, probably because the compound is sparingly soluble in pure acetonitrile. Because of the differences in polarity of CVS 738 and CVS 1123, it was felt that the two compounds would not be well represented by a single internal standard, nor would they chromatograph similarly when using an isocratic HPLC separation system. Thus our earlier assays, those analyzing toxicology samples in rats and in cynomolgus monkeys relied on the use of two internal standards. CVS 738 and its internal standard, CVS 737, were eluted using methanol–water (50:50), while CVS 1123 and its internal standard, CVS 1235, were retained. CVS 1123 and its internal standard, CVS 1235, were eluted using methanol–trifluoroacetic acid (99.8:0.2). This method gave satisfactory results for the toxicology studies, but the added amount of time involved with the sample preparation and two HPLC separations for each plasma sample motivated us to look for a method which would combine the extractions and use a single HPLC separation.

The analysis of the human plasma samples was also complicated by a substantial loss in recovery upon adapting the method used for the toxicology species directly to the human plasma. We also wanted to use a 1 ml volume for plasma analysis, to get better sensitivity for the lower doses used for the clinical study compared to the toxicology studies. Initial investigation showed that the CVS 1123 was not being retained on the extraction cartridge. A systematic study to improve the absolute recovery from human plasma was performed. We used Empore 7 mm C_{18} extraction disk cartridges for these studies. The parameters investigated included addition of various buffer solutions to the plasma. We investigated the effect of buffer pH, salt content, buffer composition and buffer concentration. We tried the addition of 1 *M* guanidine·HCl to disrupt protein binding and the addition of Biocryl to remove lipids from solution. The best results from these experiments were drug compound recoveries of about 60%, obtained from the use of addition of 0.1 *M* sodium citrate buffer, pH 3.2, to the plasma. Testing this solution with other extraction cartridges led to our choice of the Empore 7 mm SDB

Table 1
Absolute recovery of CVS 1123 and CVS 738 from rat, monkey and human plasma and from human urine

	CVS 1123	CVS 738
Rat		
Mean	0.851	0.776
Standard deviation	0.079	0.054
Cynomolgus monkey		
Mean	0.584	NDC
Standard deviation	0.028	NDC
Human urine		
Mean	0.600	0.532
Standard deviation	0.079	0.071
Human plasma		
Mean	0.730	0.607
Standard deviation	0.081	0.066

Recoveries are calculated as the ratio of the peak area for an extracted sample to the peak area for the neat standard, calculated for at least eight samples.
NDC=no data collected.

extraction disk cartridge. By combining the citrate dilution buffer with the SDB cartridge, we were able to increase the absolute recovery of CVS 1123 to about 70% from 1 ml volumes of human plasma. A summary of the absolute recoveries of CVS 738 and CVS 1123 is shown in Table 1.

The Empore extraction disk cartridges had the advantage that less solvent was used for conditioning, rinsing and eluting the columns compared to conventional packed extraction cartridges. The smaller elution solvent volume saved great amount of time in drying the eluted samples before reconstitution for HPLC separation. We used the Bond Elut 300 mg C_{18} extraction cartridges for the urine extraction because our experimentation demonstrated that we had slightly better recoveries with these columns.

The most difficult compound to assay was the determination of CVS 738 in human urine. The urine contained high levels of endogenous compounds which eluted at about the same retention time as CVS 738 (see Fig. 7). These compounds also responded to the same excitation and emission fluorescence detector settings of 390 nm excitation and 500 nm emission, even without the postcolumn derivatization system in place. These endogenous compounds elute from the extraction cartridge with a 20% organic wash, but we were not able to retain the CVS 738 on the extraction cartridge at this strength of a wash. We were not able to fully separate the CVS 738 from the interferences and were forced to accept an overlap of the tail of the pigment peak with CVS 738. This overlap forced us to accept a higher

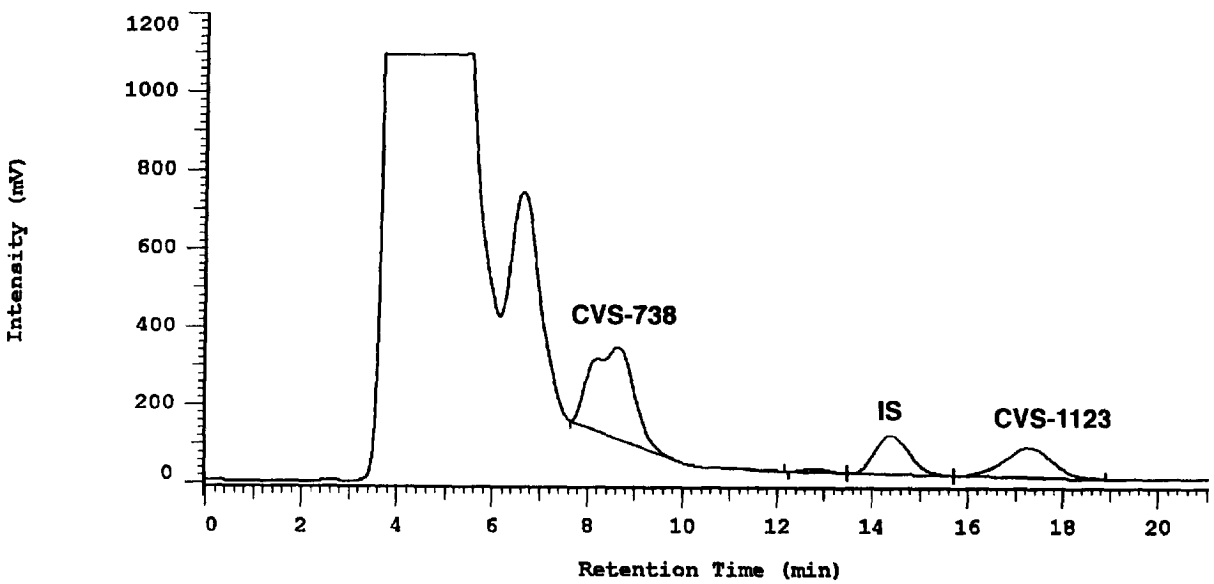

Fig. 7. Separation of CVS 738, Internal Standard (IS), and CVS 1123 extracted from human urine.

quantification limit (360 ng/ml) for CVS 738. Because the CVS 1235 peak and CVS 1123 peak were separated from the interferences by several min, their quantification was not affected.

3.4. Esterase activity in rat plasma

We found that the aspartic acid methyl ester in CVS 1123 was susceptible to hydrolysis by esterases present in rat plasma to give CVS 738. Substantial conversion of the methyl ester is observed upon incubation of CVS 1123 in citrated rat plasma for 1 h at 37°C. This conversion was not observed in the plasma of other species. The purpose of adding NaF to the frozen plasma is to prevent conversion of the aspartic acid methyl ester to aspartic acid by the inhibition of esterase activity in the plasma. Table 2 shows the effect of 10 m*M* NaF to protect CVS 1123 in rat plasma. Analysis of rat plasma which had been incubated with 10 m*M* NaF and CVS 1123 showed no conversion to CVS 738.

Table 2
Effect of NaF to protect CVS 1123 from conversion to CVS 738 in rat plasma by esterase activity

	Low	Mid	Hi
No NaF			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	50.1	324	542
Standard deviation	8.42	36.3	114
%R.S.D.	16.8	11.2	21.1
%Accuracy	49.9	56.8	54.9
With NaF			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	81	719	1120
Standard deviation	5.94	23.9	97.6
%R.S.D.	7.33	3.32	8.7
%Accuracy	19	4.16	6.49

The no NaF samples were spiked with CVS 1123, frozen, thawed and analyzed, while the With NaF samples were 10 m*M* NaF in the plasma before the plasma was spiked with CVS 1123, frozen, thawed and analyzed. The %R.S.D. is given by 100·standard deviation/mean. The %Accuracy is given by 100·(Amount Spiked−Mean)/Amount Spiked.

Table 3
Summary of the precision and accuracy determinations of CVS 1123 and CVS 738 in rat plasma

	Low	Mid	Hi
CVS 1123			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	81	719	1120
Standard deviation	5.94	23.9	97.6
%R.S.D.	7.33	3.32	8.7
%Accuracy	19	4.16	6.49
CVS 738			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	104	718	1162
Standard deviation	10	13.17	32.12
%R.S.D.	9.57	1.84	2.76
%Accuracy	−4.61	4.33	3.14

3.5. Results of validation study

The methods for analysis of CVS 738 and CVS 1123 from toxicology and clinical studies were validated in our laboratories for dynamic range, precision, accuracy, specificity, plasma stability and stability in injection solvent using accepted performance requirements for bioanalytical assays [14]. The precision and accuracy results for the quality control samples for the four assays are shown in Tables 3–6.

4. Conclusion

We developed and validated methods for the analysis of the thrombin inhibitor CVS 1123, and its desmethyl form, CVS 738, in rat, monkey and human plasma and in human urine. The problem of multiple forms of the compound was overcome by selecting chromatographic conditions which promoted rapid interconversion of the forms. Sensitivity levels of 17 ng CVS 1123/ml human plasma and good selectivity was obtained by using a postcolumn derivatization method which is specific for guanidine groups. These methods are simple, quick and selective, and allowed us to analyze samples from clinical

Table 4
Summary of the precision and accuracy determinations of CVS 1123 and CVS 738 in cynomolgus monkey plasma

	Low	Mid	Hi
CVS 1123			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	103	769	1250
Standard deviation	6.44	64.2	104
%R.S.D.	6.24	8.35	8.33
%Accuracy	−3.18	−2.51	−4.43
CVS 738			
Amount spiked (ng/0.1 ml)	100	750	1200
Mean assay result (ng/0.1 ml)	98.5	775	1253
Standard deviation	12.1	85.8	80.2
%R.S.D.	12.3	11.1	6.4
%Accuracy	1.48	−3.4	−4.43

Table 6
Summary of the precision and accuracy determinations of CVS 1123 and CVS 738 in human plasma

	Low	Mid	Hi
CVS 1123			
Amount spiked (ng/0.1 ml)	100	750	1250
Mean assay result (ng/0.1 ml)	98.8	707	1340
Standard deviation	12.6	69.7	87.5
%R.S.D.	12.8	9.86	6.51
%Accuracy	1.23	5.73	−7.53
CVS 738			
Amount spiked (ng/0.1 ml)	100	750	1250
Mean assay result (ng/0.1 ml)	106	738	1274
Standard deviation	10.6	59.5	110
%R.S.D.	10	8.07	8.66
%Accuracy	−5.81	1.64	−1.93

and toxicology studies of CVS 1123. We were able to quantify the compounds below levels which gave a measurable biological effect. The data generated using these methods allowed the determination of such pharmacokinetic parameters as area under the curve, time of maximum concentration, maximum concentration level and half life. The described bioanalytical assay is generally applicable to guanidine containing structures. It has proven to be highly useful in monitoring pharmacokinetic parameters of a large number of our inhibitor structures and helped us greatly in the selection of candidates for preclinical and clinical studies.

Table 5
Summary of the precision and accuracy determinations of CVS 1123 and CVS 738 in human urine

	Low	Mid	Hi
CVS 1123			
Amount spiked (ng/0.1 ml)	200	700	1200
Mean assay result (ng/0.1 ml)	198	580	997
Standard deviation	20.5	47.3	81.7
%R.S.D.	10.4	8.15	8.2
%Accuracy	1.17	17.2	17
CVS 738			
Amount spiked (ng/0.1 ml)	600	2000	4000
Mean assay result (ng/0.1 ml)	592	1680	3420
Standard deviation	115	173	298
%R.S.D.	19.4	10.3	8.72
%Accuracy	1.37	15.8	14.6

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