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Methods for the analysis of the new vinca alkaloid derivative, S 12363, in plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

Two sensitive analytical methods for the analysis of S 12363 in plasma are described. A highly sensitive procedure for human and dog plasma using cyanopropyl solid-phase extraction with ion pairing chromatography and fluorescence detection, has a limit of quantification of 0.1 ng ml^{-1}. The technique has an overall precision and accuracy of 4.8 and 5.4% respectively over the concentration range 0.1–20 ng ml^{-1}. A second, less sensitive, assay specifically adapted for rodent plasma, uses benzene sulphonyl cation-exchange solid-phase extraction followed by reversed-phase chromatography, with post-column fluorescence enhancement. This method has a limit of quantitation of 1.0 ng ml^{-1}, with overall accuracy and precision of 7.2 and 11.6% respectively, over the concentration range 1.0–20.0 ng ml^{-1}. Both assays have been successfully applied to dog and mouse toxicokinetic studies.

1. Introduction

The vinca alkaloid derivative S 12363, ((1*S*)-1[3-(O4-deacetyl-3-demethoxy-carbonylvincaleukoblastinyl)-carbonylamino)-2-methyl-propyl-phosphonic acid diethyl ester) (I) (Fig. 1), a new α-aminophosphonic acid derivative of vinblastine, is currently under development at Servier Research and Development. Compound I has similar tubulin polymerisation inhibition properties as vincristine but shows less inhibition than vinblastine. However, I was found to be up to 553-fold and up to 74-fold more cytotoxic than vincristine and vinblastine respectively, when tested on a panel of different murine and human tumour cell lines [1]. Some recent studies have suggested that the different pharmacological activities and cytotoxicities observed for the vinca alkaloids may, at least partly, be due to their different uptake and cellular retention [2–7]. The observation that slight alterations in their structures can have dramatic consequences on cellular retention has led to a number of modifications to the vindoline moiety with amino acid derivatives [8,9]. The α-aminophosphonate derivative was chosen for I following positive

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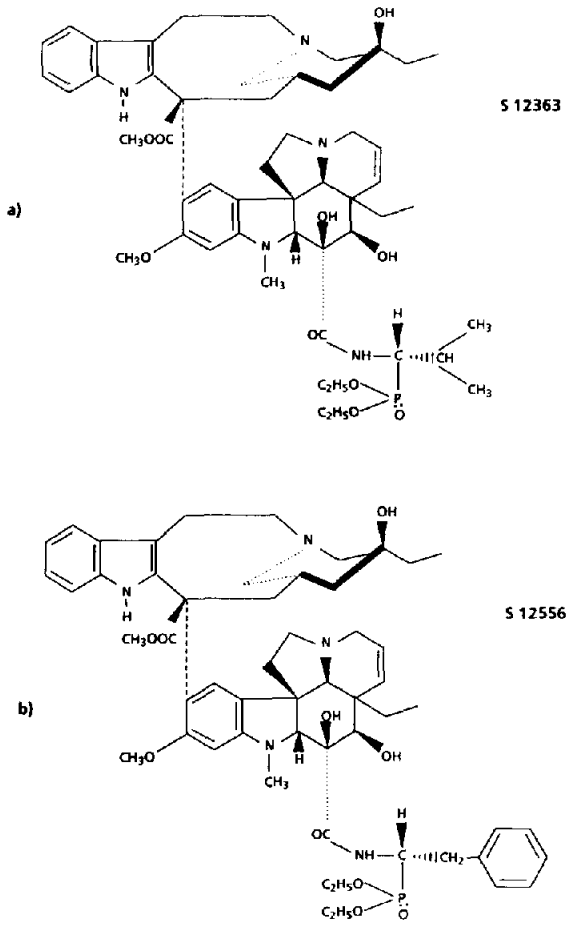

Fig. 1. The structures of (a) S 12363 (I), and (b) the internal standard, S 12556.

results obtained with a thus modified nitrosourea compound [10]. I was reported to be at least as active as vincristine and vinblastine on two murine tumours *in vivo* with the optimal dosage being some 20-fold lower [1]. The high potency of I did not seem to be due to enhanced interaction with tubulin, but rather to facilitated cellular uptake or retention conferred on the molecule by the α-aminophosphonate group [1]. The high potency of I necessitated initial dosing experiments to be performed at very low levels and therefore assays of considerable sensitivity were required.

A number of relatively sensitive methods have been published for the analysis of the vinca alkaloids by immunoassay but these methods generally lacked specificity [11,12]. Of the 40 published HPLC methods reviewed by Vendrig [13], the vast majority exhibit either poor resolution from endogenous material or poor peak shape or both, with reported quantification limits between 1.0 and 10 ng ml^{-1}. None of these assay procedures could be applied to I, since because of the anticipated low dose administration, a more sensitive method would be required.

Two new analytical procedures are therefore presented: a simple robust method based on separation through dispersion forces and dipole interactions, and analysis by ion-pairing chromatography for human and dog plasma samples. A second method for the analysis of rodent plasma utilises the dual retention characteristics of a benzene sulphonyl cation-exchange solid-phase sample enrichment, followed by reversed-phase chromatography. Both methods are based on fluorescence detection, using the natural fluorescence of the molecule conferred upon it primarily by the indole-methyl moiety of the vindolin subunit. A pH controlled environment to enhance this fluorescence was preferred rather than chemical derivatisation.

2. Experimental

2.1. Reagents and chemicals

I and the internal standard S 12556, [(1*S*)-1-(3-(O4-deacetyl-3-demethoxycarbonylvinca-leucoblastinyl)-carbonylamino)-benzyl-propyl-phosphonic acid diethyl ester], were supplied as sulphate salts by Technologie Servier (Orléans, France).

Perchloric acid, potassium dihydrogen orthophosphate, orthophosphoric acid and disodium hydrogen orthophosphate were of Aristar grade and obtained from BDH Chemicals (Poole, Dorset, UK). Heptane sulphonic acid (0.1 *M*) was supplied by Fisons Chemicals (Loughborough, Leics, UK). Acetonitrile, triethylamine (TEA), dimethylformamide (DMF) and methanol were of SpS grade and supplied by Romil (Loughborough, Leics, UK). Deionised water

was further purified on site using an Elga UHQ system (Elga, High Wycombe, Bucks, UK).

Control heparinised human plasma was obtained from healthy volunteers who were free from medication for at least three weeks prior to donation. Control heparinised dog, rat and mouse plasma were obtained from Biologie Servier (Gidy, France).

2.2. Preparation of standards

I sulphate was dissolved in methanol to give a concentration of 0.25 mg ml^{-1} expressed as the free base. Aliquots of this solution were further diluted in methanol to give secondary solutions of 100 ng ml^{-1} to 10 μg ml^{-1}. From these, aliquots were added to control plasma to produce a series of standards over the range 0.1 to 20 ng ml^{-1}. These standards were stored, together with control plasma, at −20°C. Separate standards were independently prepared for calibration, validation and quality control.

2.3. Chromatographic system

The chromatographic system consisted of a Hewlett-Packard 1090 liquid chromatograph (Bracknell, Berks, UK) with a Hitachi F2000 fluorescence detector (Nissei Sangyo, Wokingham, Berks, UK).

2.4. Human and dog plasma

All analyses were carried out under isocratic conditions on a 4-μm Nova Pak C_{18} column (15 × 0.39 cm I.D.; Waters Associates, Watford, Herts, UK). The mobile phase was prepared by adding 30 ml of 0.1 *M* heptane sulphonic acid to 500 ml of 50 m*M* KH_2PO_4 at pH 3.0. The volume was adjusted to 780 ml with methanol, and then to 1000 ml with acetonitrile. A second mobile phase was prepared to elute late running peaks from the column which may otherwise interfere with subsequent injections. This was prepared by the addition of 30 ml of 0.1 *M* heptane sulphonic acid to 350 ml of 50 m*M* KH_2PO_4 at pH 3.0. The volume was made up to 1000 ml with acetonitrile. The system was run at a column temperature of 40°C and at a flow-rate of 1 ml min^{-1}. Detection was by fluorescence with excitation at 276 nm and emission at 358 nm, each with a 20 nm bandpass.

2.5. Rodent plasma

All analyses were performed under isocratic conditions on a 5-μm ODS Hypersil column (12.5 × 0.46 cm I.D.; Shandon Scientific, Runcorn, Cheshire, UK). The mobile phase was prepared by mixing 350 ml of 25 m*M* KH_2PO_4 at pH 4.0, and 650 ml of acetonitrile followed by 50 ml of purified deionised water and 500 μl of TEA. A second mobile phase used to elute late running peaks from the column was prepared by mixing 200 ml of 25 m*M* KH_2PO_4 at pH 4.0, 800 ml of acetonitrile and 500 μl of TEA. The system was run at a column temperature of 40°C and at a flow-rate of 0.95 ml min^{-1}. Detection was by fluorescence as previously described, but in this case the fluorescence was enhanced with a post column reagent of 1.0 *M* orthophosphoric acid, adjusted to approximately pH 2.0 with sodium hydroxide, at a flow-rate of 50 μl min^{-1}.

2.6. Extraction of I

Human and dog plasma

To a 1.0-ml plasma sample were added an aliquot (7.5 μl) of the internal standard in methanol (1.0 μg ml^{-1}) and 250 μl of a 1.4% (v/v) solution of perchloric acid in water. The sample was mixed and centrifuged at approximately 1500 *g* for 7 min at ambient temperature. After centrifugation, the supernatant was aspirated and 25 m*M* KH_2PO_4 buffer (pH 3.0, 1.0 ml) was added.

The cyanopropyl solid-phase extraction cartridges (100 mg, Varian International, Harbor City, CA, USA) were solvated with methanol (5.0 ml) and conditioned with 25 m*M* KH_2PO_4 (1.0 ml, pH 3.0). The plasma/buffer mixture was then drawn through the cartridge using variable and slight vacuum at a flow-rate not exceeding 1.0 ml min^{-1}. The cartridges were washed sequentially with 25 m*M* Na_2HPO_4 (2.0 ml, pH 8.5), and 3.0 ml of a 10% (v/v) methanol

solution in water. The cartridges were dried under vacuum for one minute and then I and internal standard were eluted with 500 μl of methanol. The eluate was evaporated to dryness in a centrifugal evaporator (Life Sciences International, Runcorn, Cheshire, UK) and the residue was reconstituted in 100 μl of mobile phase. Up to 75 μl of the sample extract was injected onto the HPLC system.

Rodent plasma

To a 500-μl plasma sample were added an aliquot (25 μl) of internal standard in methanol (1.0 μg ml^{-1}) and 125 μl of a 2% (v/v) solution of perchloric acid in water. After centrifugation at ambient temperature for 10 min at approximately 1500 *g*, an aliquot (1.25 ml) of 50 m*M* KH_2PO_4 (pH 4.0) was added without disruption of the pellet.

Benzene sulphonyl (SCX) cartridges (100 mg, Varian International, Harbor City, CA, USA) were solvated with methanol (1.0 ml) prior to conditioning with 50 m*M* KH_2PO_4 (pH 4.0, 1.0 ml). The plasma/buffer mixture was applied at a flow-rate of 1.0 ml min^{-1} and the cartridges were washed sequentially with 50 m*M* KH_2PO_4 (pH 4.0)–acetonitrile (70:30, v/v, 1.0 ml), water (1.0 ml) and methanol (1 ml). I and internal standard were eluted with 1.0 ml of 0.35% (v/v) TEA in DMF. The eluent was evaporated to dryness in a centrifugal evaporator and the residue was reconstituted in a mobile phase–water mixture (50:50, v/v, 100 μl). Up to 80 μl of this extract was injected onto the HPLC system.

2.7. Validation of the method

Intra-assay variability was assessed from six plasma replicates of each standard at nominal concentrations ranging from 0.1 to 20 ng ml^{-1}. The concentration of I was calculated by reference to the peak-height ratios (I/I.S.) against an independently prepared set of calibration standards which were co-extracted and analysed in duplicate with each analytical run.

The precision of the assay was measured at each concentration according to the equation:

$$\text{Precision}(\%\text{C.V.}) = \frac{\text{Standard deviation}}{\text{Mean concentration}} \cdot 100$$

The accuracy of the assay was measured at each concentration according to the equation:

$$\text{Accuracy}(\%) = \frac{\text{Measured conc.} - \text{Actual conc.}}{\text{Actual conc.}} \cdot 100$$

Using the data from this intra-assay validation procedure the limit of quantification of each method was established as the lowest concentration which had a signal-to-noise ratio of at least three to one relative to the blank extract, and had precision and accuracy values of less than 20%.

Chromatographic selectivity was assessed from the discrimination between I and structurally related material, *i.e.* vincristine, vinblastine and vindesine. These compounds were applied to the human and dog HPLC system as chemical standards.

2.8. Toxicology studies

(a) Two groups of six Beagle dogs (3 male and 3 females) each received a single intravenous dose of I at either 0.012 mg kg^{-1} or 0.018 mg kg^{-1}. Blood (3.0 ml) was sampled at predetermined time points up to 24 h, and plasma was prepared by centrifugation at 1500 *g* for 10 min (4°C) and stored at −20°C until analysis.

(b) Sixty-six $B_6D_2F_1$ mice were divided into 2 groups, one group of which was dosed intravenously at 0.2 mg kg^{-1} and the other at 0.4 mg kg^{-1}. Three mice were sacrificed at predetermined times up to 72 h after dosing. Blood was collected into heparinised tubes and centrifuged at 1500 *g* for 10 min (4°C) to obtain plasma. The plasma samples were pooled for each time point and stored at −20°C until analysis.

3. Results

Under the conditions described for both assays, compound I was well resolved from the internal standard and endogenous plasma com-

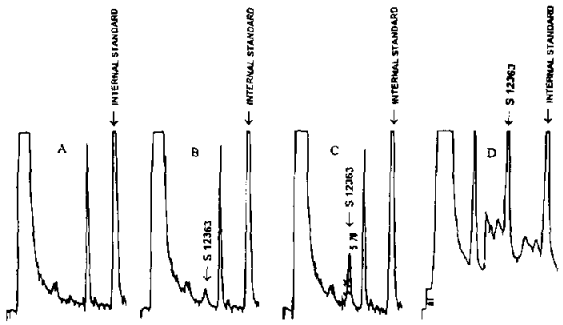

Fig. 2. Representative chromatograms of extracts of I from human plasma. I is eluted at 5.78 min, and the internal standard at 9.56 min. (A) Plasma blank with internal standard; (B) plasma standard at 0.3 ng ml^{-1}; (C) plasma standard at 1 ng ml^{-1}; (D) patient sample 20 min after infusion.

ponents (Figs. 2 and 3). Furthermore, using chemical standards, both I and internal standard were well resolved from vincristine, vinblastine and vindesine (Fig. 4), under the chromatographic conditions established for human and dog plasma.

A correlation coefficient of 0.9997 ($n = 6$, $y = 1.031x + 0.00$) was obtained for human plasma and 0.9998 ($n = 6$, $y = 0.0293x + 0.001$) in mouse plasma. Linearity was demonstrated for both assays.

Intra-assay precision and accuracy were determined from six replicates at each concentration. The results are presented in Tables 1 and 2. Inter-assay variability was assessed for a series of analytical runs, at three different concentrations.

Based on the criteria of the limit being the lowest concentration which has a baseline signal-to-noise ratio greater than three to one, and an accuracy and precision of measurement better than 20%, a limit of quantitation of 0.1 ng ml^{-1} was established for human and dog plasma, with mean accuracy and precision at this level of 11.6 and 9.8% respectively. For rodent plasma, a limit of quantitation of 1 ng ml^{-1} was established with accuracy and precision at this level of 2.4 and 17.2% respectively.

When the methods were applied to toxicokinetic studies, compound I was quantifiable for up to 24 h in the dog, with all plasma levels

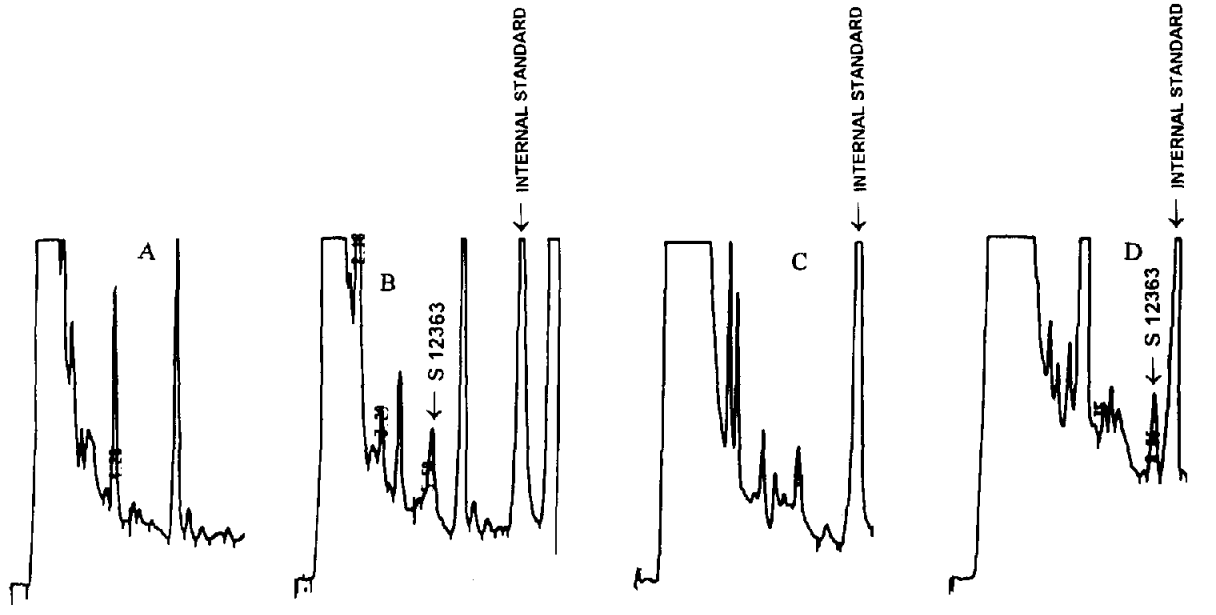

Fig. 3. Representative chromatograms of extracts of I from dog and mouse plasma. I is eluted at 6.0 and 9.3 min respectively, and the internal standard at 10.2 and 10.3 min respectively for the two assays. (A) Blank dog plasma; (B) dog plasma sample 10 min after dosing; (C) blank mouse plasma with internal standard; (D) mouse plasma sample 72 h after dosing.

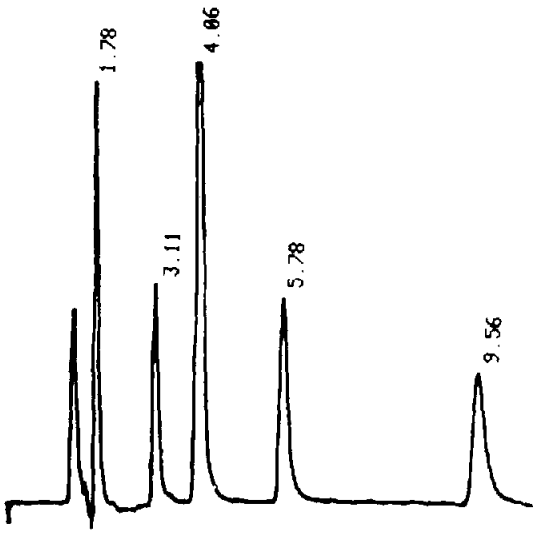

Fig. 4. Chromatogram demonstrating the selectivity of the human/dog system with the elution order vindesine (1.78 min), vincristine (3.11 min), vinblastine (4.06 min), I (5.78 min) and internal standard (9.56 min).

Table 3
Plasma levels of I (ng ml^{-1}) in the dog following intravenous administration of the compound at two different doses

Time (h)	0.012 mg kg^{-1}	0.18 mg kg^{-1}
0.167	0.857	0.988
1.0	0.577	0.662
5.0	0.447	0.354
24.0	0.258	0.152
AUC_t [a]	9.16	7.19

[a] AUC_t = Area under the plasma concentration *vs.* time curve to the last measured time point (ng ml^{-1} h).

within the calibration range of the assay (Table 3). For mice, I was quantifiable for up to 72 h, with the vast majority of samples at the higher dose (0.4 mg kg^{-1}) requiring dilution prior to analysis (Table 4). Sample dilution studies had demonstrated that dilution of high concentration samples with control matrix to a concentration within the calibration range of the assay, gave results within 10% of the original

Table 1
Precision and accuracy results at six replicate concentrations over the range 0–20 ng ml^{-1} in human plasma

	Nominal concentration (ng ml^{-1})						
	0.1	0.3	0.6	1.0	3.0	10.0	20.0
Mean ($n = 6$)	0.11	0.34	0.59	0.99	2.96	9.76	20.87
S.D.	0.01	0.03	0.01	0.03	0.09	0.35	0.63
%C.V.	9.8	9.5	1.8	3.0	3.0	3.6	3.0
% Acc.	+11.6	+13.0	−2.8	−1.8	−1.7	−2.7	+4.0

Table 2
Precision and accuracy results at six replicate concentrations over the range 0–20 ng ml^{-1} in mouse plasma

	Nominal concentration (ng ml^{-1})						
	0.2	0.3	0.5	1.0	2.0	10.0	20.0
Mean ($n = 6$)	0.09	0.37	0.59	1.02	2.34	10.44	21.03
S.D.	0.18	0.05	0.13	0.18	0.31	0.47	2.37
%C.V.	200	13.3	21.2	17.2	13.4	4.5	11.2
% Acc.	+54.5	+24.6	+18.0	+2.4	+17.0	+4.4	+5.1

Table 4
Plasma levels of I (ng ml^{-1}) in the mouse following intravenous administration of the compound at two different doses

Time (h)	0.2 mg kg^{-1}	0.4 mg kg^{-1}
0.083	193	330
0.167	128	211
0.25	73.3	169
0.5	41.0	159
1.0	31.6	114
4.0	33.6	73.9
8.0	26.3	48.2
24.0	7.7	29.9
48.0	3.8	16.6
72.0	2.4	5.5
AUC_t [a]	718	2062

[a] AUC_t = Area under the plasma concentration *vs.* time curve to the last measured time point (ng ml^{-1} h).

concentration on back-calculation of the diluted sample results.

Inter-assay variability was assessed for the human and dog plasma assay during analysis of the dog toxicology samples. Of the six analytical runs performed, only one run was rejected due to underestimation of all quality control (QC) samples. As a consequence, the mean precision particularly at the lower QC level, was effected (Table 5).

4. Discussion

4.1. Chromatography

Many methods have been published concerning the chromatographic analysis of vinca alkaloids from a variety of matrices [13]. However, most of these exhibit either poor resolution, poor peak shape or both. Bearing in mind the anticipated dosing levels of I and the requirement therefore to maximise the sensitivity, it was necessary to optimise all aspects of the chromatographic system.

Table 5
Inter-assay validation results performed on dog plasma on six separate occasions. Samples were run as quality control samples

	Target concentrations (ng ml^{-1})		
	0.3	1.0	10.0
Mean ($n = 6$)	0.28	0.92	9.97
S.D.	0.07	0.16	1.54
%C.V.	23.7	17.9	15.5
% Acc.	−5.3	−7.9	−0.3

For the human and dog plasma assay procedure resolution and peak symmetry were achieved by combination of a hydrogen-bonding organic modifier in an ion-pairing system with heptane sulphonic acid. In a reversed-phase mode with the mobile phase at pH 3.0, both I and the internal standard are protonated and elute at or near V_0. In the ion-pairing mode at this pH the electrophilic groups on the analytes appear to be ionically bound to the sulphonyl moieties of the heptane sulphonate and they thus partition into the bonded phase essentially as a neutral species, the ionisation of the acidic groups being suppressed at this pH, giving retention times of approximately 6 and 10 min for I and the internal standard respectively.

An alternative means of maintaining peak shape and therefore sensitivity would have been to increase the pH of the mobile phase to suppress the ionisation of the amine groups, or to incorporate an amine modifier such as triethylamine into the mobile phase. It has been observed that the fluorescence of I, other vinca alkaloids and other indole containing molecules is highly pH dependent [15] with a maximum quantum yield being achieved at a pH of *ca.* 3 to 3.5. Consequently, with the ionisation suppressed at a pH of *ca.* 6.0, there was little if any usable fluorescence.

In I, the internal standard and the parent molecule vinblastine, the electron releasing groups of the indole-methyl moiety of the vindoline subunit confer a fluorescence on these compounds some sixty-fold greater than that observed with the indole-formyl electron withdrawing group present at the same location on vincristine. The conjugated ring system in the catharanthine sub unit probably also contributes to the fluorescence, since its dehydroxylation and double bond formation, as observed in navel-

bine, causes a 20% decrease in fluorescence intensity at this acidic pH compared to vinblastine and I. Changes in the conformation, ionisation or saturation of these groups together with changes in mobile phase composition may therefore account for the pH dependency of vinca fluorescence. The control of pH in liquid chromatography is important, not only for the column parameters k', α and peak symmetry, but also for optimisation of the detection.

The rodent plasma extracts were not as clean as those obtained with the human and dog samples. In the ion-pairing system I could not be resolved from all the endogenous material. It was therefore necessary to utilise the differential selectivity obtained by the adjustment of the mobile phase pH. Raising the pH to 4.0 together with the addition of triethylamine (0.05%, v/v) was sufficient to achieve the desired resolution but, as expected, this led to a marked decrease in sensitivity through the loss of fluorescence. This problem was overcome by the post-column addition of phosphoric acid at pH 2.0 at a flow-rate of 50 μl min^{-1}. The resultant reduction in the pH of the column eluant yielded a maximised fluorescence whilst maintaining the separation conferred on the system by a higher pH.

4.2. Sample preparation

A number of solid-phase extraction matrices are reported for the selective enrichment of the vinca alkaloids from biological samples. These usually use ionic or Van der Waals forces to facilitate retention. Such systems, particularly the latter, tend to demonstrate a lack of selectivity as evidenced by the analyte eluting on the down slope of the unretained material at V_0. This often causes problems caused by inconsistent integration, particularly for samples with very low analyte concentrations.

Vendrig [14] described a method for the enrichment of vinblastine and vincristine using the same cyanopropyl (CN) bonded phase reported here and postulated an ionic primary retention mechanism between the free silanols on the bonded phase and the protonated form of the analytes, with hydrophobic interaction as secondary interaction. In the method reported here we believe that dipole–dipole interactions predominate and confer selectivity to the system.

The first part of the extraction procedure, which was the same for both methods, was treatment of the plasma sample with 1.4% (v/v) perchloric acid. This particular concentration of reagent was used to sufficiently disrupt the tertiary structure of the proteins in the sample without causing precipitation. When the acid concentration was increased in order to precipitate the protein then it was found that, although the extracts were considerably cleaner, there was a very marked reduction in recovery of the analytes presumably through entrapment in the protein pellet.

The CN moiety has a relatively strong dipole moment of ~ 3.9 which appears to be sufficient to either induce a dipole or to react with a permanent dipole on the alkaloids, thus facilitating retention by electrostatic interaction. It is assumed that silanol interactions do not play a significant role in this method since the bonds so formed are resistant to a 2-ml wash with 25 mM Na_2HPO_4 at pH 8.5. Compound I has pK_a's of 5.0 and 6.9 and it is reasonable to assume that the internal standard has pK_a's similar to those of I or even those of vinblastine (-0.8, 3.8, 6.1 and 7.0). Consequently, at pH 8.5 the neutral form of the molecules predominate thus diminishing their propensity for significant ion-exchange with the free silanols. Furthermore, if it is assumed that the main retention was by ion-exchange, which would be negated at pH 8.5, then the relatively weak Van der Waals forces associated with a CN functionality are unlikely to retain the analytes during the 10% methanol wash.

Whilst this extraction method proved satisfactory for human and dog plasma it did not provide sufficiently clean extracts for the rodent plasma. This situation was overcome by the use of a mixed-mode cation-exchange non-polar system. The samples were loaded and washed in their protonated forms at pH 4.0 under which conditions they were resistant to 30% acetonitrile and 100% methanol washes. The selective elution of I and the internal standard was effect-

ed by the use of a volatile basic solvent mixture–35% (v/v) triethylamine in dimethylformamide. An alternative elution mixture of 2% (v/v) ammonia in methanol was used initially with some success but was found to be associated with an intermittent total loss of the analyte fluorescence signal after evaporation and reconstitution. A UV trace confirmed the presence of the analytes at the high concentration levels, and radioactivity confirmed their presence at all standard concentrations. The use of the TEA/DMF mixture gave consistent fluorescence although the background noise was slightly increased.

Both assay procedures have been successfully applied to toxicokinetic studies in the dog and mouse respectively. For both species I was quantifiable up to the last measured time point.

5. Conclusions

Under the conditions described, it is possible to quantify I in human and dog plasma over the range 0.1–20 ng ml^{-1} and in rodent plasma over the range 1.0–20 ng ml^{-1}. The differences between the matrices of each species and the need for high sensitivity required a particular and different technique to achieve the desired results. These techniques have been successfully applied to animal studies.

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