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Short Communication

Determination of DMP 728, a IIb/IIIa receptor antagonist, in rat and dog plasma by high-performance liquid chromatography with fluorimetric detection

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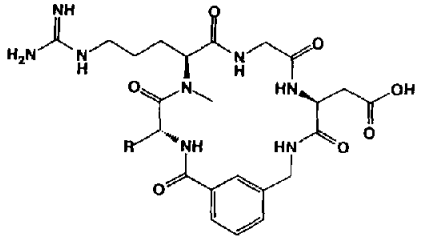
Abstract

A specific and sensitive HPLC assay for the determination of DMP 728 in dog and rat plasma has been developed. The method involves solid-phase extraction of DMP 728 and the internal standard from plasma using a C_2 column. The extracted compounds are derivatized with benzoin under alkaline conditions. Using a mixture of acetonitrile and 0.1 *M* potassium phosphate buffer (25:75, v/v, pH 7.4) as mobile phase, the derivatized products are separated on a Regis semipermeable surface C_8 column and monitored fluorometrically using 325 nm and 425 nm as excitation and emission wavelengths, respectively. The assay is linear from 2.5 to 1000 ng/ml in dog plasma and from 5 to 1000 ng/ml in rat plasma. The limit of quantitation is 2.5 ng/ml using 0.5 ml of dog plasma and 5 ng/ml using 0.5 ml of rat plasma. The assay has been used in pharmacokinetic studies of DMP 728 in dogs and rats.

1. Introduction

Cyclo(D-2-aminobutyryl-L-N-methylarginyl-glycyl-L-aspartyl-*m*-aminomethylbenzoic acid) (DMP 728 I) (Fig. 1), is a potent platelet glycoprotein IIb/IIIa receptor antagonist [1,2]. A sensitive assay method is required for pharmacokinetic studies. I does not have a highly absorbing chromophore and thus high performance liquid chromatography (HPLC) with ultraviolet detection is not sensitive enough (detection limit = 25 ng/ml using 1 ml plasma) to achieve reliable quantitation of low ng/ml ranges from biological matrices. However, the guanidine or the carboxylic acid moiety can be derivatized to give a fluorescent product which would allow for a more sensitive detection. This



DMP 728: R = ethyl
DMP 757: R = isopropyl

Fig. 1. Chemical structures of I (DMP 728) and the internal standard (DMP 757).

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paper describes a HPLC-fluorescence detection method.

2. Experimental

2.1. Materials

I and internal standard, DMP 757 (Fig. 1), both as mesylate forms, were synthesized at The DuPont Merck Pharmaceutical Company (Wilmington, DE, USA. Benzoin, 2-mercaptoethanol, 2-methoxyethanol, Tris-HCl were purchased from Aldrich (Milwaukee, WI, USA). Dibasic potassium phosphate was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade acetonitrile was purchased from E.M. Science (Gibbstown, NJ, USA). All other reagents were of analytical reagent grade and were used without further purification.

2.2. Instrumentation

The HPLC system consisted of two Model 510 pumps, a gradient controller (Waters, Milford, MA, USA), a Regis semi-permeable surface octyl reversed-phase column (5 μm, 150 × 4.6 mm I.D.; Morton Grove, IL, USA) and a Shimadzu RF 551 fluorescence detector (Columbia, MD, USA). The mobile phase consisted of acetonitrile–0.1 *M* potassium phosphate (25:75, v/v, pH 7.4). The flow-rate was 1.5 ml/min. The excitation and emission wavelengths of the detector were set at 325 nm and 425 nm, respectively. The data system consisted of an A/D converter (Model 960, Nelson Analytical, Cupertino, CA, USA), a 16-bit desk-top computer (Model 9816, Hewlett-Packard, Avondale, PA, USA), a hard disk drive (Model HP 9133C, Hewlett-Packard), a dot-matrix printer (Hewlett-Packard, Thinkjet Model 2225A), and Xtrachrom 4400X data acquisition software (version 7.2, Nelson Analytical).

2.3. Preparation of plasma samples

A C_2 solid-phase extraction column (Bond Elut, 100 mg obtained from Varian, Sunnyvale, CA, USA) was used to extract the compounds from plasma. The column was conditioned sequentially with 2 × 1 ml of methanol and 2 × 1 ml of water. Plasma (0.5 ml) containing I and the internal standard was then loaded onto the column. The column was washed with 2 × 1 ml of water followed by 3 × 1 ml of methylene chloride–acetonitrile (20:80, v/v). The column was gently vacuum-dried for 5 min and the analytes were then eluted with methanol (2 × 1 ml). The combined eluates were evaporated to dryness under nitrogen. The residue was subjected to pre-column derivatization.

2.4. Extraction recovery

The extraction recovery from plasma was determined by comparing the peak area of the extracted sample with that of an unextracted reference standard of identical concentration. The extracted and unextracted aliquots were analyzed on a Waters μBondapak phenyl column (300 × 3.9 mm I.D.) using acetonitrile–0.01 *M* phosphoric acid (11:89, v/v) as mobile phase and monitored for UV absorbance at 210 nm. With a 1.5 ml/min flow-rate, the retention time of I was approximately 12.8 min.

2.5. Pre-column derivatization procedure

To the dried extracted sample, 50 μl of water, 25 μl of 4 m*M* benzoin in 2-methoxyethanol, 25 μl of an aqueous solution containing 0.1 *M* mercaptoethanol, 0.2 *M* sodium sulfite, and 50 μl of 0.4 *M* potassium hydroxide were added. The mixture was briefly vortex-mixed and heated at 45°C for 20 min. The mixture was then cooled in ice water for 1 min after which 20 μl of a mixture of 0.5 *M* Tris HCl and 0.8 *M* HCl (1:1, v/v) were added. The resulting mixture was vortex-mixed for 1 min; a 75 μl aliquot was injected onto the HPLC column and analyzed by fluorometric detection.

2.6. Calibration

The peak-area ratios of I relative to the internal standard of the extracted unknown sam-

ples were evaluated against those of spiked standards. Two unweighted linear regression curves were used to determine slopes, intercepts, and correlation coefficients for each standard curve. One regression curve was used to evaluate standard concentrations ranging from 2.5 (or 5) to 100 ng/ml (low standard curve) and the second regression curve was used to evaluate standard concentrations ranging from 100 to 1000 ng/ml (high standard curve). The concentration of I in the unknown sample was calculated by the following equation:

Concentration
= (Peak-area ratio − intercept)/slope

2.7. Pharmacokinetic study in dogs

Female beagle dogs, approximately 8–10 months old weighing 9–13 kg at the time of dosing (Hazleton Research Products, Kalamazoo, MI, USA) were used in the studies. The animals were housed in stainless steel metabolic cages equipped with automatic watering valves. The animal room environment was controlled (temperature 20 ± 3°C; humidity 50 ± 20%; 12 h light and 12 h dark cycle; 10–15 air changes per h). The animals received standard certified commercial dog food (400 g Wayne certified Dog Chow No. 8727) 4 h post-dose over a 2-h feeding period.

Four female dogs received I as a single intravenous dose via jugular vein at 1.0 mg/kg in normal saline (0.1 ml/kg). Blood samples (5 ml) were collected from the jugular vein by venipuncture at pre-dose and at various time periods into tubes containing heparin as anticoagulant. The plasma was harvested following centrifugation (for 15 min at 150 *g*). Platelet-rich plasma samples were stored frozen at −20°C prior to analysis for I.

3. Results

3.1. Chromatography and specificity

Typical chromatograms obtained from control dog and rat plasma and plasma spiked with I are shown in Figs. 2 and 3, respectively. The retention times for I and internal standard were approximately 10 and 12 min, respectively. No interfering peaks were observed in the chromatograms obtained from either dog or rat plasma blanks.

3.2. Linearity

The calibration functions of I in rat plasma for low (5–100 ng/ml) and high (100–1000 ng/ml) curves were as follows: $y = 0.0007x + 0.002$, $r^2 = 0.998$ and $y = 0.0007x - 0.0116$, $r^2 = 0.999$, re-

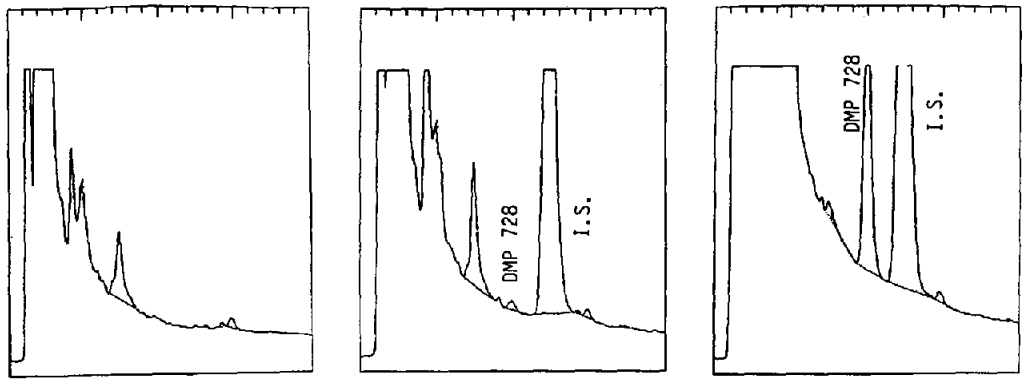

Fig. 2. Representative chromatograms of blank dog plasma (A), blank dog plasma spiked with 5 ng/ml of I (B), and a dog plasma sample obtained 2 h after an i.v. bolus dose of I at 1.0 mg/kg (C).

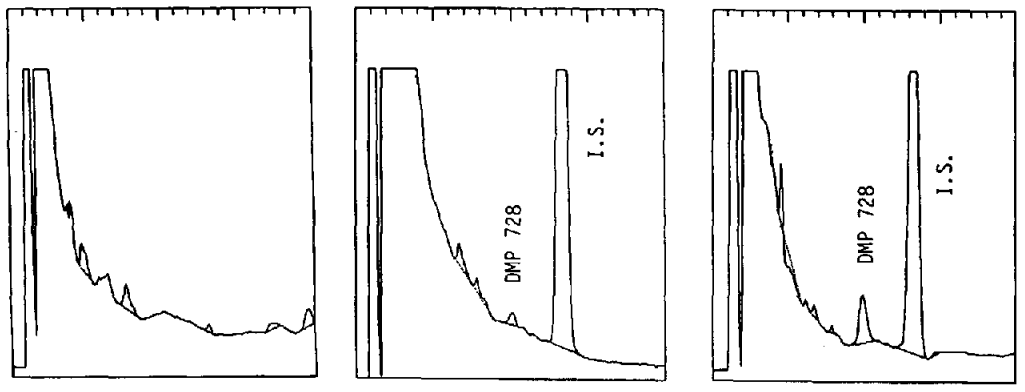

Fig. 3. Representative chromatogram of blank rat plasma (A), blank rat plasma spiked with 5 ng/ml of I (B), and a rat plasma sample obtained 2 h after i.v. bolus dose of I at 1.0 mg/kg (C).

spectively. The calibration functions in dog plasma for low (2.5–100 ng/ml) and high (100–1000 ng/ml) curves were as follows: $y = 0.0025x + 0.0071$, $r^2 = 0.998$ and $y = 0.0031x - 0.0828$, $r^2 = 0.999$, respectively. Excellent linearity was exhibited in both types of plasma samples.

3.3. Intra- and inter-day reproducibility

Six sets of rat or dog samples at four I concentrations were analyzed on the same day to obtain intra-day reproducibility data. The results are summarized in Table 1. The coefficients of variation ranged from 3.3 to 8.6% and from 4.9 to 12.6% for rat and dog plasma, respectively. Four concentration levels in rat or dog plasma were analyzed on three different days to obtain interday reproduciblity data. The results are summarized in Table 2. The coefficients of variation ranged from 4.9 to 12.4% and from 6.9 to 9.0% for rat and dog plasma, respectively.

3.4. Extraction recovery

The extraction recovery of I was determined from four extracted samples and three unextracted reference standards. The results are

Table 1
Intra-day precision data for I in plasma

Spiked concentration (ng/ml)	Found concentration (ng/ml) (Mean ± S.D., $n = 6$)	C.V. (%)
Rat		
5	5.2 ± 0.4	8.6
25	25.3 ± 0.8	3.3
100	101.0 ± 3.8	3.8
500	503.3 ± 33.7	6.7
Dog		
2.5	2.4 ± 0.1	4.9
25	24.6 ± 1.9	7.9
100	106.0 ± 7.5	7.1
500	494.6 ± 62.3	12.6

Table 2
Inter-day precision data for I in plasma

Spiked concentration (ng/ml)	Found concentration (ng/ml) (Mean ± S.D., $n = 3$)	C.V. (%)
Rat		
5	4.5 ± 0.6	12.4
25	22.9 ± 2.1	10.1
100	91.6 ± 8.4	9.2
500	522.0 ± 25.7	4.9
Dog		
2.5	2.5 ± 0.2	8.7
25	24.1 ± 1.7	6.9
100	100.2 ± 9.0	9.0
500	491.4 ± 35.1	7.1

Table 3
Extraction recovery of I from plasma

Spiked concentration (ng//ml)	Recovery (%)	
	Dog	Rat
5	54.48	63.75
250	58.47	54.04
1000	62.24	52.06

shown in Table 3. The recovery was in the range of 50 to 65% in both rat and dog samples.

3.5. Optimization of the reaction temperature and length of reaction time

Using 0.8 *M* potassium hydroxide, the derivatization was performed at 45, 55, 65, and 85°C for different periods of time. The results, as shown in Fig. 4, indicated that 45°C for 20 min was the most favorable set of conditions.

3.6. Optimization of the concentration of potassium hydroxide

To determine the concentration of potassium hydroxide to use in the reaction, the derivatization was carried out at 45°C for 20 min using four different concentrations (ranged from 0.2 to 0.8 *M*) of potassium hydroxide solution. The results, as shown in Fig. 5, indicated that 0.4 *M* appeared to be the optimal concentration.

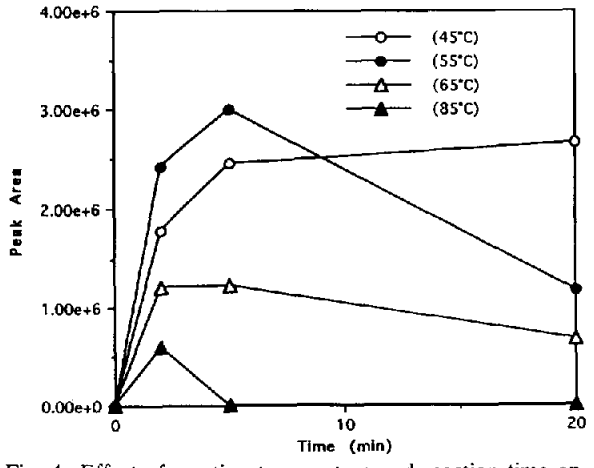

Fig. 4. Effect of reaction temperature and reaction time on fluorescence intensity of derivatized I.

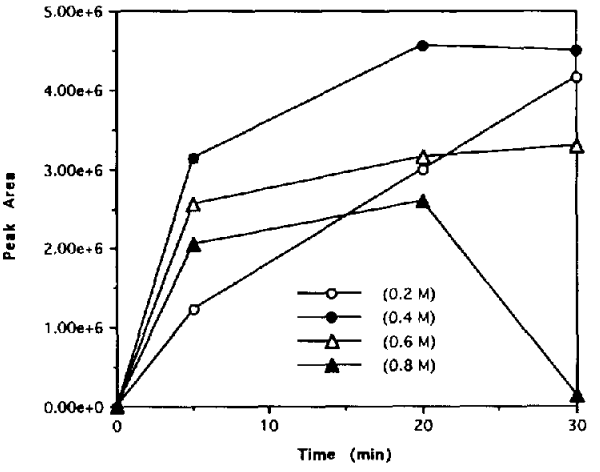

Fig. 5. Effect of potassium hydroxide concentration on the fluorescence intensity of derivatized I at 45°C.

3.7. Pharmacokinetic study in dogs

The mean plasma concentrations of I in beagle dogs after a single intravenous bolus dose of 1 mg/kg I (equivalent to zwitterion) are illustrated in Fig. 6. The mean (±S.D.) terminal elimination half-life, steady-state volume of distribution, and systemic plasma clearance were 4.7 ± 1.4 h, 0.8 ± 0.1 l/kg and 4.3 ± 0.7 ml/min/kg, respectively. The plasma concentration could be quantitated (>10 ng/ml) 16 h after dosing. No interfering peaks were observed in the pre-dose samples. The results indicated that the assay was sensitive and specific.

4. Discussion

The very hydrophilic nature of I and the internal standard excludes the use of liquid–liquid extraction as a sample cleanup procedure from plasma. Solid-phase extraction, therefore, in the method of choice for this assay. Among various solid-phase columns tested, C_2 columns give the cleanest background while maintaining a reasonable extraction recovery.

The HPLC-UV detection method does not

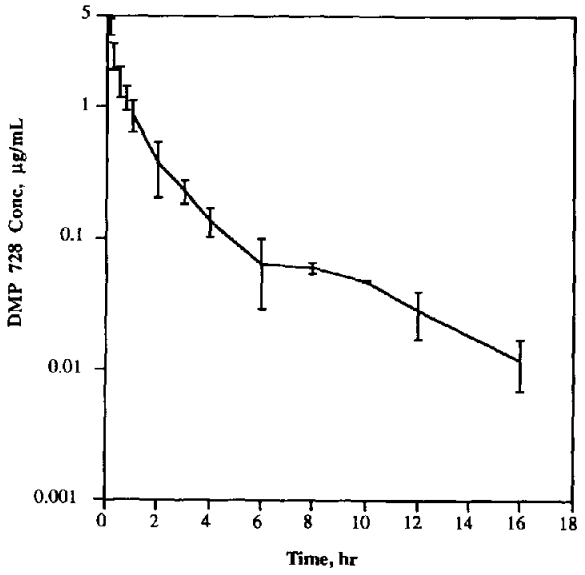

Fig. 6. Mean (±S.D.) plasma concentration–time profile of I following an i.v. bolus dose of 1 mg/kg in female beagle dogs ($n = 4$).

give adequate sensitivity for pharmacokinetic studies. The enhancement of detection sensitivity is achieved by fluorescence detection of the derivative of I. Either the guanidine or the carboxylic acid group of I can be derivatized. The guanidine group was selected, as many endogenous carboxylic acids exist in plasma and the guanidine group can specifically react with benzoin to give a fluorescent compound. The reaction specificity in conjunction with HPLC separation and fluorescence detection would ensure that the present assay is specific.

Since I is a tripeptide and the derivatization is performed in alkaline conditions, it was anticipated that the peptide linkage of I would be susceptible to hydrolysis. Therefore, mild reaction conditions are essential in order to preserve the chemical integrity of I. The reaction between guanidine and benzoin is highly pH and temperature dependent. The optimization of the derivatization was performed by varying the concentration of potassium hydroxide solution, the length of reaction time, and the reaction temperature. It was observed that performing the reaction at 45°C for 20 min using 0.4 *M* of potassium hydroxide gave the most favorable results. The benzoin derivatization forms a highly fluorescent 2-substituted amino-4,5-diphenylimidazole compound [3–8]. The fluorescence intensity of the derivatization product is also pH dependent. It fluoresces most intensely in a weak alkaline solution (pH 8.5–10.0) [5]. Among the reversed-phase columns tested, the Regis semi-permeable surface C_8 column was found to give the best resolution. A pH of 7.4 for the mobile phase was a compromised selection to achieve a high fluorescence intensity while maintaining column stability. As some other lipophilic compounds were also formed during derivatization, the column was washed with 60% aqueous acetonitrile for 4 min after the desired products were eluted; the column was re-equilibrated with mobile phase before next analysis. The total analysis time was approximately 35 min per sample. The present assay method was linear between 5 and 1000 ng/ml using 0.5 ml of rat plasma and from 2.5 to 1000 ng/ml using 0.5 ml of dog plasma. The quantitation limit was 5 and 2.5 ng/ml using 0.5 ml of rat and dog plasma, respectively. The present method has been applied to the analysis of preclinical samples after i.v. and oral administration.

5. Conclusion

The present assay, using a C_2 column for solid-phase extraction and employing benzoin as a pre-column derivatization reagent, has successfully enhanced the detection sensitivity of I. The assay is also specific and reproducible and has been used to analyze I in rat and dog plasma samples from various pre-clinical studies.

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