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Analytical methods for the determination of vinca alkaloids in biological specimens: a survey of the literature*

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Abstract: The bio-analysis and pharmacokinetics of vinca alkaloids have been the subject of many investigations. In most cases radiolabelled compounds have been used for quantification purposes. Although this method lacks selectivity, it has provided valuable information on tissue distribution of unchanged drug and metabolites in an early stage of clinical and preclinical investigations. During the last few years, methods based on high-performance liquid chromatography have been presented. This paper reviews the methods described in the literature for the bio-analysis of vinca alkaloids, supplemented with our own experience in this field. Special attention is paid to the problems that may arise during the analytical processes.

Keywords: *Vinca alkaloids; metabolites; radio-labelled drugs; HPLC; bio-analysis; review.*

Introduction

Vinblastine (VBL) and vincristine (VCR) are naturally occurring vinca alkaloids derived from the periwinkle plant *Catharantus roseus G. Don.* These drugs have been employed in a variety of human neoplastic disorders for over 25 years. The minor structural differences between VCR and VBL lead to differences in anti-tumour spectra, potency and toxicity. Several analogues have been synthesized in order to enhance the therapeutic efficacy and/or to change the anti-tumour spectrum. Vindesine (VDS) was the first of these semi-synthetic derivatives shown to be clinically useful. Other derivatives are being or have been investigated in pre-clinical and clinical studies [1–5].

With the increasing interest into the pharmacokinetic properties of these compounds, the need arose for analytical methods for the measurement of vinca alkaloids in biological materials. High demands are made upon the sensitivity of these techniques, because the compounds are administered in low dosages. During the late sixties, when this type of drug was introduced into clinical practice, investigators lacked any analytical equipment which met this requirement. Consequently, most pharmacokinetic studies were performed by the administration of radiolabelled drugs. Only a few years ago, the first papers appeared in the literature, dealing with the bio-analysis of vinca alkaloids, based on high-performance liquid chromatography (HPLC) in combination with on-line sensitive detectors.

After 2 years of experience with the bio-analysis of this family of compounds, in particular investigational vinca derivatives, we learned that the analysis of most vinca alkaloids in biological material can be reduced to a manageable problem, provided certain analytical requirements are respected; although it should be noted that the analytical behavior of these compounds sometimes remains unexpected and demands continuous attention. This paper reviews several methods described in the literature for the bio-analysis of the dimeric vinca alkaloids, supplemented with our own experience in this field. Other pharmaceutically active alkaloids derived from other periwinkle plants (vinca minor) are not

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included. Detailed information concerning the procedures of sample handling and chromatographic analysis are presented.

Bio-analysis using Radiolabelled Compounds or Radio-immuno Assays

Formerly, most animal and human pharmacokinetic studies with vinca alkaloids have been carried out by using radiolabelled compounds [6–15, 21]. The amount of radioactivity present in samples were determined by liquid scintillation counting after extraction or combustion of the biological sample [radiolabelled assay (RA)]. The use of this method is simple, but it lacks selectivity because it monitors the destiny of the radiolabel, which is not necessarily identical to that of the investigated compound. Therefore most investigators refined the RA method by combining it with a chromatographic method (TLC or HPLC) to discriminate between unchanged drug and other sources of radiolabel (metabolites or tritiated water). In spite of the fact that these studies provided mainly qualitative rather than quantitative results, the method has certainly expanded the knowledge about tissue distribution of unchanged drug and metabolites [7, 8, 10–12, 14]. However, characterization of the plasma kinetics was only possible for a short period of time after administration, as undetectable levels were attained within a few hours [6, 7, 11].

With the introduction of radio-immunoassays (RIA) for vinca alkaloids it became possible to monitor drug plasma levels for up to 72–96 h [16–21]. Although RIA, in general, can be very specific, the specificity of the assay depends upon the characteristics of the antiserum used. As long as the identity and concentration of metabolic conversion products and their interactions with the antiserum is not known, the results obtained by RIA should be interpreted with caution.

The recent availability of radiolabelled drug with a very high specific activity enabled the use of RA for the measurement of plasma levels for an extended period of time (240–360 h) [13, 15], but large discrepancies were reported between the results obtained by the RA and those obtained by more specific RIA methods. This result clearly invalidates the use of the RA for the establishment of the plasma pharmacokinetics.

HPLC Methods

With the growing popularity of HPLC in the bio-analytical field, several methods capable of analysing vinca alkaloids and metabolites in low concentrations have been developed [22–31]. The first report was made by de Smet *et al.* [22] using reversed-phase chromatography with UV detection at 220 nm. A rather laborious sample pretreatment procedure was needed to obtain sufficient selectivity, while the detection limit (6 ng ml^{-1}) was still inadequate for plasma pharmacokinetic purposes. These limitations are inherent to the use of UV detection at 220 nm. Current more sophisticated UV detectors may perform better in terms of sensitivity but selectivity will remain a major problem as many endogenous organic substances absorb in the low UV range.

A major step forward in gaining selectivity (and sensitivity) was obtained by the observation that this class of compounds is electrochemically active under typical reversed-phase HPLC conditions [23], and most HPLC studies presented recently have employed reversed-phase HPLC with electrochemical detection (ECD) [22–24, 26, 29]. Although it is not described in most papers, it is our experience that persistent problems can be encountered during these chromatographic processes (peak broadening and nonlinearity [29]), that are most likely due to the presence of residual silanol activity on the reversed phase packing material. These problems, that vary from column to column (even when factory packed in one lot from one supplier), appeared difficult to control and frequently complicate the analysis. In our laboratory this has led to the use of a totally different mode of separation [28, 30, 31], based on ion-exchange chromatography on unmodified silica (IESPC), which was described for alkaloid substances by Lingman *et al.* [32] and adapted later by Vendrig *et al.* [25] for the analysis of VBL and VDS. In our hands IESPC has proved to be very useful for the analysis of vinca alkaloids and investigational derivatives, with no complications in terms of stability and column to column reproducibility. Retention times may slowly decrease upon aging of the column, but this does not affect the applicability of the assay. The relatively high percentage of organic solvent present in the mobile phase enhances the native fluorescence properties of

the vinca alkaloids, permitting the use of a fluorescence detector (FD) for sensitive detection, instead of ECD, which further increases the stability of the system.

As was shown by Lingman *et al.* [32], the retention of alkaloid compounds in a IESPC system can be regulated by the pH, the counter-ion and the organic modifier concentration of the mobile phase. Optimization of these parameters resulted in a system, that is suitable for the analysis of a variety of vinca alkaloids and/or their metabolites using uniform chromatographic conditions (Table 1).

Sample Pre-treatment Procedures

As with many HPLC applications a sample clean-up step prior to the chromatographic separation is essential. Vendrig *et al.* have described solid phase extraction (SPE) procedures, by which concentrations in the ng ml^{-1} range in plasma could be quantitated. Bond Elut® diol columns [23] gave poor results due to non-reproducible column packings, while Bond Elut® CN columns [24] did not show these problems and appeared to be suitable. Apart from the fact that these SPE methods are very laborious, which can be circumvented by the use of an automatic sample processor, SPE has also the disadvantage that it can only handle clear 'non-turbid' samples, because particles can block the SPE column. As we were interested in a method for the analysis of low concentrations of vinca alkaloids in turbid samples (e.g. tissue and faeces homogenates), liquid–liquid extraction was selected as sample pretreatment procedure.

Extraction procedures with chloroform or methylene chloride have been used, but were only successful if preceded by a deproteinization step with ethanol or acetonitrile [12, 13]. We found that these procedures can be reduced to a single-step by diluting the sample with phosphate buffer of appropriate pH (Fig. 1). This strategy can be used for most vincas except for navelbine (NVB) and its main metabolic product desacetylnavelbine (DNVB), as these compounds showed a poor recovery in chloroform under all conditions tested. However these compounds are extracted effectively with diethylether and a combination of diethylether extraction and IESPC-FD is now routinely used for the bio-analysis of NVB and DNVB in our laboratory.

Complications in the Bio-analysis of Vinca Alkaloids

As mentioned before, problems with the

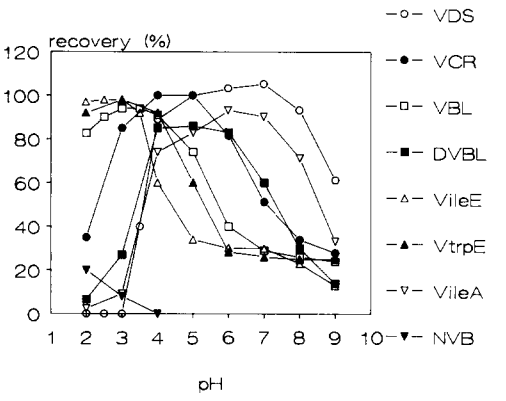

Figure 1
Extraction recoveries of vinca alkaloids from plasma. A 500 µl volume of plasma (containing 0.5 µg of each vinca alkaloid) was mixed with 2.5 ml 0.5 M phosphate buffer and extracted with 5 ml of chloroform. The pH of the buffer ranged from 2 to 9.

Table 1
Capacity factors (k') of vinca alkaloids in the chromatographic system based on ion-exchange normal phase chromatography

Compound	k'
N-(Deacetyl-*O*-4-vinblastoyl-23)-L-ethyl-tryptophane (vintriptol, VtrpE)	1.6
N-(Deacetyl-*O*-4-vinblastoyl-23)-L-ethyl-isoleucinate (VileE)	1.6
5′-Noranhydrovinblastine (navelbine, NVB)	1.8
Vinblastine (VBL)	2.2
4-*O*-Deacetylnavelbine (DNVB)	2.4
4-*O*-Deacetylvinblastine (DVBL)	2.9
N-(Deacetyl-*O*-4-vinblastoyl-23)-L-tryptophane (VtrpA)	3.1
N-(Deacetyl-*O*-4-vinblastoyl-23)-L-isoleeucinate (VileA)	4.0
Vindesine (VDS)	5.1

Chromatographic conditions — column: 5 µm Spherisorb-Si (250 × 2 mm); mobile phase: acetonitrile:citrate buffer (pH 3.0; 10 mM) containing 10 mM tetrabutylammonium bromide (85:15, % v/v); flow rate: 0.2 ml min^{-1}.

analysis of this type of compounds are frequently encountered but not often discussed. In the next paragraphs an overview is presented of the obstacles, that we were confronted with during our studies.

Adsorption to the material of the test tube is a general feature of vinca alkaloids. The nature of the material will be of importance, but we have not discovered one to be superior to the others. The extent of adsorption is mainly determined by the solvents used and is most prominent when the compounds are dissolved at relatively low concentration (<20 μg ml^{-1}) in pure water. In organic solvents like acetonitrile and chloroform there are no losses in concentrations down to 1 ng ml^{-1}. The same occurs when vinca alkaloids are dissolved in biological fluids, which is probably caused by interactions with endogenous compounds that prevent adsorption to the container material. We found that 40 g l^{-1} bovine serum albumin (BSA) offers an adequate protection against adsorption. As a result of these findings, stock solutions of 1 mg ml^{-1} are prepared in water and all further dilutions are prepared with the appropriate matrix (e.g. plasma or urine). Furthermore, tissue and faeces samples are homogenized in a solution of 40 g l^{-1} BSA in water.

For most studies chloroform is used for sample clean-up. Vigorous mixing of the test tubes is essential for a good recovery, but can lead to the formation of an emulsion, which reduces the recovery. Centrifugation at high speed (2500g) for 10–15 min eliminates these emulsions and provides a good separation between the aqueous and organic phases. A consecutive step in the sample pretreatment procedure is the evaporation of the organic solvent with subsequent dissolution (by sonication) of the residue in a solvent compatible with the HPLC system (e.g. acetonitrile). Dissolution appears quantitative if the organic phase is obtained from the extraction of a sufficient amount (500 μl) of plasma or urine. Extraction, however, with less biological material leads to an incomplete dissolution of the analytes. It is hypothesised that a critical amount of co-extracting compounds should be present in the residue to prevent irreversible adsorption after evaporation. Consequently, the analysis of plasma or urine is always performed with a 500 μl sample volume, whereas other solid specimens (e.g. tissue or faeces homogenates) require the addition of 500 μl blank plasma to each extraction tube.

Application of the Method for Tissue and Faeces Samples

In earlier reports the validity of these methods for the analysis of vinca alkaloids in plasma and urine has been demonstrated [28, 30, 31]. The usefulness of the assay for the analysis in other biological matrices is presented here. Figure 2 depicts the chromatograms obtained by extraction of homogenized faeces samples from mice (FVB strain) who received 6.0 mg kg^{-1} VBL. Apart from the peaks corresponding to VBL and DVBL, two other major peaks are present. Their identities and/or origin (hepatic or intra-intestinal) have not yet been established.

Figure 3 displays the chromatograms obtained after extraction of several tissues from FVB mice, who received 12 mg kg^{-1} NVB. The advantage of this HPLC method over RA or RIA methods is obvious, as significant amounts of DNVB were detected in all tissues.

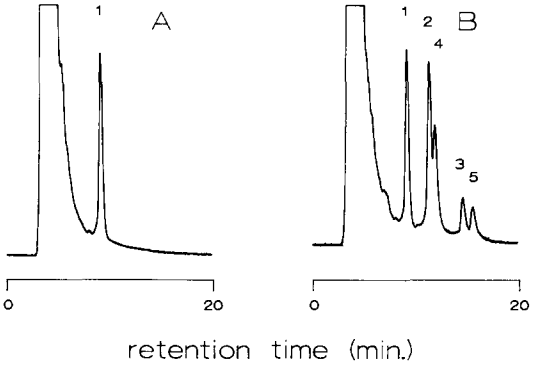

Figure 2
Analysis of VBL and metabolites in faeces from FVB strain mice collected before (A) and 0–24 h after administration of 6.0 mg kg^{-1} VBL (B). A volume of 100 μl of faeces homogenates (approximately 0.1 g ml^{-1}) supplemented with 500 μl blank human plasma, 10 μl (50 mg l^{-1}) VtrpE (internal standard) and 2.5 ml 0.5 M phosphate buffer pH 4.0 were extracted with 5 ml chloroform/2-propanol (95/5). The organic phase was evaporated to dryness and the residue dissolved in 100 μl acetonitrile. Samples (20–80 μl) were chromatographed on a 5 μm Spherisorb-Si column (250 × 2 mm) eluted at a flow rate of 0.20 ml min^{-1} with acetonitrile:citrate buffer (10 mM: pH 3.0) containing 10 mM tetrabutylammoniumbromide (85:15, % v/v). Fluorescence detection was used with the excitation monochromator fixed at 270 nm, while emission was monitored using a 320 nm long-pass filter. [1 = VtrpE (internal standard), 2 = VBL, 3 = DVBL, 4 and 5 = unknown.]

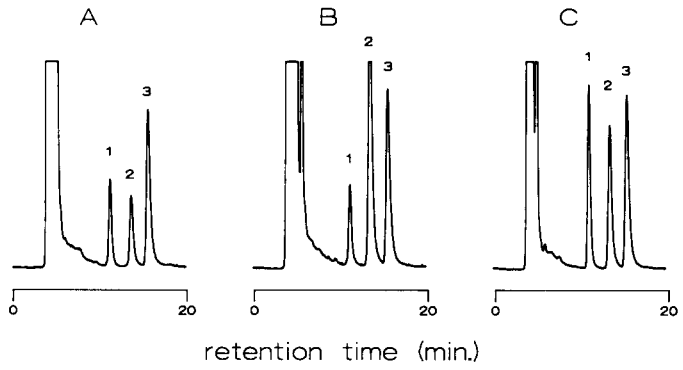

Figure 3
Analysis of NVB (1) and DNVB (2) in tissues from mice (FVB strain) obtained 24 h after the administration of 12.0 mg kg^{-1} NVB. (A = small intestine, B = liver, C = kidneys.) A volume of 500 µl tissue homogenate together with 500 µl blank plasma and 10 µl (15 µg ml^{-1}) DVBL [internal standard (3)] was extracted with 4 ml diethylether. The organic phase was evaporated to dryness and the residue dissolved in 100 µl acetonitrile. For chromatographic conditions see the legend of Fig. 2.

Conclusions

In conclusion, RA or RIA methods for the analysis of vinca alkaloids have been important methodologies in the past. Validated analytical procedures based on HPLC have now been developed by several laboratories and HPLC should be preferred as the method of choice in further studies, as they allow a better discrimination of unchanged drug from metabolites. Liquid–liquid extraction in combination with ion-exchange normal phase chromatography and fluorescent detection provides a selective, flexible and sufficiently sensitive method for the determination of these compounds in various biological matrices.

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