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Determination of a platelet activating factor antagonist (CV-3988): methodology and clinical application

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SUMMARY

A high-performance liquid chromatographic method was developed for determination of the platelet activating factor antagonist CV-3988 in human plasma and urine After development of a column extraction procedure without an internal standard, a more satisfactory organic extraction procedure was set up with amiodarone as internal standard. Linearity of the calibration curves was found in the range 0 0625–10 μg/ml CV-3988. Reproducibility was higher than 10% for the column extraction and lower than 10% for the organic extraction procedure. Recovery of CV-3988 from plasma averaged 81.7% for the column procedure and 40% for the organic extraction. Urine samples could be extracted only by the organic extraction procedure. The organic extraction procedure was applied to the determination of CV-3988 in plasma and urine samples after intravenous administration to normal volunteers.

INTRODUCTION

RS-2-Methoxy-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazolio)ethyl-phosphate (CV-3988, I) is a specific antagonist of platelet activating factor (PAF), a putative mediator in various conditions such as inflammation, allergy and thrombosis [1,2].

Recently we administered I intravenously to human volunteers [3]. The

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substance was shown to inhibit PAF-induced platelet aggregation ex vivo. We describe here the determination of I in human plasma and urine samples by selective, sensitive and accurate high-performance liquid chromatography with ultraviolet detection (HPLC–UV) and discuss the advantages of the organic extraction procedure versus the preliminary column extraction method.

EXPERIMENTAL

Reagents

Compound I was obtained from Takeda Chemical Industries (Osaka, Japan) and amiodarone from Labaz-Sanofi (Brussels, Belgium). Methanol and chloroform were HPLC grade and supplied by Alltech (Deerfield, IL, U.S.A.). All other chemicals were analytical-reagent grade.

Stock solutions of 1 g/l I and 1 g/l amiodarone were prepared in absolute methanol and stored at −20°C. This solution of I was used to prepare daily fresh dilutions in methanol for adding to plasma samples in the range 0.0625–10 mg/l. The stock solution of amiodarone was diluted to 1 or 2 mg/l in methanol, and these dilutions remained stable for several weeks stored at 4°C. The structures of I and amiodarone are illustrated in Fig. 1.

Bond-Elut C_{18} columns (column capacity 3 ml) and a Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Instrumentation and chromatographic conditions

A modular SP 8700 liquid chromatograph (Spectra Physics, San Jose, CA, U.S.A.) was used in combination with a WISP (Waters Intelligent Sample Processor) automatic injector (Waters Assoc., Milford, MA, U.S.A.). Sepa-

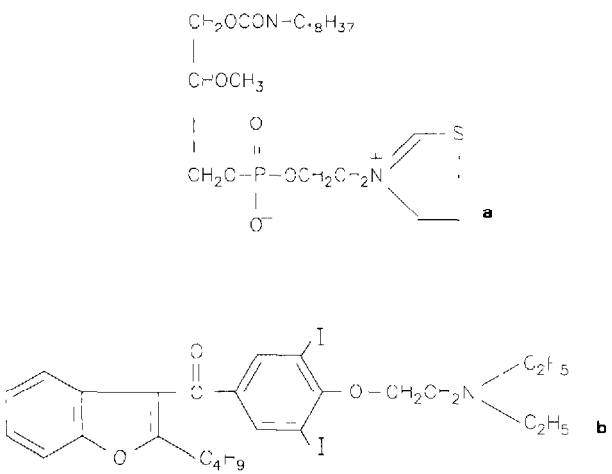

Fig. 1. Structures of (a) I and (b) amiodarone.

rations were achieved by use of a 125×4 mm I.D. reversed-phase Lichrosorb RP-2 column, 5 μm particle size (Merck, Darmstadt, F.R.G.).

The mobile phase was methanol–acetonitrile–10 m*M* phosphate buffer (pH 5.6) (62:13:25, v/v). The eluent was filtered through a Millipore-type FH 0.5-μm filter and degassed continuously with helium. The flow-rate was 1 ml/min.

An SPD-6A variable-wavelength UV detector (Shimadzu, Kyoto, Japan) was set to 0.002 a.u.f.s., and a wavelength of 238 nm was used. The detector was attached to an SP-4270 integrator (Spectra-Physics) for peak-height recording and data reductions.

Extraction procedure for plasma

Column extraction. Compound I was extracted from plasma using Bond-Elut C_{18} extraction columns (3 ml). The columns were positioned in the luer fittings in the Vac-Elut cover. Ten columns could be used at a time. A vacuum of 33–40 kPa was applied to the manifold to elute the different washes. After activation of the columns with two 3-ml column volumes of methanol and water, respectively, the diluted plasma sample [1 ml of plasma and 5 ml of a mixture of hydrochloric acid 0.1 *M* and methanol (1:1, v/v)], was applied to the column. The sample was passed through the column under vacuum, and the column was washed twice with 3 ml of water and twice with 3 ml of acetonitrile, with the column being allowed to empty between each wash. Following the last wash, the vacuum was maintained until the column was dry. The cover of the manifold was then removed, and the stainless-steel needles of the Vac-Elut cover were wiped to remove drops of washing solution. Appropriately labelled glass collection tubes were positioned under each column. The compound was then eluted by applying two 1-ml volumes of methanol. The combined eluents were evaporated to dryness with nitrogen in a water-bath at 40°C. The residue was dissolved in 200 μl of the mobile phase, and portions between 50 and 80 μl were injected into the analytical column.

Organic extraction. To 1 ml of plasma were added 2 ml of 0.1 *M* hydrochloric acid, 35 μl of the internal standard solution (amiodarone, 1 mg/l) and 10 ml of chloroform–methanol (8:2, v/v). After shaking for 15 min and centrifuging for 10 min at 2000 *g* at 4°C, the aqueous layer was aspirated and discarded. The organic phase was transferred to a conical glass tube and evaporated to dryness with nitrogen in a water-bath at 40°C. The residue was dissolved in 200 μl of the mobile phase, mixed on a vortex-mixer for 15 s and centrifuged for 10 min at 2000 *g*. The supernatant was transferred to the injection vials, and portions of 20–50 μl were injected into the column.

Extraction procedure for urine

Compound I was extracted from urine samples by a procedure similar to that for plasma samples with slight modifications, to obtain cleaner chromatograms

without interfering endogenous urine compounds. To 1 ml of urine were added 1 ml of 0.05 *M* hydrochloric acid, 35 μl of the internal standard solution (amiodarone, 2 mg/l) and 7 ml of chloroform–methanol (7:3, v/v). After shaking for 10 min and centrifuging for 10 min at 2000 *g*, the upper aqueous layer was discarded and the organic layer washed with 2 ml of a mixture of methanol–water (5:4, v/v). After shaking for 5 min and centrifuging for 5 min at 2000 *g*, the organic layer was transferred to a conical glass tube and evaporated at 40 °C in a water-bath with nitrogen. The residue was dissolved in 200 μl of the mobile phase, and aliquots of 5–40 μl were injected into the column.

Preparation of working standards

For plasma and urine standards, 50 μl of dilutions of 100, 40, 20, 10, 5, 2.5 and 1.25 mg/l I in methanol were added to drug-free human plasma or urine, resulting in concentrations of I between 5 and 0.0625 mg/l. For amiodarone, working solutions of 1 or 2 mg/l in methanol were prepared.

Quantitative analysis

Quantitative analysis was based on peak-height ratios of the signal of I versus that of the internal standard amiodarone. Ratios for the standards were plotted against the known amounts of I, and an unweighted least-squares linear regression analysis was performed. Using the regression parameters of the calibration curve, the concentrations of I were calculated.

Sample collection and storage

Plasma and urine samples were obtained from healthy male volunteers who had fasted overnight and had not taken any medication for the previous two weeks. Each volunteer received on two treatment days, separated by a washout period of at least seven days, an intravenous (i.v.) infusion of I during 1 h, respectively 1500 and 2000 μg/kg body weight.

Blood samples for pharmacokinetics were taken at the end of the 1-h infusion (0 h), and at 1, 4, 8 and 24 h post-infusion. Urine was collected at fractionated intervals during 24 h post-infusion. Plasma and urine samples were stored at −20 °C until analysis.

RESULTS

Plasma analysis

Figs. 2 and 3 show chromatograms obtained from a drug-free plasma sample and from a plasma sample with added I, extracted by the column procedure and by the organic extraction procedure. As can be seen, there are no interfering peaks at retention times corresponding to those of amiodarone and I.

Calibration curves of I for both extraction methods were linear in the range from 62.5 μg/l to 10 mg/l. Linear regression equations and correlation coeffi-

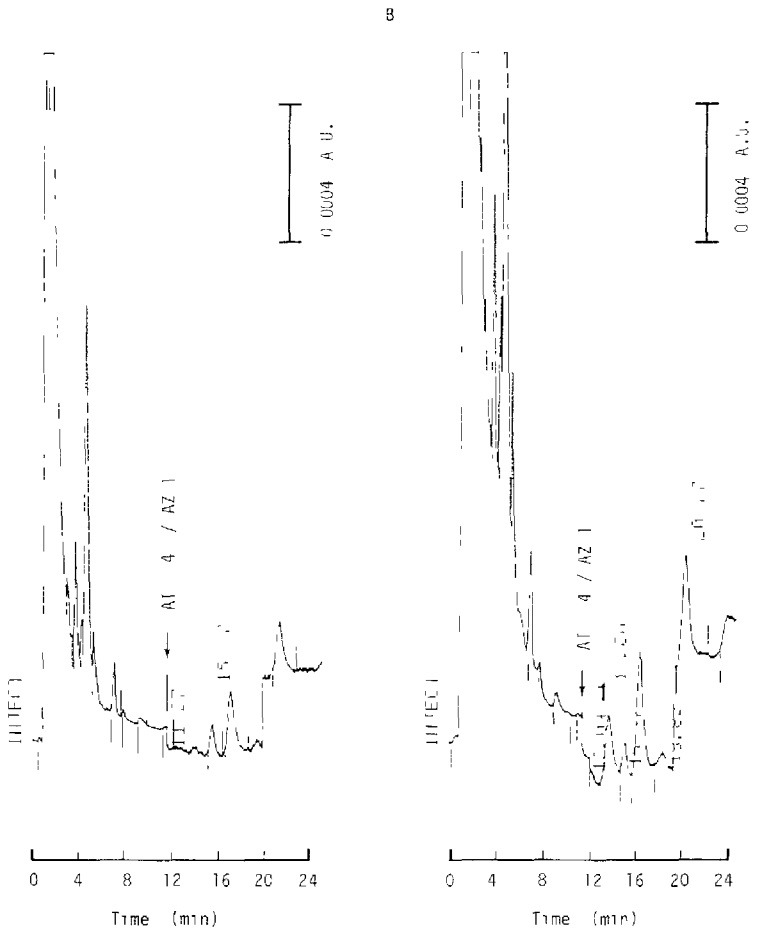

Fig. 2. Chromatograms of (A) drug-free plasma and (B) plasma with added I (0.125 mg/l) (peak 1), following the column extraction procedure. AT = attenuation, AZ = autozero.

cients for the column procedure and the organic extraction procedure were, respectively: $y = 81.2 + 3.9x$, $r = 0.9957$ (peak height versus concentration) and $y = -0.04 + 0.0006x$, $r = 0.9994$ (peak-height ratio versus concentration). Under these conditions, the lower limit of quantitation is 30 μg/l, with a signal-to-noise ratio of 3.

The reproducibility was determined, for the column procedure, by calculating the response factors (peak height/ng I) over a concentration range from 62.5 μg/l to 5 mg/l, for plasma samples analysed in duplicate (Table I).

For the organic extraction procedure, the reproducibility was determined by assaying six plasma samples, with concentrations of 0.125, 0.5 and 2 mg/l (Table I). The data in Table I show that for the column extraction procedure the

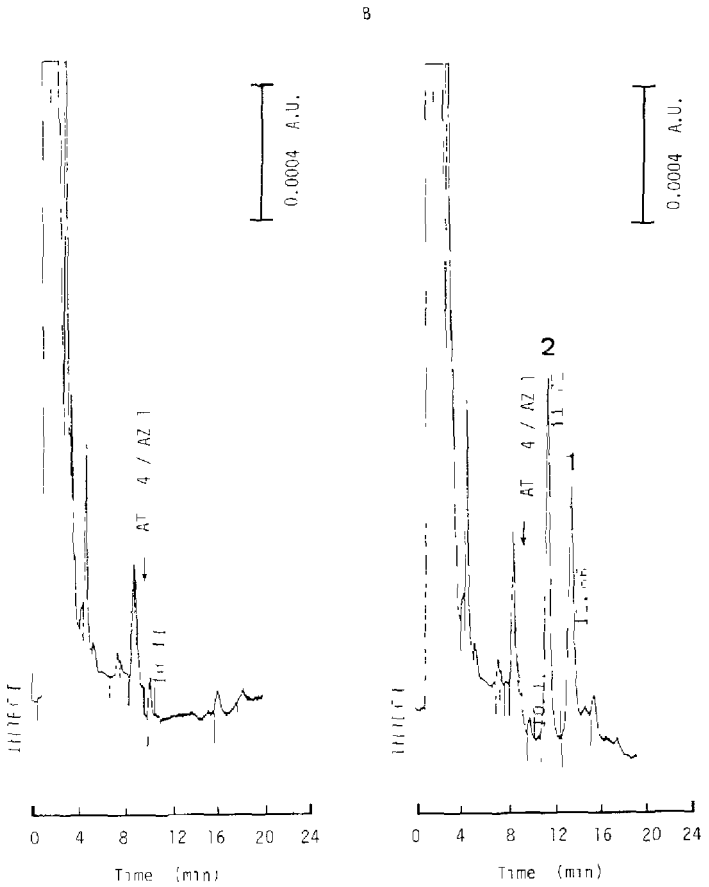

Fig. 3. Chromatograms of (A) drug-free plasma and (B) plasma with added I (1 mg/l) (peak 1) and amiodarone (0.07 mg/l) as internal standard (peak 2) following the organic extraction procedure. AT = attenuation, AZ = autozero.

coefficient of variation (C.V.) was greater than 10%, but for the organic extraction procedure it was less than 10%.

The recovery of I in the range from 62.5 μg/l to 10 mg/l was obtained by comparing peak heights after injection of non-extracted and extracted solutions. For the column extraction the recovery averaged 81.7%, and for the organic extraction it was ca. 40%.

Urine analysis

Fig. 4 shows chromatograms of drug-free urine and of urine spiked with I. As for plasma, good linearity of response and good reproducibility (Table II)

TABLE I

REPRODUCIBILITY OF THE EXTRACTION OF I FROM PLASMA

Column extraction		Organic extraction ($n=6$)	
Concentration (mg/l)	Response factor (peak height, ng)	Concentration (mg/l)	C.V. (%)
0.0625	2.66	0.125	7.7
	3.07	0.5	3.8
0.125	3.67	2.0	5.9
	2.52		
0.25	4.02		
	2.78		
0.5	3.63		
	3.90		
1.0	3.70		
	4 36		
2.0	4.31		
	4.65		
5.0	4.00		
	3.63		
Mean	3.64		
S.D.	0.66		
S.E M.	0.18		
C.V. (%)	18.1		

were obtained. The linear regression equation and correlation coefficient were: $y=0.043+0.0012x$, $r=0.9957$ (peak-height ratio versus concentration).

Clinical application

The organic extraction procedure described above was applied to the determination of I in plasma and urine after its i.v. administration to normal volunteers. Fig. 5 shows the plasma concentration profiles in four volunteers after i.v. infusion over 1 h of 1500 and 2000 μg/kg I.

Compound I was not detected in the urine, because concentrations were very low and not detectable by our extraction procedure.

DISCUSSION

Although column extraction of I is a fast and simple procedure, we preferred to use the organic extraction procedure for a number of reasons. For the column procedure no suitable internal standard was found, resulting in higher C.V. for the extraction (see Table I). The organic extraction procedure is more time-consuming but has the advantage of an internal standard (amiodarone),

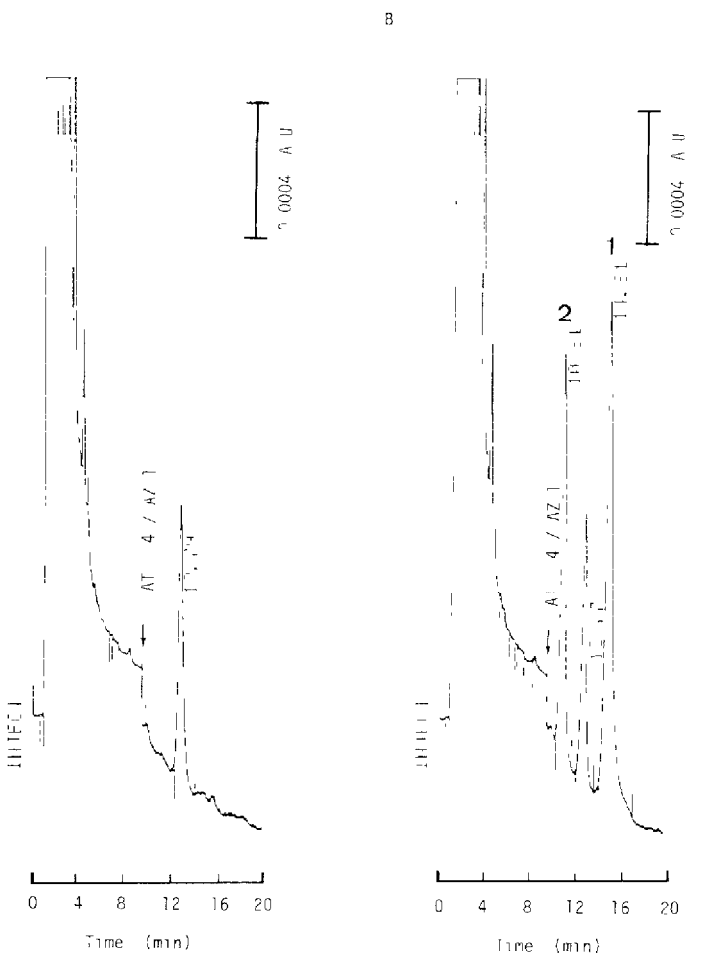

Fig 4. Chromatograms of (A) drug-free urine and (B) urine spiked with I (1 mg/l) (peak 1) and amiodarone (0.07 mg/l) as internal standard (peak 2), following the organic extraction procedure. AT = attenuation, AZ = autozero.

TABLE II

REPRODUCIBILITY OF THE ORGANIC EXTRACTION OF I FROM URINE ($n=6$)

Concentration (mg/l)	C.V. (%)
0.25	6.3
1.0	4.2
5.0	2.4

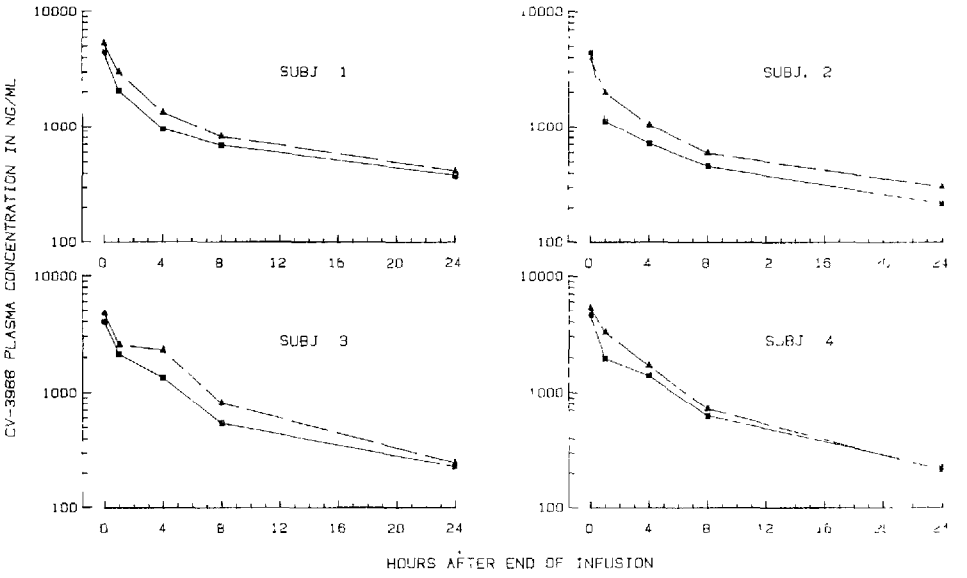

Fig. 5. Plasma concentration profiles after i v administration of 1500 (■) and 2000 (▲) μg/kg I to four healthy volunteers (0 h = end of 1-h infusion).

giving accurate and reproducible results (Table I). In addition, the organic extraction procedure for plasma resulted in cleaner chromatograms requiring ca. 30 min runtime while the column extraction required ca. 45 min of runtime before washing out all interfering compounds from the column. Although the recovery using organic extraction is only ca. 50% of the recovery using column extraction, the same sensitivity can be obtained by increasing the injection volumes.

Urine samples could be assayed only by organic extraction owing to the very low recoveries obtained with the column procedure. Despite the sensitivity of the organic extraction procedure in urine, I could not be detected in urine samples. This confirms preliminary data in animals, which show that urinary recovery is less than 1% of the administered dose [4].

CONCLUSION

The organic extraction procedure was chosen for analysis of batches of samples, allowing analysis of ca. 30 samples per 24 h. Use of an internal standard made the method accurate and precise. The column extraction procedure could become the method of choice, because of its speed and simplicity of manipulations, if an appropriate internal standard could be used.

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