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Sensitive method for the determination of vincristine in human serum by high-performance liquid chromatography after on-line column-extraction

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ABSTRACT

A column-switching high-performance liquid chromatographic method was developed for the determination of vincristine in serum. Sample preparation was carried out by means of on-line column-extraction, using a C_{18} reversed-phase preconcentration column. This technique is simple (minimizing manual sampling errors), rapid (reduction of time and costs) and can be easily automated. Both ultraviolet and electrochemical detection are possible, but the latter shows a cleaner chromatogram and is, by the use of a new electrochemical detector, far more sensitive (detection limit 0.3 μg/l at a signal-to-noise ratio of 3). A matrix study was carried out (using human serum and urine and two kinds of calf's serum). Although it appeared that the system was matrix-dependent, no difference in matrix effects could be found in the serum or plasma of different patients. Controls for human serum analysis should be prepared in human serum. With the method described, pharmacokinetic studies of vincristine in children can be performed.

INTRODUCTION

Vincristine (VCR) is an antineoplastic vinca alkaloid, isolated from *Catharanthus roseus* G. Don, and is used in the treatment of various childhood and adult malignancies, *e.g.* acute lymphocytic leukaemia, lymphoma, sarcoma, Wilms' tumour, rhabdomyosarcoma and neuroblastoma [1]. The cytotoxic action of the agent results from binding to tubulin, an intracellular protein that polymerizes to

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form the mitotic spindle. VCR blocks the polymerization process and inhibits the progression of cells through mitosis [2]. An inhibiting effect on phospholipid synthesis has also been reported [3].

Although VCR is a component of established therapy for many childhood malignancies, only one pharmacokinetic study of VCR in four children has been published [4]. In part, this lack of extensive pharmacokinetic data can be explained by limitations of the analytical methodology for measuring VCR. A radioimmunoassay [5] has been the only assay with sufficient sensitivity to support pharmacokinetic studies in patients, but this assay is relatively non-specific, as metabolites may cross-react with the antibody. Therefore, there is a need for a sensitive and specific procedure to allow further studies of VCR pharmacokinetics in pediatric patients. Recently, a high-performance liquid chromatographic (HPLC) method has been published, describing off-line solid-phase extraction and electrochemical detection (detection limit 1.0 μg/l at a signal-to-noise ratio of 3.0) [6]. Our objective was to develop a sufficiently sensitive, simple and rapid method for determining VCR in serum so as to perform pharmacokinetic studies of VCR in children. In this assay VCR is determined in serum by HPLC after on-line solid-phase extraction, using an automatic column-switching technique. The great advantage of this method is the possibility of total automation, as VCR and the internal standard (vinblastine) are both stable in serum or plasma at 4°C for over 24 h [7]. So the sample and internal standard mixture is pipetted in the automatic injector, kept at 4°C. Besides, the manual time-consuming sample-extraction step can be omitted, the number of analyses per time unit can be increased (overall reduction of costs), and manual sampling errors are minimized. The detection limit could be decreased to 0.3 μg VCR/l serum at a signal-to-noise ratio of 3.0, by using a new type of amperometric detector, *i.e.* an optimized wall-jet design.

The plasma elimination of VCR after intravenous administration is usually described by a three-compartment model. VCR is substantially metabolized by the liver. Two metabolites, N-deformyl vincristine [8] and desacetyl vincristine [1], have been identified but their activities have not been characterized. VCR is eliminated primarily in the faeces (70%) as a result of biliary excretion, and to a lesser extent in the urine (12%). The dose-limiting toxicity of VCR is neurotoxicity. A relationship has been established between the area under the plasma concentration–time curve (AUC) and neurotoxicity in adults [1].

EXPERIMENTAL

Chemicals and samples

Vincristine sulphate was commercially obtained from Cyanamid (Wayne, NJ, USA, Batch No. K-8902). Distilled water was purified by a Milli-Q water purification system (Millipore). Methanol (pro analysi, Merck, Art. No. 6009) and acetonitrile (HPLC, Rathburn, No. RH 1016) were used without further puri-

fication. Phosphate buffer (25 m*M*, pH 7.0) was prepared by dissolving 3.41 g of potassium dihydrogenphosphate (KH_2PO_4) in 1.0 l of water and adjusting with 1.5 *M* potassium hydroxide to pH 7.0.

The stock solution of 2.0 mg/l VCR in water (stored at −80°C for a maximum of one year) was made by dissolving 11.2 mg of vincristine sulphate in 100.0 ml of water and diluting 1.00 ml of this solution with water to 50.0 ml. As internal standard, a 2.0 mg/l stock solution of vinblastine was used (stored at −80°C for a maximum of one year). This solution was prepared by dissolving 22.0 mg of vinblastine sulphate (Eli Lilly Netherlands, Batch No. C12-7AE-195) in 100.0 ml of water and diluting 1.00 ml of this solution with water to 100.0 ml.

Blood samples were collected in a polypropylene tube. Directly after collection, the samples were centrifuged and the serum (or plasma) was transferred to a second polypropylene tube and stored in the dark at −80°C (for a maximum of one year). Samples were analysed immediately after thawing.

Apparatus and chromatography

The column-switching HPLC system (Fig. 1) consisted of two HPLC pumps from ABI Analytical (Kratos Division Holland), a Rheodyne hand injector or a Promis II automatic injector (Spark Holland) both with a 1.0-ml loop, a solvent selector valve (Multiport stream switch, MUST, Spark Holland) with a Rheodyne valve 1010 (load time 10 min, desorption time 5 min, wash time 10 min; back-flush mode) and a Kratos Model 783 UV–VIS detector (set at 300 nm, range 0.002 a.u.f.s.) connected in series with an AMOR electrochemical detector (Spark Holland), which essentially is an amperometric (conversion 4–10%) thin-layer cell with a glassy carbon working electrode (WE), a carbon-filled Teflon auxiliary electrode (AE) and a so-called "*in situ*" (1 m*M* Cl required in mobile phase) Ag/AgCl reference electrode (RE). The 50-μm spacer was clamped between and partly covered the WE and the AE. The cut-out in the spacer settled the cell volume to *ca.* 0.5 μl. The flow path and performance were determined mainly by

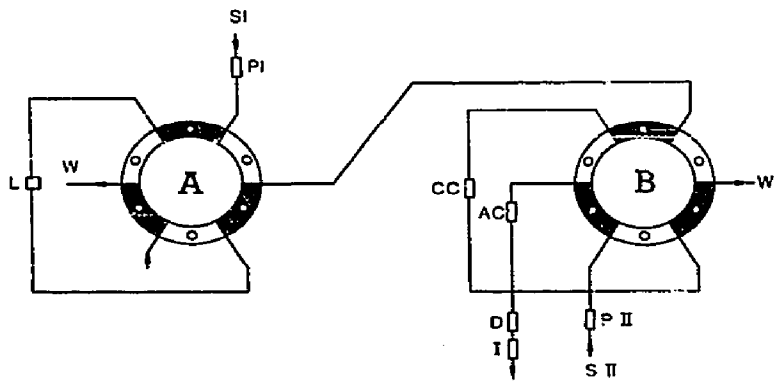

Fig. 1. Column-switching configuration, using backflush mode. SI = wash fluid; SII = mobile phase; PI and PII = HPLC pumps; L = loop; W = waste; CC = preconcentration column; AC = analytical column; D = detector; I = integrator. ■, Wash phase; □, desorption phase.

the smoothness of this cut-out and by the flatness of the WE and the AE (leakage!). The potential on the WE was +0.75 V vs. Ag/AgCl. In this way, possibly interfering serum peaks were eliminated (response ratio UV/electrochemical detector must be constant). In order to improve the detection limit, a recently developed electrochemical detector (type VT-03, ANTEC Leyden, Leiden, Netherlands), was used instead of the AMOR detector. This detector cell is amperometric (conversion 5–10%), with a WE of 3 mm diameter. The eluent was directed perpendicular to the centre of the glassy carbon WE ("wall-jet") and the concentrically positioned stainless-steel AE tightly enclosed the WE in this half of the Kel-F cell body [9]. The 50-μm spacer, clamped between this half and the block, containing the Ag/AgCl RE and the cell inlet, was positioned far (*i.e.* 20 mm) outside the flow path, to ensure an undisturbed, radiant flow over the WE. The cell outlet was mounted in the cell block containing the WE and the AE. Essentially, in such a position the cell volume is determined by the area of the WE and the thickness of the spacer and amounts to 330 nl. Critical in this design are the finish and the enclosement of the WE. The potential on the WE was +0.83 V vs. Ag/AgCl [9].

The preconcentration column, used for the prepurification of the serum samples, was a reversed-phase C_{18} column (Chrompack Netherlands, Art. No. 28675, 10 mm × 3.0 mm I.D.), dry-filled in our laboratory with Whatman pellicular octadecylsilane (ODS) material (particle size 30–38 μm, Chrompack, Netherlands, Art. No. 4102-010). Each day a new inexpensive preconcentration column was used.

The analytical column, used for the separation of the compounds eluted from the preconcentration column, consisted of a reversed-phase Microspher C_{18} column (particle size 3 μm, 100 mm × 4.6 mm I.D., Chrompack, Netherlands, Art. No. 28410).

The UV detector and electrochemical detector were both connected to an SP 4290 computer integrator (Spectra-Physics Holland), which was used for measuring peak heights (paper speed 0.5 cm/min, AT = 8).

The mobile phase (solvent II) consisted of methanol–acetonitrile–25 m*M* phosphate buffer (pH 7.0) (48:20:32, v/v/v). This solution was filtered and degassed by sparging with helium. The wash fluid (solvent I) was a solution of methanol 5% (v/v) in water. The flow-rate of both pumps (solvents I and II) was 1.25 ml min. The operating pressure with this system was *ca.* 12 MPa.

Assay

In a 2.0-ml conical polypropylene tube, 1.2 ml of serum or plasma was pipetted. Then 100 μl of internal standard (2.0 mg/l vinblastine in water) were added. The samples were mixed and centrifuged for 5 min at 3000 rpm (*ca.* 1500 *g*) in order to remove cells and other particulate matter. Subsequently, 1.0 ml of clear serum was injected directly into the preconcentration column. This column was then washed for 10 min with solvent I. During this time, VCR was concen-

trated on the preconcentration column, and proteins and other impurities were washed off (wash phase, see Fig. 1). After 10 min the solvent selector valve was switched.

The mobile phase (solvent II) was pumped through the preconcentration column in the opposite direction (backflush-mode) and transported VCR to the analytical column (desorption phase, see Fig. 1). The compounds were eluted within 8 min. After 5 min the solvent selector valve was switched back. Both columns were then conditioned for 10 min.

Calculation

The VCR concentrations were calculated from the peak-height ratio of VCR to internal standard for samples and standards, by using the integrator.

Calibration and recovery

A calibration graph was prepared as follows: 1.0 ml of drug-free pooled human serum was pipetted into a 2.0-ml conical polypropylene tube. Then 0, 5, 10, 20, 40 or 80 μl of the stock solution of VCR (2.0 mg/l) was added. This resulted in a calibration graph of 0, 10, 20, 40, 80 and 160 μg of VCR per litre of human serum. The extraction procedure as described above was used for these standards.

The extraction recoveries of VCR from serum samples were calculated by comparing the peak heights of VCR after injection of non-extracted VCR spiked solutions directly on the analytical column, to peak heights obtained after injection of extracted spiked serum samples containing equal concentrations of VCR. Extraction recoveries were determined at concentrations of 20 and 6.0 μg/l for VCR and at a concentration of 100 μg/l for vinblastine.

Precision

Series of serum controls spiked with VCR and the internal standard were determined on the same day (at concentrations of 5.0, 15.6 and 31.2 μg/l for VCR and 100 μg/l for vinblastine with $n = 10$) and over twenty days (at a concentration of 15.6 μg/l for VCR).

Matrix studies

Four different matrices, *i.e.* human serum, two kinds of calf's serum (foetal bovine serum, Flow Labs. Irvine, UK, Lot. No. 028138 and neonatal bovine serum, ICN Biochemicals, USA, Lot. No. 158327) and drug-free human urine, were used to study the matrix influences on reproducibility. For each matrix, calibration graphs were made by spiking 1.1 ml of the matrix with 0, 20, 40 or 80 μl VCR stock solution (0.50 mg/l). This resulted in calibration graphs of 0, 8.9, 17.5 and 33.9 μg VCR. To study the influence of the human matrix itself, eight serum samples, each from a different patient, collected from samples submitted for routine therapeutic monitoring, were spiked to yield a concentration of 50 μg VCR/l in serum and were assayed on the same day.

RESULTS AND DISCUSSION

Chromatography

Representative chromatograms are shown in Fig. 2. Fig. 2A shows a chromatogram of a spiked human serum sample detected by the UV detector, and Fig. 2B shows a chromatogram of the same sample detected by the wall-jet cell. The retention times of VCR and the internal standard are *ca.* 3.7 and 4.9 min, respectively.

From these values it is seen that electrochemical detection results in a cleaner (fewer possibly interfering serum peaks) chromatogram. Besides, with the wall-jet cell the detection limit is reduced considerably (see *Precision*).

Calibration and recovery

The calibration graph for the determination of VCR in serum after amperometric detection covered the range 0–160 μg/l and was linear: $y = 1.87x + 0.02$ (y = response ratio; x = μg/l VCR) and a correlation coefficient of 0.9990.

The recoveries of VCR in human serum, determined at concentrations of 20 and 6 μg/l, were 68.2 ± 2.1 and 70.0 ± 6.2% (n = 6). The recovery of the internal standard was 75.0 ± 6.0%, determined at a concentration of 100 μg/l vinblastine in human serum.

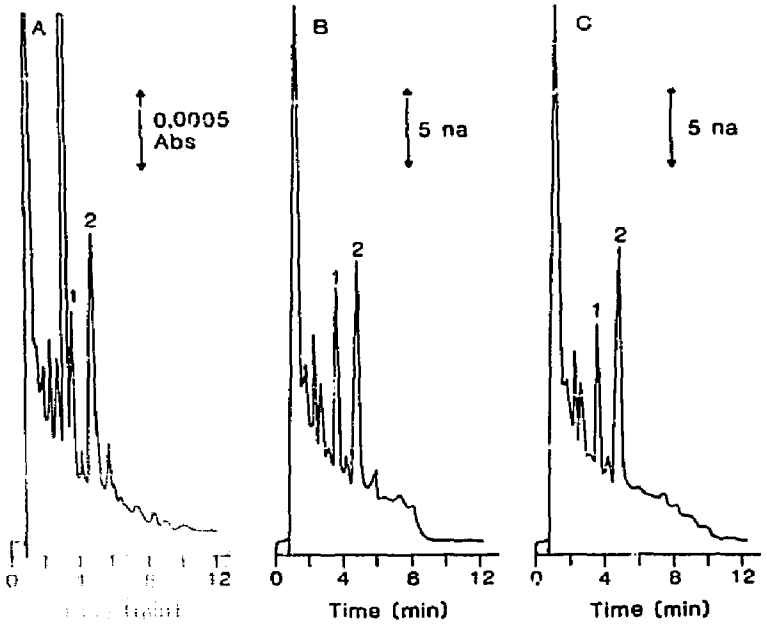

Fig. 2. (A) and (B) Chromatograms of a spiked human serum sample (9.0 μg/l VCR), detected by (A) the UV detector and (B) the wall-jet cell. (C) Chromatogram of a sample taken 30 min after intravenous bolus injection of 1.5 mg/m² VCR using the wall-jet cell (7.1 μg/l VCR). Vinblastine (20.0 μg/l) was used as the internal standard (peak 2). UV detection does not adequately resolve the peaks of interest from the early-eluting peaks, but amperometric detection does.

Precision

The within-day (n = 10) coefficients of variation (C.V.) for this method (with controls of human serum used) were 4.7% (5.0 μg/l), 3.2% (15.6 μg/l) and 2.9% (31.2 μg/l) for VCR and 3.5% (200 μg/l) for vinblastine, using UV detection. The day-to-day reproducibility for the method was 4.4% (15.6 μg/l; UV detection) and 5.1% (15.6 μg/l; electrochemical detection) for VCR (n = 20).

The limit of detection for VCR, defined as a signal-to-noise ratio of 3, was 0.3 μg/l, using the wall-jet cell. With the AMOR detector and with the UV detector the detection limits were both 1.0 μg/l.

Matrix studies

After the assay of VCR spiked serum, urine, and two kinds of calf's serum, a distinct matrix-dependency of the system became clear. Fig. 3 shows the calibration graphs in the various matrices. Obviously, there is a great difference in response between the different matrices. No difference in response was noticed between eight different human serum samples (spiked with 10 μl of 5.0 mg/l VCR in water, data not shown), so the human serum matrix itself has no influence on the recovery of VCR. It is obvious that if urine samples have to be determined, the calibration graphs should also be made using urine.

Kinetics and interferences

This assay is now used for clinical pharmacokinetic studies of VCR in children. Fig. 4 shows the serum concentration–time curve obtained after intravenous bolus injection of vincristine (1.5 mg/m^2) to a 3-year-old boy with acute lymphocytic leukemia. In this patient the estimated total body clearance of vincristine was 13.5 l/h/m^2; the terminal serum half-life was 20.5 h.

Further kinetic studies are currently being performed. No analytical interferences were noticed with drugs regularly administered to children suffering from cancer. Vendrig [10] has found that desacetylvincristine (DVCR, the main metabolite of VCR) is formed at pH 1.2. We also found at this pH a decrease of the VCR peak and no interference between VCR and the metabolite.

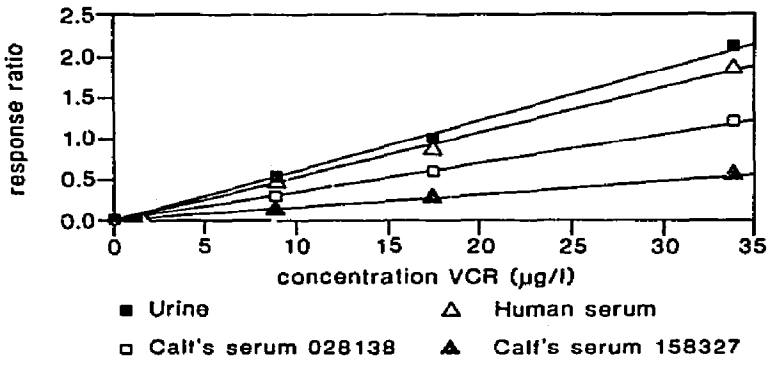

Fig. 3. Calibration curves of VCR, using four different matrices. The response ratio is expressed as the peak-height ratio of VCR to VCR in a spiked calf's serum standard of 31.2 μg/l.

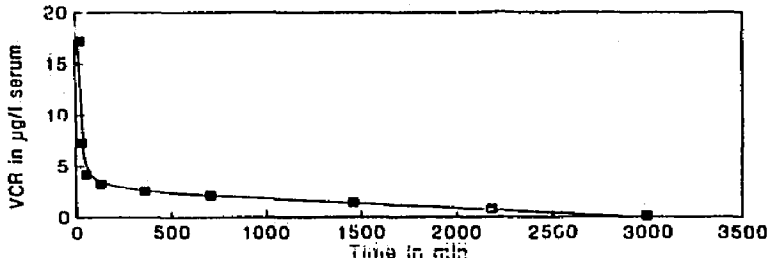

Fig. 4. Log plasma VCR concentration–time curve (intravenous bolus injection of 1.5 µg/m² VCR, in a child of 3.9 years and 14.6 kg).

CONCLUSIONS

After on-line column-extraction it is possible to determine VCR at the µg/l level using HPLC with UV and electrochemical detection. By using two detection methods, the problem of interfering peaks is minimized. The advantages of this kind of extraction compared with liquid–liquid extraction or off-line solid-phase extraction are numerous, the most important being the posibility of total automation. The detection limit is 0.3 µg/l when a recently developed electrochemical detector is used. This high sensitivity allows reliable pharmacokinetic studies even in a low concentration range. It also makes a smaller sample size feasible for studies in very young children.

The system is matrix-dependent so, for preparing standards, human and calf's serum are not interchangeable. The human serum matrix can be considered as a constant matrix: no difference in response between different spiked human serum samples was noticed.

The precision of this method is good with low C.V. (less than 5%). The low detection limit of 0.3 µg/l at a signal-to-noise ratio of 3.0 makes this method sufficiently sensitive for use in clinical pharmacokinetic studies in children.

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