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DETERMINATION OF VINCA ALKALOIDS IN PLASMA AND URINE USING ION-EXCHANGE CHROMATOGRAPHY ON SILICA GEL AND FLUORESCENCE DETECTION

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SUMMARY

The development of a method for the determination of the antineoplastic vinca alkaloids vinblastine and vindesine in biological samples is described. The selectivity of the assay is high owing to the use of solid-phase extraction on a cyanopropyl extraction column prior to isocratic chromatography on unmodified silica gel with fluorescence detection. The influence of acetonitrile concentration and mobile phase pH on the capacity factors of the drugs was studied in order to optimize the separation between the drugs and endogenous components. The effect of varying the type and concentration of competing cations in the mobile phase was also examined. The limit of determination (signal-to-noise ratio = 3) for vinblastine is 0.5 ng/ml in plasma and urine and for vindesine 2.5 ng/ml. The assay is suitable for determining the concentrations of both compounds in plasma and urine samples from patients.

INTRODUCTION

High-performance liquid chromatography (HPLC) of basic compounds (mainly drugs) is generally performed on reversed-phase columns. When this kind of stationary phase is used for basic compounds the chromatograms often show broad and tailing peaks owing to unwanted adsorption of the compounds on free silanol groups. The chromatography of such compounds can be improved by the addition of ion-pair reagents to the mobile phase, but the results are not always satisfactory. Another possibility for improving the chromatography of basic drugs is the use of silica gel as the stationary phase.

Unmodified silica gel can be used in the normal-phase mode [1] or in the ion-exchange mode [2]. In the ion-exchange mode a mobile phase consisting of an

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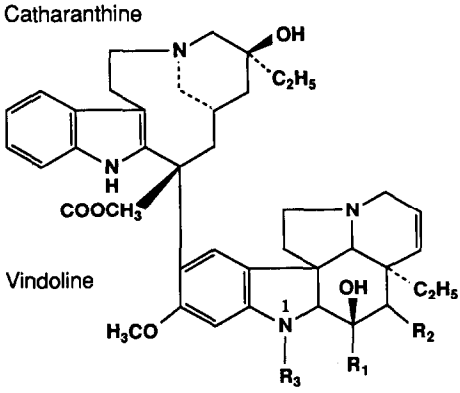
organic modifier and an aqueous buffer solution containing ions competing with the protonated drug for free silanol groups is used. Both systems can be used for the determination of alkaloids, although the second system often yields better peak shapes and results in shorter retention times.

Until recently, very few reports on the practical application of unmodified silica gel in the ion-exchange mode for the determination of drugs in biological fluids had appeared [3]. The use of different retention mechanisms for solid-phase extraction (reversed-phase) and the analytical chromatography (ion-exchange) offers new possibilities for the determination of drugs in biological fluids in general.

We have investigated such a combination for the antineoplastic vinca alkaloids, a group of basic drugs of which the analysis and clinical pharmacology is under investigation. Vincristine (VCR), vinblastine (VBL) and vindesine (VDS) (Fig. 1) are clinically important vinca alkaloids. In cancer chemotherapy the normal dose of these compounds is 1–6 mg/m^2 resulting in active plasma concentrations below 10 ng/ml several hours after administration. For the determination of these compounds in plasma and urine, sensitive methods are needed in order to study the clinical pharmacokinetics. For drug level monitoring, which is useful for restricting toxic side-effects and improving dosage schedules, one needs to be able to determine the drug concentrations at the ng/ml level.

This paper describes the determination of VBL and VDS in plasma and urine using an HPLC method with fluorescence detection. Chromatography is performed on silica gel in the ion-exchange mode with acetonitrile as organic modifier. We studied the influence of the concentration of organic modifier and the pH of the mobile phase on the capacity factors of the drugs in order to optimize the resolution. The effect of the type and concentration of tetraalkylammonium bromide salts was also investigated. In addition, the influence of these parameters on chromatograms of blank plasma and urine extracts was examined. The alkaloids were isolated from the biological fluid by solid-phase extraction on a cyanopropyl column.



	R_1	R_2	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. Structures of vincristine, vinblastine, vindesine and desacetylvinblastine.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Spectroflow 400 HPLC pump (Applied Biosystems, Ramsey, NJ, U.S.A.) equipped with a Model U6K injector or a Model 710 WISP autoinjector (Waters Assoc., Milford, MA, U.S.A.). The detector was a Hitachi F 1000 fluorescence detector (Merck, Darmstadt, F.R.G.). The optimum excitation wavelength for VBL and VDS was 285 nm and the optimum emission wavelength was 365 nm in the mobile phase used. In addition, a Spectroflow 773 UV absorbance detector was used at 254 nm to detect VCR.

The stainless-steel column (300 mm×3.9 mm I.D.) was slurry-packed with LiChrosorb Si-60, particle size 10 μm (Merck). Before use the column was activated for 12 h with a mixture of methanol–water containing 10 m*M* tetramethylammonium bromide (TMABr) and 10 m*M* disodium citrate (pH 5.9) (60:40, w/w) [4]. The temperature was 50°C and the flow-rate 0.5 ml/min.

The buffer for the preparation of the mobile phase contained 50 m*M* tetrabutylammonium bromide (TBABr) and 10 mM sodium dihydrogencitrate, adjusted to pH 3.0 with sodium hydroxide. The mobile phase was acetonitrile–buffer (85:15, w/w) and was degassed ultrasonically. The flow-rate was 3.0 ml/min at 20°C. Chromatograms were recorded on a BD41 flat-bed recorder (Kipp and Zonen, Delft, The Netherlands).

Extractions were carried out on a Vac Elut processing station (Analytichem International, Harbor City, CA, U.S.A.).

Chemicals and solutions

Analytical-reagent grade chemicals were used as received, unless stated otherwise. Acetonitrile and methanol were obtained from Merck and were distilled before use. Deionized water was filtered using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) when it was to be used for the preparation of the mobile phase, otherwise it was distilled twice before use. VBL sulphate (Velbe®) and VDS sulphate (Eldisine®) were kindly provided by Eli Lilly Nederland (Utrecht, The Netherlands). Standard solutions of VBL and VDS were prepared in methanol and stored at −20°C. Tetraalkylammonium salts were purchased from Fluka (Buchs, Switzerland).

Pooled plasma from healthy volunteers was obtained from the blood bank of the Red Cross in Utrecht, The Netherlands. Blank urine was collected from laboratory workers. The fluids were stored at −20°C until used.

Extraction procedure

VBL was used as the internal standard (I.S.) when VDS was determined; VDS was the I.S. in the determination of VBL. The I.S. was added to 1.0 ml of plasma or urine. The samples were mixed on a vortex mixer and centrifuged for 10 min at 3000 *g*. Extraction was carried out using Bond Elut CN extraction columns, capacity 1 ml (Analytichem International). The cartridge was washed with 5 ml of distilled methanol and 5 ml of 10 m*M* sodium phosphate buffer (pH 5.5) containing 50 m*M* TMABr.

After activation of the column the plasma or urine sample was passed through, then the column was washed with 5 ml of methanol–10 m*M* sodium phosphate buffer (pH 5.5) (25:75, v/v) and 2 ml of 25 m*M* phosphate buffer (pH 7.0). The solid phase was kept wet between the different steps. Finally, the extraction column was thoroughly dried by the passage of air. Both compounds were eluted with 500 μl of methanol into a conical polypropylene tube (1.5 ml). The eluate was gently mixed on a vortex mixer and the methanol was evaporated under nitrogen at 30°C. The extract was dissolved in the mobile phase (100 μl) by vortexing for 30 s. After centrifuging (10 min, 3000 *g*) a volume of 10–80 μl was injected into the chromatographic system.

Calibration and recovery

The linearity was studied in the range 0.2–2000 ng for VBL and 1.3–2000 ng for VDS (absolute amount injected). The linearity of the extraction from plasma and urine was investigated in the range 0.5–100 ng/ml for VBL and 10–200 ng/ml for VDS. Calibration graphs were obtained by spiking drug-free plasma and urine with methanolic standard solutions (a maximum of 20 μl/ml of methanol was added).

Extraction recoveries from spiked plasma and urine samples were determined 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 a standard solution. This solution was prepared by spiking 500 μl of methanol with the same amount of drugs as the biological samples. This solution was treated in the same way as the eluate had been treated following extraction of the drugs from plasma and urine [5].

Reproducibility of the method

The extraction procedure used in this study was almost identical with the isolation described in a previous paper [5]. Only the pH of the column and the concentration of methanol and the pH of the washing fluids differed slightly from those in the previous method. The differences resulted from the optimization of the sample preparation in relation to another chromatographic system and method of detection. Because of these changes we evaluated the intra-assay precision for VBL at levels of 5 and 50 ng/ml and for VDS at 50 and 200 ng/ml in both plasma and urine. A batch of 6.0 ml was spiked with VBL or VDS. The I.S. was added to five 1.0-ml portions and the samples were extracted as described.

Applicability to patients' samples

The assay developed was used to determine VBL and VDS concentrations in plasma and urine samples obtained from patients treated with one of the drugs. Samples containing different concentrations were analysed. A series of calibration samples were extracted together with each set of patients' samples. A number of cytostatic agents used in combination therapies with VBL or VDS, e.g. bleomycin and methotrexate, were investigated for possible interferences with the vinca alkaloids during chromatographic analysis.

RESULTS AND DISCUSSION

Fluorescence detection

We recently developed a sensitive and selective HPLC method using electrochemical detection for the determination of VCR, VBL and VDS in plasma and urine [5]. Another very selective technique for the determination of drugs is HPLC in combination with fluorescence detection. Fluorescence detection in HPLC is often easier to handle than electrochemical detection and is generally used more often than electrochemical detection in the determination of drugs. Fluorescence detection can be a very selective and sensitive method for detecting vinca alkaloids [6]. However, the fluorescence yield of VCR is about 40 times lower than that of VBL. Therefore, it is impossible to determine VCR at the ng/ml level using fluorescence detection. The only difference between the structures of VBL and VCR is the replacement of the methyl group in VBL by the formyl group in VCR at N-1 of the vindoline part of the molecule. From this it can be concluded that the formyl group is responsible for quenching the fluorescence yield of VCR. Capacity factors of this drug were determined with UV detection at 254 nm. The reversed-phase system we used for electrochemical detection was not suitable for fluorescence detection because interfering peaks were present in the chromatograms of extracts of blank plasma and urine. The solid-phase extraction of the vinca alkaloids had already been optimized [5,7]. Therefore, greater selectivity could only be achieved by changing the chromatographic system.

Van Duuren [8] reported that the fluorescence of indole alkaloids increased in the presence of increasing amounts of organic modifier. The use of silica gel in the ion-exchange mode offered the possibility of using a mobile phase with a high concentration of organic phase, which was favourable for the fluorescence detection of the vinca alkaloids. The fluorescence in acetonitrile–water mixtures was higher than that in methanol–water mixtures with the same concentration of the organic phase. The combination of solid-phase extraction on a reversed-phase extraction column and HPLC with fluorescence detection on a silica column led to a selective and sensitive method for the determination of the vinca alkaloids.

Effect of organic modifier and pH of the mobile phase

The retention behaviour of VBL and VDS was studied as a function of the concentration of the organic modifier in the range 70–85% (w/w). Chromatograms of extracts of blank plasma and blank urine were used in combination with retention times and peak shapes of the drugs in order to determine the most suitable eluent. The influence of the amount of organic modifier was studied with buffer of pH 3.0. This low pH was chosen because the fluorescence of indole alkaloids increased with decreasing pH [8]. TMABr (50 mM) was used as the competing ion.

When the concentration of organic modifier ranged from 70 to 85% (w/w), the capacity factor (k') of VBL increased from 0.7 to 4.0 (Fig. 2). The strongest influence was seen at concentrations between 80 and 85%.

The pK_a values of VBL are −0.8, 3.8, 6.1 and 7.0 [9]. At pH 3.0 at least two nitrogen atoms of the vinca alkaloids were protonated. A decrease in the buffer

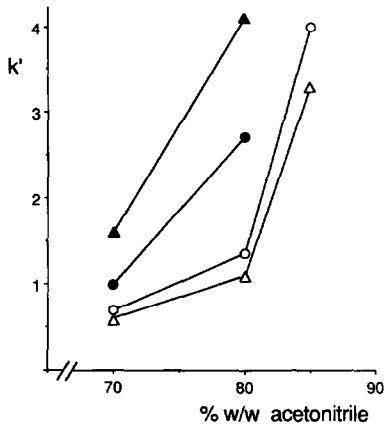


Fig. 2. Influence of acetonitrile concentration on the k' values of vincristine (△), vinblastine (○), vindesine (▲) and desacetylvinblastine (●). Chromatographic conditions: 50 mM TMABr, 10 mM sodium dihydrogencitrate (pH 3.0); flow-rate, 2.0 ml/min. VCR detection: UV (254 nm).

content of the mobile phase from 20 to 15% caused a 25% decrease in the concentration of competing ions. Reduced competition between the TMA ions and the protonated alkaloids for the deprotonated silanol groups led to greater retention of the vinca alkaloids.

The pH of the buffer was varied between 2.5 and 3.5. The acetonitrile concentration was 80% (w/w) and the TMABr concentration in the buffer was 50 mM. The k' value increased when the pH of the buffer was increased from 2.5 to 3.5. At pH 2.5 more silanol groups were protonated, so fewer positively charged alkaloids were bound. However, the increase in k' was small.

Effect of the type and the concentration of competing ions

The type and concentration of cations were varied at a concentration of 80% (w/w) acetonitrile. The pH of the buffer in the mobile phase was 3.0. The capacity factors of VBL obtained after adding TMABr, TBABr and tetrapentylammonium bromide (50 mM) to the mobile phase were 1.3, 1.7 and 2.1, respectively. Tetrahexylammonium bromide could not be used because of its low solubility in acetonitrile. Smaller ions were better able to compete with the alkaloids for the deprotonated silanol groups. Smaller ions showed a stronger interaction with the ion-exchange sites of the stationary phase than larger ions owing to the shorter distance between the two charges and less solvation by the mobile phase [10,11].

Increasing the concentration of competing ions reduced the k' values of the compounds (Fig. 3). The competition between TBABr and protonated vinca alkaloids for the deprotonated silanol sites was enhanced by increasing the concentration of TBABr. This led to shorter retention times for the alkaloids. At TBABr concentrations above 50 mM the k' values of the drugs remained constant. Probably all available silanol groups were occupied at TBABr concentrations above 50 mM.

The final choice of the concentration of organic modifier (85%, w/w), type (TBABr) and concentration of competing cations (50 mM) and pH of the buffer

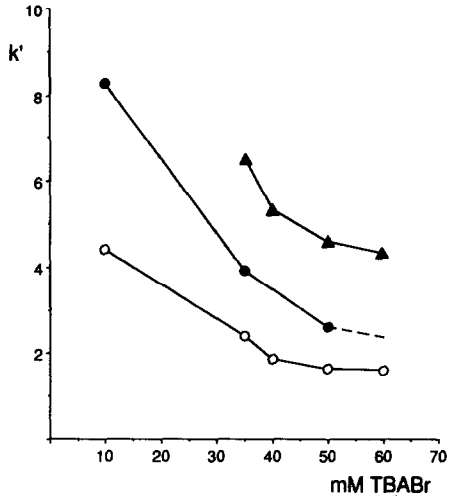


Fig. 3. Influence of the concentration of TBABr on the k' values of vinblastine (○), desacetylvinblastine (●) and vindesine (▲). Chromatographic conditions: acetonitrile–10 m*M* sodium dihydrogencitrate (pH 3.0) (80:20, w/w); flow-rate, 2.0 ml/min.

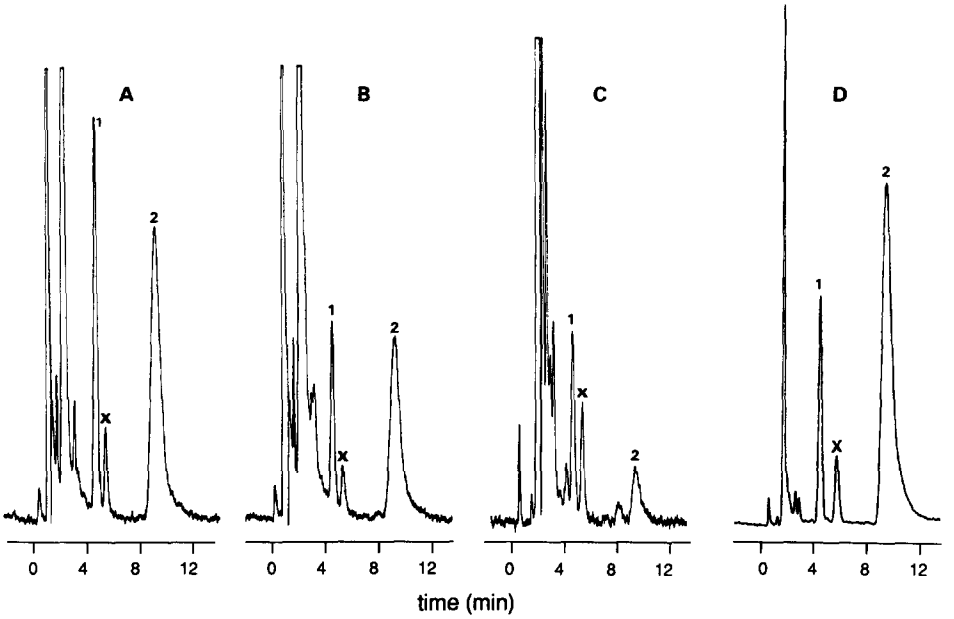


Fig. 4. Chromatograms of plasma and urine extracts. (A) Spiked plasma: 50 ng/ml vindesine, 12.5 ng/ml vinblastine; 40 µl of the plasma extract were injected; sensitivity 20. (B) Spiked urine: 5 ng/ml vinblastine, 25 ng/ml vindesine; 60 µl injected; sensitivity 20. (C) Patient's plasma: 13 ng/ml vinblastine, 20 ng/ml vindesine; 30 µl injected; sensitivity 20. (D) Patient's urine: 72 ng/ml vinblastine, 500 ng/ml vindesine; 20 µl injected; sensitivity 5. Peaks: 1 = vinblastine; 2 = vindesine; x = unknown compound.

(3.0) was made on the basis of the chromatograms of blank plasma and urine extracts. The retention times and peak shapes of VBL and VDS were acceptable under these conditions. The use of the mobile phase described above enabled us to separate the alkaloids from each other and to separate the drugs from endogenous material (Fig. 4).

Linearity, reproducibility and limit of detection

The detector response was linear in the ranges 0.2–2000 ng of VBL and 2.5–2000 ng of VDS. The calibration graphs (Table I) showed good correlation coefficients ($r^2 \geqslant 0.9997$). The intercepts were not significantly different from zero ($p < 0.05$).

The reproducibility of the system was determined by replicate injections of absolute amounts of 0.8 and 8 ng of VBL ($n = 10$) and 8 and 80 ng of VDS ($n = 8$). The relative standard deviations (R.S.D.) of the peak heights measured were 2.3 and 1.1% for VBL and 1.2 and 3.0% for VDS, respectively.

The detection limits were 200 pg (220 fmol) for VBL and 1 ng (1170 fmol) for VDS at a signal-to-noise ratio of 3.

Calibration, recovery and reproducibility of the extraction procedure

Table I shows the calibration graphs for VBL and VDS obtained following their extraction from plasma and urine. All graphs were linear with acceptable correlation coefficients ($r^2 \geqslant 0.9955$). The intercepts were not significantly different from zero ($p < 0.05$). The limits of determination were 0.5 ng/ml for VBL and 2.5 ng/ml for VDS in both plasma and urine.

Fig. 4 shows chromatograms of plasma and urine extracts. The peak shape of VDS (wide and tailing) was not optimal. The tailing was probably caused by the

TABLE I

CALIBRATION GRAPHS FOR VINBLASTINE AND VINDESINE

Fluid	Drug	Range	Internal standard (ng)	Slope	Intercept	Correlation coefficient (r^2)
Methanol	Vinblastine	0 - 125*	–	3.3894	0.010	0.9997
		0 -2000	–	2.9139	8.490	0.9999
	Vindesine	0 - 100	–	0.9903	−0.092	0.9998
		0 -2000	–	1.0002	−3.360	0.9998
Plasma	Vinblastine	0.5- 10**	25	0.2330	0.005	0.9984
		10 - 100	250	0.0162	0.008	0.9997
	Vindesine	10 - 200	10	0.0357	−0.169	0.9996
Urine	Vinblastine	0.5- 10**	25	0.2039	0.016	0.9989
		10 - 100	250	0.0158	0.007	0.9994
		25 - 750	500	0.0088	0.023	0.9981
	Vindesine	10 - 200	10	0.0411	−1.117	0.9991

*ng injected.
**ng/ml.

presence of a primary amide function. All chromatograms showed a peak (x) at a retention time of 5.7 min. This compound was probably introduced into the extracts by one of the reagents as it was also present in the chromatogram of the reagent blank (Fig. 5A). The chromatogram of an extract of the reagent blank strongly resembled those of blank plasma and urine extracts (Fig. 5). This showed that the separation of VBL and VDS from endogenous material was very selective.

The solid-phase extraction column only retained basic and neutral compounds. Acid compounds ($pK_a < 6$) were removed by washing the column with phosphate buffer (pH 7). Neutral compounds present in the extract were eluted with the solvent front on the analytical column owing to the high concentration of acetonitrile in the mobile phase. Therefore, the separation between the vinca alkaloids and other drugs was also very selective. In addition, we examined the chromatography and detectability in the system described for adriamycin, bleomycin, cyclophosphamide, dactinomycin, daunorubicin, etoposide, methotrexate, mitomycin C and thiotepa. None of these compounds interfered with the vinca alkaloids. A small change in the amount of acetonitrile caused a relatively large change in the retention of VBL and VDS (Fig. 2). The same effect was seen in the retention time of peak x (Fig. 5). The resolution between peak x and VBL and VDS remained good for all mobile phase mixtures used.

Desacetylvinblastine (DVBL), a major metabolite of VBL, had a retention time of 5.7 min in the chromatographic system used. It was not possible to separate peak x, present in all extracts, from DVBL. Therefore, this HPLC method could not be used to determine small amounts of DVBL in urine samples.

Table II shows the recoveries of VBL and VDS following their extraction from plasma and urine. To remove as many plasma and urine constituents as possible

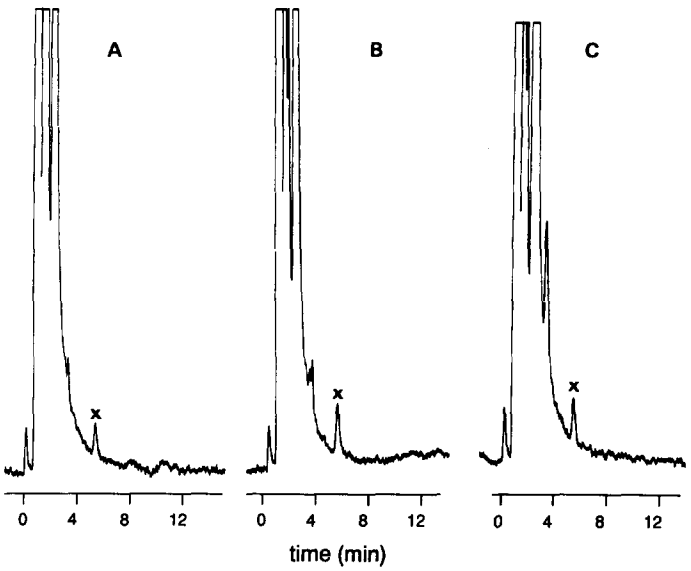


Fig. 5. Chromatograms of extracts (80 μl injected, sensitivity 20). (A) Reagent blank; (B) blank plasma; (C) blank urine. Peak x = unknown compound.

TABLE II

RECOVERIES OF VINBLASTINE AND VINDESINE AFTER EXTRACTION FROM PLASMA AND URINE

Drug	Fluid	Concentration (ng/ml)	Recovery (%)	*n*
Vinblastine	Plasma	5	72.8	3
		50	84.6	5
	Urine	5	81.6	3
		50	84.1	5
Vindesine	Plasma	50	81.0	2
		200	76.6	5
	Urine	50	80.1	2
		200	82.5	5

TABLE III

RELATIVE STANDARD DEVIATIONS (R.S.D.) OF PEAK-HEIGHT RATIOS (DRUG/I.S.) AFTER EXTRACTION FROM PLASMA AND URINE

Drug	Fluid	Concentration (ng/ml)	I.S. (ng)	R.S.D. (%)	*n*
Vinblastine	Plasma	5	25	3.9	5
		50	250	3.0	5
	Urine	5	25	0.7	4
		50	250	1.7	5
Vindesine	Plasma	50	10	2.1	5
		200	100	5.4	5
	Urine	50	10	3.5	5
		200	100	2.8	5

under the conditions used, we changed some parameters of the washing fluids previously used for the extraction [5]. The pH of the buffer for the equilibration of the cartridge was chosen by comparing the chromatograms of blank plasma and urine extracts. The concentration of methanol in the washing fluid was mainly determined by the drug recoveries. With this extraction procedure the recoveries of the drugs after extraction were slightly lower than those using the extraction procedure described previously [5]. Blank chromatograms, however, showed fewer interfering peaks.

The results for the reproducibility of the extraction are presented in Table III. The R.S.D.s were low ($\leqslant 5.4\%$), which proved that the method is highly reproducible.

Fig. 4 also shows chromatograms of extracts of samples from patients who had received VBL. The chromatogram of a patient's urine showed one extra peak. Other chromatograms for the same patient and those for other patients showed no more peaks than chromatograms obtained with spiked urine samples.

CONCLUSION

VBL and VDS can be chromatographed on unmodified silica gel using a mobile phase containing a high concentration of acetonitrile. With this system the peak shapes and retention times are acceptable. The high content of organic solvent makes the system suitable for fluorescence detection. Solid-phase extraction on a reversed-phase extraction column followed by chromatography on unmodified silica gel constitutes a selective and sensitive method for the determination of VBL and VDS in plasma and urine. The major advantage in this method is the use of fluorescence detection. A fluorescence detector is generally more stable and less susceptible to interferences than an electrochemical detector. The limit of determination for VBL in plasma and urine proved to be lower than when HPLC is carried out on reversed-phase material with electrochemical detection [5]. However, for VDS the determination limit is higher. It is not possible to determine low concentrations of VCR and DVBL with this method.

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