Journal of Chromatography, 424 (1988) 83–94
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3938

ANALYSIS OF VINCA ALKALOIDS IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

D.E.M.M. VENDRIG*, J. TEEUWSEN and J.J.M. HOLTHUIS

Department of Pharmaceutical Analysis, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)

(First received June 25th, 1987; revised manuscript received September 2nd, 1987)

SUMMARY

The reversed-phase high-performance liquid chromatography with electrochemical detection was used to quantify plasma and urine levels of vinblastine, vincristine, vindesine and a metabolite of vinblastine, desacetylvinblastine. Sample clean-up consisted of solid-phase extraction with a Bond Elut CN column. The extracts were separated on a Hypersil ODS column. The mobile phase consisted of a mixture of methanol and 10 m*M* phosphate buffer (pH 7.0). The limit of sensitivity using electrochemical detection was 100 pg on-column for all compounds with a signal-to-noise ratio of 3. Quantification of the compounds in human plasma and urine was possible down to 1 ng/ml (ca. 1 pmol). Pharmacokinetic results show that the sensitivity of the method is adequate for drug monitoring in clinical research.

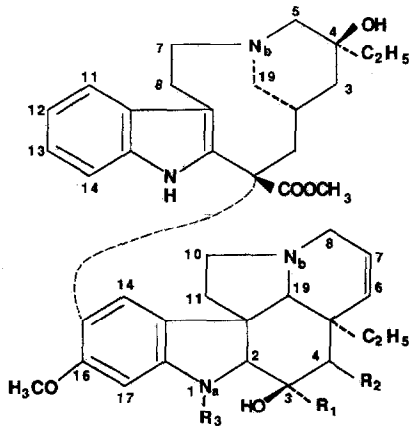
INTRODUCTION

The vinca alkaloids are dimeric indole derivates (Fig. 1), isolated from the periwinkle plant, *Vinca rosea* Linn. Vinblastine and vincristine are naturally occurring alkaloids and vindesine (4-desacetylvinblastine amide) is a semisynthetic derivative of vinblastine. The vinca alkaloids are used in the treatment of various neoplastic diseases. Although they differ only slightly in chemical structure and biological action, there is a wide variation in their clinical toxicity, activity and pharmacokinetic parameters [1–3].

Up till now, radioimmunoassay and liquid scintillation counting of radioactively labelled drugs have been the principal methods used to determine the compounds in plasma and urine for the study of metabolism and pharmacokinetics [4–6].

Recently, we published preliminary results of the bioanalysis of vinblastine and vincristine using solid-phase extraction [7]. In that paper we also revealed that

0378-4347/88/$03.50



	R_1 (3)	R_2 (4)	R_3
VINCRISTINE	$COOCH_3$	$OCOCH_3$	CHO
VINBLASTINE	$COOCH_3$	$OCOCH_3$	CH_3
VINDESINE	$CONH_2$	OH	CH_3
DESACETYLVINBLASTINE	$COOCH_3$	OH	CH_3

Fig. 1. Chemical structures of vinblastine, vincristine, vindesine and desacetylvinblastine.

the extraction recovery of the vinca alkaloids decreased enormously when the last of three batches of columns was used. It was not possible to increase the recovery of the vinca alkaloids by alteration of the extraction parameters. This paper presents a reproducible and accurate method for the analysis of vincristine and vinblastine in plasma and urine at the picomole level. The extraction procedure used also proved suitable for the determination of vindesine and desacetylvinblastine, a major metabolite of vinblastine.

EXPERIMENTAL

Chemicals and solutions

Vinblastine sulphate (Velbe®),vincristine sulphate (Oncovin®) and vindesine sulphate (Eldisine®) were supplied by Eli Lilly Nederland. Vinblastine sulphate and vincristine sulphate were supplied by Pharmachemie (Haarlem, The Netherlands). Desacetylvinblastine sulphate was a gift from Gedeon Richter (Budapest, Hungary).

Methanol (Merck, Darmstadt, F.R.G., pro analysi) and deionized water were distilled and stored in glass containers. Other chemicals were of analytical grade and were used as received.

Standard solutions of vinblastine (1.00 mg of vinblastine sulphate in 5.0 ml of methanol), vincristine (10.00 mg of Oncovin or 1.00 mg of vincristine sulphate in 5.0 ml of methanol), vindesine (6.00 mg of Eldisine in 5.0 ml of methanol) and desacetylvinblastine (1.0 mg of desacetylvinblastine sulphate in 5.0 ml of methanol) were prepared in disposable polypropylene tubes and stored at −20°C. From these solutions dilutions were made in methanol.

Plasma (heparinized or citrated respectively) and urine from patients and volunteers were stored in polypropylene containers at −20°C.

Apparatus and chromatographic conditions

The chromatographic system used was identical with that reported previously [7]. In addition to the described Hypersil ODS column, we also used a Chromsep

Hypersil ODS column (100×4.0 mm I.D., 5 μm) (Chrompack, Middelburg, The Netherlands). The mobile phase consisted of a mixture of methanol and 10 m*M* phosphate buffer at pH 7.0 (65:35 to 55:45, w/w, depending on the column used).

Sample collection

Vinblastine sulphate (Velbe) was dissolved in normal saline prior to administration. The drug was administered in an intravenous bolus injection or a short infusion. Blood samples were collected in polypropylene tubes containing heparin. Immediately after collection the samples were centrifuged and the heparinized plasma was transferred to a second polypropylene tube. Samples were taken before administration of the drug and at the following times: 0, 2, 4, 8, 15, 30 min and thereafter at 1, 2, 4, 8, 16, 24, 48 and 72 h after administration. Urine samples were collected over periods of 6 h up to 36–52 h after adminstration. After the urine samples had been mixed and the total volume determined, an aliquot of 100 ml was taken. All samples were stored at −20°C.

Extraction procedure

Vincristine was used as the internal standard (I.S.) in the assay of vinblastine, vindesine and desacetylvinblastine. Vinblastine was used as I.S. in the vincristine analysis. To 1.0 ml plasma or urine were added 5–25 μl of the I.S. solution (1–50 ng/μl in methanol, Table I). The samples were mixed on a vortex mixer for 5 s and centrifuged for 10 min (2500 *g*). The vinca alkaloids were isolated by solid-phase extraction with a Bond Elut CN extraction column (1 ml capacity) (Analytichem International, Harbor City, CA, U.S.A.). The column was washed with 5 ml of methanol and 5 ml of 50 m*M* tetramethylammonium bromide in 10 m*M* phosphate buffer at pH 4.5. The biological fluid was passed through the column attached to a Vac Elut processing station (Analytichem International). After the column had been washed consecutively with 5 ml of methanol–water (20:80, v/v) and 2 ml of 25 m*M* phosphate buffer at pH 7.0, the column was dried by the passage of air through it. The vinca alkaloids were eluted with 500 or 750 μl of methanol. The eluate was collected in a conical polypropylene tube (capacity 1.5 ml). The eluate was mixed on a vortex mixer, and the methanol was evaporated under a nitrogen stream at room temperature. The residue was dissolved in 100 μl of the mobile phase by vortexing for 30 s. After centrifugation for 10 min, a volume of 10–80 μl was injected into the chromatograph.

Calibration and recovery

Calibration graphs were constructed to demonstrate the linear relationship between the peak-height ratio and the concentration of the samples. The range covered 1–100 ng/ml for plasma and 1–750 ng/ml for urine (Table I). The calibration graphs were derived by spiking drug-free plasma and urine samples with vinblastine, vincristine, vindesine or desacetylvinblastine dissolved in methanol (a maximum of 25 μl of methanol was added). Spiked calibration samples were extracted with each set of samples from patients.

The extraction recoveries of the vinca alkaloids from spiked plasma samples

TABLE I

CALIBRATION GRAPHS OF THE VINCA ALKALOIDS

For all graphs, $n=5$.

Concentration range (ng/ml)	Matrix	Amount of I.S.* (ng)	Correlation coefficient (r^2)
Vinblastine			
1-10	Plasma	8	0.9978
10-100		80	0.9996
10-100	Urine	80	0.9991
50-750		400	0.9998
Vincristine			
1-10	Plasma	4	0.9966
10-100		40	0.9998
5-100	Urine	40	0.9980
Vindesine			
1-10	Plasma	10	0.9979
10-100		100	0.9976
10-100	Urine	100	0.9998
50-750		500	0.9980
Desacetylvinblastine			
2.5-10	Plasma	10	0.9972
10-100		100	0.9941
2.5-10	Urine	10	0.9967
10-100		100	0.9999

*In each case the I.S. was vincristine except for the determination of vincristine, in which case the I.S. was vinblastine.

were calculated by comparing the peak-height ratio of the drug to be determined and the external standard (added to the eluate after the column extraction) with the peak-height ratio obtained with methanolic standards that had been dried under nitrogen and dissolved in the mobile phase (Table II).

Intra-assay and inter-assay variability

Intra-assay and inter-assay precision were determined at a concentration of 10 ng/ml of plasma for all compounds (Table III). The intra-assay precision for the extraction from urine was determined at a concentration of 50 ng/ml for vinblastine, vincristine and vindesine, and 10 ng/ml of urine for desacetylvinblastine (Table IV). The samples were spiked and stored at $-20°C$ for 24 h before analysis. The samples were analysed six times in one run.

The inter-assay precision and accuracy were estimated over five days (Table III).

TABLE II

EXTRACTION RECOVERY OF THE VINCA ALKALOIDS

The extraction recovery of vinblastine, vincristine, vindesine and desacetylvinblastine has been determined for concentrations of 10 and 100 ng/ml in plasma and urine ($n=1$). The Bond Elut CN columns used were from different batches.

Drug	Matrix	Recovery (%)	
		10 ng/ml	100 ng/ml
Vinblastine	Plasma	101.7	91.0
Vincristine		105.6	79.8
Vindesine		77.1	69.7
Desacetylvinblastine		102.7	84.6
Vinblastine	Urine	98.5	77.7
Vincristine		96.4	76.4
Vindesine		83.2	78.2
Desacetylvinblastine		89.5	82.0

TABLE III

INTRA- AND INTER-ASSAY VARIABILITY OF THE EXTRACTION PROCEDURE FOR PLASMA

Plasma samples were spiked with 10.0 ng/ml; $n=6$.

Day	Observed concentration (mean ± S.D.) (ng/ml)			
	Vinblastine	Vincristine	Vindesine	Desacetylvinblastine
1	10.1 ± 0.51	9.9 ± 0.17	9.7 ± 0.55	11.1 ± 0.31
2	10.1 ± 0.45	10.3 ± 0.42	10.8 ± 0.53	11.0 ± 0.56
3	10.1 ± 0.45	9.9 ± 0.29	9.8 ± 0.15	10.1 ± 0.39
4	10.9 ± 0.49	10.2 ± 0.29	10.9 ± 0.46	11.0 ± 0.44
5	10.6 ± 0.47	10.7 ± 0.40	10.2 ± 0.53	10.1 ± 0.47
Mean over five days	10.2 ± 0.44	10.2 ± 0.34	10.3 ± 0.56	10.7 ± 0.51

TABLE IV

INTRA-ASSAY VARIABILITY OF THE EXTRACTION PROCEDURE FOR URINE

Urine samples were spiked with 10.0 or 50.0 ng/ml; $n=5$.

Drug	Observed concentration (mean ± S.D.) (ng/ml)
Vinblastine	49.7 ± 1.4
Vincristine	49.5 ± 1.7
Vindesine	49.3 ± 3.4
Desacetylvinblastine	10.4 ± 0.3

Inter-batch variability

Because we had experienced several different recoveries with Bond Elut Diol extraction columns [7], we determined the extraction recovery of vinblastine at a concentration of 100 ng/ml of plasma for every new batch of CN columns.

Recycling of the solid-phase extraction columns

The possibility of using the same column several times was investigated for plasma samples at two concentration levels of vinblastine. Batches of 7.0 ml of plasma were spiked with 10 and 200 ng/ml vinblastine and vincristine (I.S.). After the standard extraction procedure the same procedure was repeated with a sample of the same plasma batch, but without extra washing steps. The influence of the concentration on the peak-height ratio vinblastine/vincristine was tested by extracting alternately plasma samples spiked with 10 and 200 ng/ml vinblastine. The concentration of vincristine was 50 ng/ml for both vinblastine concentrations. Peak-height ratios of vinblastine and vincristine were compared to interpret the results.

RESULTS AND DISCUSSION

Calibration and recovery

Table I presents the calibration graphs for the determination of the compounds in plasma and urine in the range 1–750 ng/ml. To determine plasma concentrations higher than 100 ng/ml the patient's plasma is diluted to the desired concentration with blank plasma prior to analysis. The calibration graphs were all linear with acceptable correlation coefficients ($r^2 \geqslant 0.9941$). In no graph was the intercept on the y-axis significantly different from zero ($P < 0.05$). An equation of a typical calibration line is: $y = 0.0211x - 0.0009$ (10–100 ng vinblastine per ml plasma). Table II shows the extraction recovery of all compounds in plasma and urine determined at concentration levels of 10 and 100 ng/ml. The recovery was not complete in all cases, but high enough to determine the vinca alkaloids at the nanogram level.

When the columns are washed with a higher percentage of methanol (30% instead of 20%, v/v), the extraction recovery decreases drastically (from 80 to 40%) (results not shown).

Intra-assay and inter-assay variability of the extraction procedure

We determined the intra-assay variability in order to evaluate the precision of the extraction procedure. The results for the variability of the assay are presented in Tables III and IV. The relative standard deviations (R.S.D.) are low (<6%). The inter-assay variability is also good. The accuracy of the method can be judged from the small differences between the theoretical concentrations and the observed concentrations over five days. The accuracy proves to be high and reproducible for all days.

The detection limit is 1 ng/ml of plasma or urine for all compounds.

Figs. 2 and 3 show chromatograms of extracted samples from blank, spiked and patient's plasma and urine. The blank plasma and urine chromatograms show no

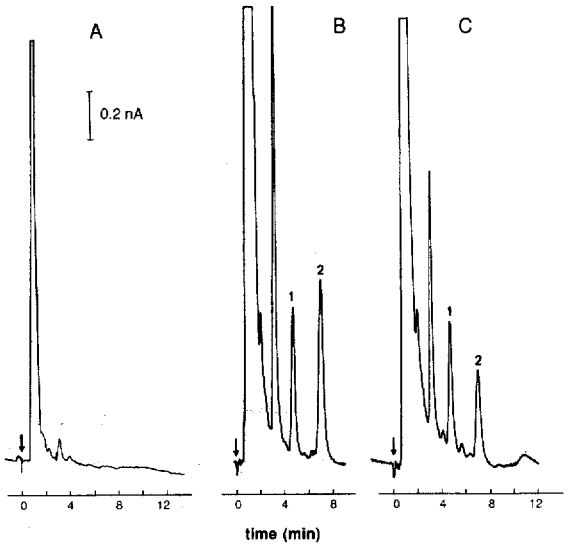


Fig. 2. Chromatograms of plasma extracts. (A) Blank plasma; (B) spiked plasma, containing 7.5 ng/ml vinblastine; (C) patient's plasma, containing 4.3 ng/ml vinblastine. Peaks: 1 = vincristine; 2 = vinblastine.

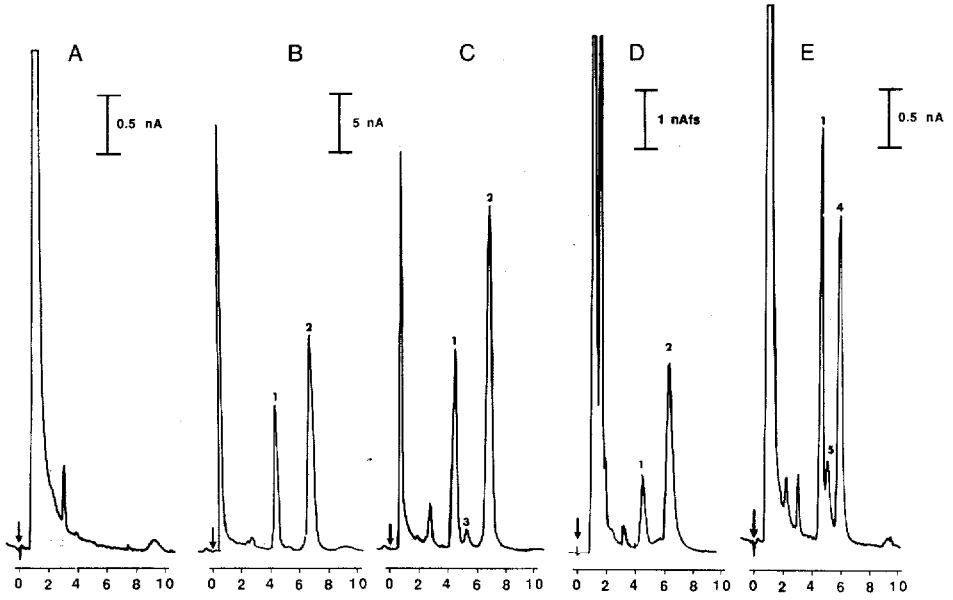


Fig. 3. Chromatograms of urine extracts. (A) Blank urine; (B) spiked urine, containing 612 ng/ml vinblastine; (C) patient's urine, containing 685 ng/ml vinblastine; (D) patient's urine, containing 18.4 ng/ml vincristine; (E) patient's urine, containing 90.4 ng/ml vindesine. Peaks: 1 = vincristine; 2 = vinblastine; 3 = desacetylvinblastine; 4 = vindesine; 5 = possible metabolite.

TABLE V

INTER-BATCH VARIABILITY OF BOND ELUT CN COLUMNS

Extraction recovery of plasma samples spiked with 100 ng/ml vinblastine.

Batch No.	Methanol volume (μl)	Recovery (mean ± S.D.) (%)	n
130 197	500	82.5 ± 5.1	4
131 729	500	84.2 ± 2.7	4
131 719	500	83.3 ± 1.8	4
133 548	500	56.3 ± 12.1	3
133 548	750	84.5 ± 2.8	3
133 548	1000	84.2 ± 6.2	3

peaks that interfere with any of the compounds. The chromatogram of a urine sample from a patient who had received vinblastine shows the presence of desacetylvinblastine. The urine extract of a patient who received vindesine also shows the presence of a possible metabolite. The chromatogram of a patient's urine with vincristine shows no extra peaks.

Inter-batch variability

Table V shows the extraction recoveries of vinblastine in plasma, using different batches of CN columns. The recoveries determined with the first three batches were highly reproducible. However, after extraction with the last batch received (No. 133548) the recovery of vinblastine had decreased to 50%. For vincristine the recovery was of the same order as the recovery determined with other batches. Because more cyanopropyl groups are bound to silica gel, the recovery of vinblastine from that batch is poor. The higher amount of cyanopropyl groups increases the column capacity, therefore 750 μl of methanol is necessary to elute vinblastine from this column. The consequence is that whenever vinblastine is used as I.S., at least 750 μl of methanol are required for the elution.

Possible retention mechanism of the vinca alkaloids on the CN-bonded phase

The extraction columns used for the isolation of the vinca alkaloids contain silica modified with cyanopropyl groups. Owing to incomplete coverage of the silica there are still silanol groups present. The retention of the vinca alkaloids on the CN column is a complex mechanism, possibly involving the interaction of the protonated and deprotonated species with both silanol groups and the bonded phase [8,9]. The retention of the protonated vinca alkaloids (VA^+) can be attributed to the interaction with ionized silanol groups (SiO^-). Protonated vinca alkaloids and tetramethylammonium ions compete for the cation-exchange sites of the column material. Tetramethylammonium bromide can be seen as a tailing suppressing agent, which improves the elution profile of the vinca alkaloids resulting in a low amount of methanol necessary to elute the vinca alkaloids [8–10].

The very strong interaction between SiO^- and VA^+ makes it possible to wash the column with a solution containing 20% of methanol to remove interfering

TABLE VI

PEAK-HEIGHT RATIOS OF VINBLASTINE AND VINCRISTINE OBTAINED AFTER REPETITIVE ANALYSIS OF IDENTICAL PLASMA SAMPLES USING THE SAME EXTRACTION COLUMN

Analysis No.	Peak-height ratio			
	10 ng/ml vinblastine and vincristine		200 ng/ml vinblastine and vincristine	
	Column A	Column B	Column C	Column D
1	0.744	0.770	0.805	0.837
2	0.782	0.758	0.799	0.826
3	0.812	0.769	0.790	0.837
4	0.781	–*	0.802	0.814
5	0.791	0.660	0.772	0.801
6	0.750	0.576	0.778	0.788
7	0.764	0.636	0.783	0.799
8	0.667**	0.560**	0.671**	0.582**
9	0.721	0.697	0.790	0.807
10	0.795	0.663	0.785	0.812
n	9	5	9	9
Mean	0.771	0.646	0.789	0.813
S.D.	0.029	0.045	0.011	0.017
R.S.D. (%)	3.8	8.4	1.4	2.1

*After this extraction a hole was visible in the packing material.
**The deviation of the mean is more than twice the S.D. and therefore the values are not used in the calculation of the mean.

endogenous compounds. Subsequently the column is washed with phosphate buffer at pH 7.0 to deprotonate the vinca alkaloids. The retention is maintained by the hydrophobic interaction between the cyanopropyl groups and the vinca alkaloids. Finally the vinca alkaloids are eluted with a low amount of methanol. It can be concluded that it is possible to isolate the vinca alkaloids from biological matrices very selectively using CN-bonded phase for the extraction.

Recycling of the extraction columns

To reduce the cost of routine monitoring of vinca alkaloids the recycling of the solid-phase columns was investigated. Table VI shows the peak-height ratios of vinblastine and vincristine after extraction ten times with the same column. The peak heights (not shown) and peak-height ratios did not change significantly after the same column had been used more than once. In column B a visible hole had been formed in the column material due to our attempts to remove the last residue of buffer solution before elution. After this event, not only were the peak-height ratios of column B different from those before the hole formed, but the R.S.D. had more than doubled.

When the concentration of vinblastine alternated between 10 and 200 ng/ml plasma (Table VII) the peak height (not shown) and peak-height ratios did not

TABLE VII

PEAK-HEIGHT RATIOS OF VINBLASTINE AND VINCRISTINE AFTER ALTERNATE EXTRACTION OF PLASMA SAMPLES CONTAINING 10 AND 200 ng/ml VINBLASTINE WITH THE SAME COLUMN

A B, C and D are the different columns used, and the following numbers denote the sequence of analysis.

10 ng/ml vinblastine and 50 ng/ml vincristine				200 ng/ml vinblastine and 50 ng/ml vincristine			
A1	0.170	C1	0.199	B1	3.212	D1	3.092
B2	0.163	D2	0.189	A2	3.083	C2	3.067
A3	0.176	C3	0.192	B3	3.287	D3	3.105
B4	0.296*	D4	0.184	A4	N.D.**	C4	3.088
A5	0.188	C5	0.186	B5	3.185	D5	3.196
B6	0.171	D6	0.176	A6	2.903*	C6	2.953
A7	0.181	C7	0.278*	B7	3.085	D7	3.044
B8	0.179	D8	0.191	A8	3.192	C8	3.006
A9	0.187	C9	0.185	B9	3.141	D9	2.721*
B10	0.183	D10	0.263*	A10	3.079	C10	3.165
n	9		8		8		9
Mean	0.178		0.188		3.152		3.080
S.D.	0.008		0.007		0.063		0.075
R.S.D. (%)	4.5		3.7		2.0		2.4

*The deviation of the mean is more than twice the S.D. and therefore the values are not used in the calculation of the mean.
**N.D. = not determined.

change. The R.S.D.s were no higher than the R.S.D.s in Table VI. The deviation of ca. 12% of the peak-height ratios is more than twice the standard deviation of the mean. We cannot explain these different values. In general, care has to be taken when using the same column for (unknown) different concentrations. Within the same concentration range a column can be used at least ten times. When the column was used for longer the extraction time increased considerably, owing to blocking of the column by plasma components.

Preliminary pharmacokinetic study of vinblastine

We tested the applicability of the described method in a preliminary pharmacokinetic study of vinblastine in three patients. Pharmacokinetic data analysis was performed with a computer extented with a non-linear least-squares program. The curve was fitted to an open three-compartment model. The half-life times (mean ± S.D.; $n = 3$) of the three phases were 0.033 ± 0.038, 0.702 ± 0.429 and 15.88 ± 7.68 h.

Fig. 4 shows the log plasma concentration versus time curve of vinblastine after 16 mg of Velbe had been administered to a patient in a bolus injection. The vinblastine was still detectable 48 h after administration at a concentration of 2.7 ng/ml plasma.

The method was also tested by determining the amount of vinblastine in the

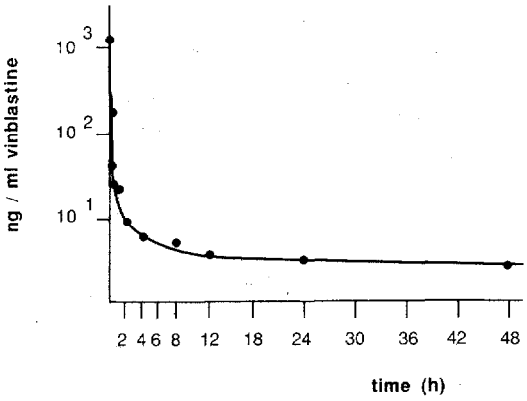
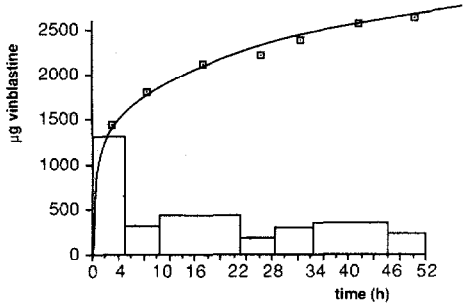


Fig. 4. Log plasma concentration versus time curve of vinblastine after administration of 16 mg of Velbe to a patient.



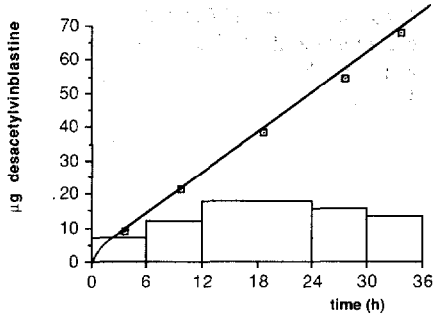


Fig. 5. Urinary excretion curve of vinblastine after administration of 23 mg of Velbe to a patient.

Fig. 6. Urinary excretion curve of desacetylvinblastine after administration of 16 mg of Velbe to a patient.

urine. Fig. 5 shows the cumulative excretion curve of vinblastine after 23 mg of Velbe had been administered. Within 52.5 h following the injection 11.5% of the dose was excreted unchanged in the urine. Fig. 6 shows the excretion of desacetylvinblastine in urine after 16 mg of Velbe had been administered. After 36 h, 0.4% of the dose was excreted as desacetylvinblastine.

CONCLUSION

The combination of HPLC and electrochemical detection after solid-phase extraction makes it possible to determine clinically used vinca alkaloids in plasma and urine. A major metabolite of vinblastine can also be analysed with this assay. All compounds can be detected at the ng/ml level.

The extraction recoveries obtained with the CN columns are high and reproducible, although some batches need little adaptation of the elution volume. Such adaptation of the procedure for the Diol column that we described previously [7] was not possible.

Since the extraction recovery is not totally independent of the batch of columns

used, the extraction recovery has to be determined each time a new batch of columns is used. The variation in recovery between columns of the same batch is overcome by using a structurally related I.S. The method has proved to be sufficiently sensitive and selective to be used in clinical pharmacokinetic experiments [11], at least up to 48 h after administration.

ACKNOWLEDGEMENTS

We thank Mr. J.-P. Boon and Mr. A. Mekking for technical support.

REFERENCES

1 W.A. Creasey, Cancer and Chemotherapy, Vol. III, Academic Press, New York, 1981, p. 79.
2 W. Brade, in W. Brade, G.A. Nagel and S. Seeber (Editors), Vindesine, Proceedings of the International Vinca Alkaloid Symposium, Frankfurt am Main, November 1980, S. Karger, Basle, 1981, p. 96.
3 R.A. Bender and B.A. Chabner, in B.A. Chabner (Editor), Pharmacological Principles in Cancer Treatment, W.B. Saunders, Philadelphia, PA, 1982, p. 256.
4 J.A. Houghton, P.M. Torrance and P.J. Houghton, Anal. Biochem., 134 (1983) 450.
5 K. Lu, H.-Y. Jap and T.L. Loo, Cancer Res., 43 (1983) 1405.
6 M.C. Castle and J.A.R. Mead, Biochem. Pharmacol., 27 (1978) 37.
7 D.E.M.M. Vendrig, J.J.M. Holthuis, V. Erdélyi-Tóth and A. Hulshoff, J. Chromatogr., 414 (1987) 91.
8 K. Sugden, G.B. Cox and C.R. Loscombe, J. Chromatogr., 149 (1978) 377.
9 B.B. Wheals, J. Chromatogr. 187 (1980) 65.
10 H. Lingeman, H.A. van Munster, J.H. Beijnen, W.J.M. Underberg and A. Hulshoff, J. Chromatogr., 352 (1986) 261.
11 D.E.M.M. Vendrig, J. Teeuwsen, J.J.M. Holthuis, E.G.E. de Vries and N.H. Mulder, Proc. Am. Assoc. Cancer Res., 28 (1987) 190.