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Note

Analysis of the anticancer drugs etoposide (VP 16-213) and teniposide (VM 26) by high-performance liquid chromatography with fluorescence detection

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In a previous paper [1] we reported on an assay for the important anticancer drugs etoposide (VP 16-213) and teniposide (VM 26) (Fig. 1) by reversed-phase high-performance liquid chromatography (HPLC) using fixed-wavelength UV detection at 254 nm, and utilizing each drug as the internal standard for the other. The assay allowed the analysis of clinical drug plasma levels to 24 h, at which time drug levels are commonly about 500 ng/ml (850 pmol/ml) [2].

The potential of using fluorescence detection of these compounds for increased sensitivity was also demonstrated [1]. Detection of these relatively weakly fluorescing compounds was accomplished by placing a 30-μl flow-cell in the cuvette block of a typical spectrofluorometer, exciting the compounds at

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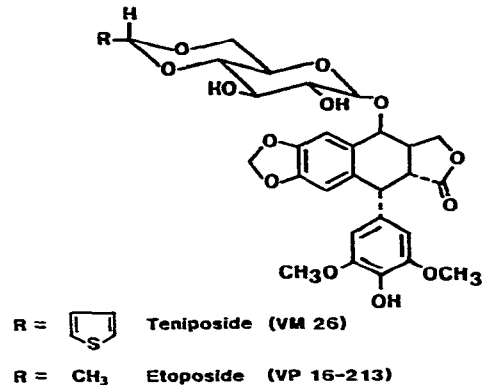

Fig. 1. Structures of etoposide (VP 16-213) and teniposide (VM 26).

288 nm and observing the emission at 328 nm. The transition from this experiment to that described here of using a commercial dedicated HPLC fluorescence detector was not straighforward, particularly regarding optimum conditions for sensitivity and selectivity. However, an improved assay for these drugs to 50 ng/ml (85 pmol/ml) of plasma was developed and is reported here. The advantages of this lower detection limit are demonstrated with a patient plasma concentration profile for etoposide over 48 h in which drug concentrations of considerably less than 500 ng/ml of plasma were encountered, primarily because a small dose of the drug was administered. The example also illustrates the advantage of the selectivity of fluorescence detection since the patient also received numerous other drugs during the 48-h time course and some of these interfered with the UV assay.

EXPERIMENTAL

Materials

Etoposide and teniposide were generously provided by Drs. H. Friedli and H. Stähelin (Sandoz, Basle, Switzerland), and by Dr. R.L. Buchanan (Bristol Labs., Syracuse, NY, U.S.A.). Chloroform and methanol (distilled in glass) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All water used was distilled. Extraction of plasma was carried out in 16 × 125 mm culture tubes with PTFE-lined caps. All glassware used was routinely washed in chromic acid.

Apparatus

The HPLC system consisted of a Model M-6000A pump, a Model U6K injector, and a prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column, all from Waters Assoc. (Milford, MA, U.S.A.). A short precolumn (7 cm × 2.1 mm I.D.) packed with Co:Pell ODS (30 μm) from Whatman (Clifton, NJ, U.S.A.) protected the analytical column. Detection was accomplished using the Model FS 970Q dedicated HPLC fluorescence detector with a quartz window photomultiplier tube, and the Model SF-770 dedicated

variable-wavelength UV detector, both from Kratos, Schoeffel Instrument Division (Westwood, NJ, U.S.A.). Cut-off filters at 300 and 320 nm, and a 7-54 broad band filter (ca. 200 nm wide centered at ca. 320 nm) were supplied by Schoeffel. A narrow band pass interference filter, 8.2 nm wide at half height and centered at 328 nm, was supplied by Spectrofilm (Winchester, MA, U.S.A.).

HPLC conditions

Analyses were performed with a flow-rate of 1.0 ml/min at an inlet pressure of about 48 bars. For samples from plasma extracts the flow-rate was increased after the compounds of interest were eluted, in order to elute retained background peaks as quickly as possible. The solvent used was methanol—water (60:40), and was filtered through a 0.5-μm MF millipore filter (Millipore, Bedford, MA, U.S.A.) and briefly degassed prior to use. The injection volume was 25 μl.

Calibration and analysis of etoposide from plasma

A stock solution of about 5 mg (accurately weighed) of etoposide per ml was made up in methanol. Ten-fold dilutions were made through 5 μg/ml (5 ng/μl). Teniposide was made up in a similar manner at 1 and 0.1 mg per ml of methanol. In spiking plasma, no more than 20 μl total methanol were added to 1 ml of plasma. One μg of teniposide was used as internal standard in spiked 1-ml plasma samples containing up to 5 μg of etoposide. For spiked samples containing more than this amount of etoposide per ml, 10 μg of teniposide were used as internal standard and this was taken into account in the calibration curve. These concentrated samples were also diluted ten-fold with methanol prior to injection in order to prevent overloading of the detector photomultiplier tube. All clinical samples analysed for the patient profile contained 1 μg of teniposide as internal standard. The samples were extracted and prepared for injection as described previously [1].

The excitation wavelength was set at 215 nm and the 10-nm band pass interference filter was mounted centrally in a piece of 2.5 × 2.5 cm cardboard, which was placed in the emission filter holder of the fluorescence detector. The sensitivity setting corresponded to about 1500 V applied to the photomultiplier tube.

Calibration points were determined in triplicate over the range 50 ng to 50 μg of etoposide per ml of plasma. No significant background was observed for the internal standard teniposide at 1.0 μg/ml of plasma. Therefore, the observed background at the retention time of etoposide, which was significant at the 50 ng/ml plasma level, was able to be internally standardized in blank samples as a peak height ratio with respect to the internal standard teniposide. This ratio was substracted from the observed peak height ratio of etoposide to teniposide in spiked samples. The zero time sample served as the blank in clinical samples.

RESULTS AND DISCUSSION

In determining the optimum instrumental configuration of the fluorescence

detector for this analysis, various excitation wavelength and emission filter combinations were employed to examine samples of pure etoposide and teniposide, as well as extracts of plasma samples spiked with these drugs. It was found that the previously used [1] excitation wavelength of 288 nm provided insufficient sensitivity for analysis. For example, with a 320-nm cut-off filter on the emission side, a 200-ng injection of pure etoposide only provided a signal-to-noise ratio of about 15:1. However, because the light source was now a deuterium lamp instead of the previously used xenon lamp, advantage could be taken of the stronger absorption of these drugs at 215 nm to gain sensitivity. A xenon lamp provides insufficient light intensity at this wavelength. Thus, with a 300-nm cut-off filter along with a 7-54 broad band filter on the emission side (to reduce stray white light reaching the PMT), 15 ng of etoposide provided a signal-to-noise ratio of about 40:1 using 215 nm excitation. Using these filters, sensitivity in the assay from plasma was ultimately limited by the background from the biological matrix. A 10-nm band pass emission filter provided only slightly better selectivity and sensitivity for plasma analysis.

The calibration curve from plasma was determined in triplicate at 51.85, 181.5, 285.2, 5185 and 51,850 ng of etoposide per ml of plasma by plotting the peak height ratio of etoposide/teniposide versus plasma concentration of

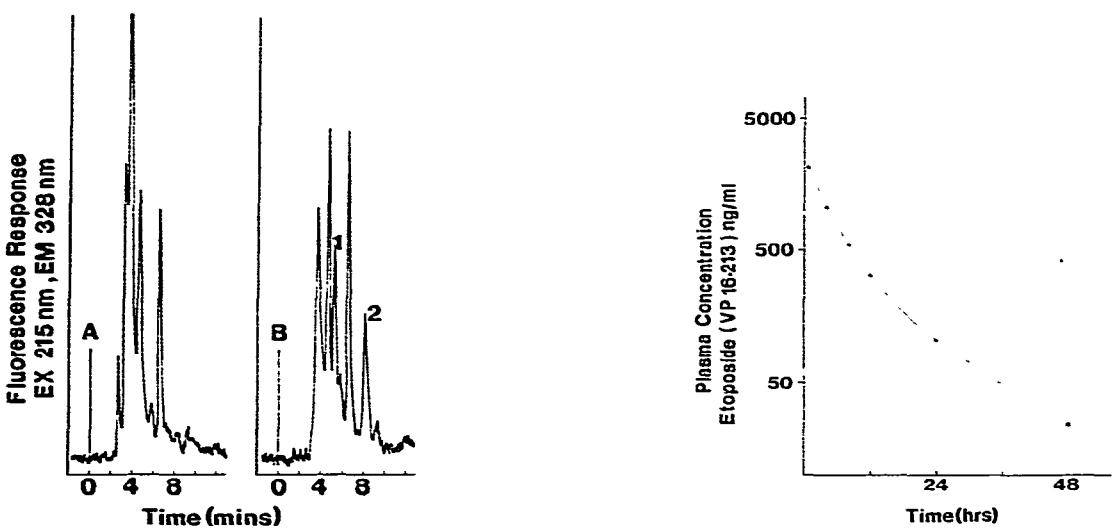

Fig. 2. HPLC fluorescence traces of plasma samples spiked with 50 ng/ml etoposide and teniposide, and treated by the chloroform extraction procedure. Chromatograms of (A) blank sample and (B) spiked sample. The endogenous substances eluting prior to 4 min varied from sample to sample but peaks after this time were reproducible. Chromatographic conditions: 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column; solvent, methanol—water (60:40); flow-rate, 1.0 ml/min at an inlet pressure of ca. 48 bars. Peaks: 1 = etoposide (51.8 ng); 2 = teniposide (50 ng). EX = excitation wavelength, EM = emission wavelength.

Fig. 3. Post-infusion plasma decay curve of etoposide for a patient receiving the drug. All points represent the average of two determinations. The point labelled * was obtained from two separate determinations and is shown as an estimate since the actual calibration was performed down to 50 ng etoposide per ml plasma.

TABLE I

COMPARISON OF UV ABSORBANCE VS. FLUORESCENCE DETECTION OF ETOPOSIDE (VP 16-213) EXTRACTED FROM PLASMA WITH TENIPOSIDE (VM-26) AS INTERNAL STANDARD (I.S.)

Sample plasma concentration	Detector*	n	Average peak height ratio, $\bar{x}$ etoposide/ teniposide	S_x	Coefficient of variation $S_x/\bar{x}$ (%)	95% Confidence limit (ng)
500 ng/ml etoposide 10 μg/ml teniposide (I.S.)	UV (254 nm)	6	0.0747	0.0042	5.6	± 75
500 ng/ml etoposide 1 μg/ml teniposide (I.S.)	Fluorescence (215 nm EX, 328 nm EM)	6	0.797	0.0124	1.5	± 21
50 ng/ml etoposide 1 μg/ml teniposide (I.S.)	Fluorescence (215 nm EX, 328 nm EM)	7	0.0767	0.0063	8.2	± 10

*EX = excitation wavelength, EM = emission wavelength.

etoposide. The correlation coefficient was $r^2 = 0.9999$ with a y intercept of −0.006 and a lowest peak height ratio determined at 0.080.

From Table I it can be seen that the fluorescence assay at 500 ng of etoposide per ml of plasma provides a considerable improvement versus the UV assay [1]. The fluorescence assay can be used with confidence to 50 ng of etoposide or teniposide per ml of plasma (Fig. 2), and the presence of the drug can be confirmed at concentrations as low as 25 ng/ml plasma.

The analysis of etoposide in the plasma of a 62-year-old woman with oat cell carcinoma of the lung is shown in Fig. 3. The patient received a constant intravenous infusion of only 51 mg of etoposide over 0.5 h. The patient also received concurrently the other antineoplastic drugs doxorubicin (adriamycin), carmustine (BCNU), and procarbazine. Over the 48-h plasma collection period the patient also received doses of the diuretic furosemide, the antiemetic prochlorperazine (compazine), and the sedative—analgesic meperidine (demerol). This patient profile (Fig. 3) demonstrates the need to be able to detect plasma levels of less than 500 ng of etoposide per ml, which would not have been measurable by the UV method. The peak identity for etoposide at the first time point was confirmed by stopped flow UV scanning while at 4 h interferences occurred in the UV traces. Injection of appropriate extracts of the pharmaceutical preparations of the drugs coadministered with etoposide, showed that some produced peaks that could have interfered with the analysis of etoposide using UV detection. However, none of these drug extracts showed any peaks by fluorescence detection and this specificity permitted accurate analysis of etoposide even at long times in patient samples (Fig. 4). The excellent profile for etoposide actually obtained reassures that no interfering compounds were co-eluted with etoposide or teniposide and detected by fluorescence.

Because of the high sensitivity capable with fluorescence detection, extractions were also carried out on 100 μl of spiked plasma samples using 500 μl of chloroform. At 500 ng etoposide per ml of plasma, about 20 ng is actually loaded on column. Recoveries were still nearly quantitative but the 95%

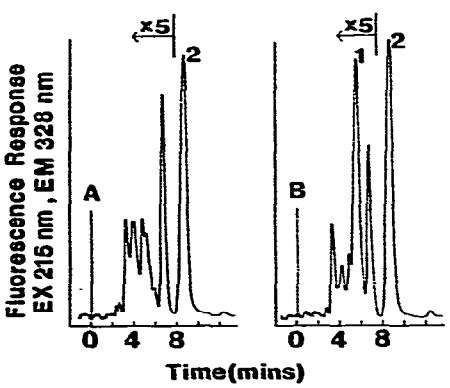

Fig. 4. HPLC fluorescence traces of patient plasma samples treated by the chloroform extraction procedure. (A) pre-infusion and (B) 24-h post-infusion. Peaks: 1 = etoposide (109 ng/ml); 2 = teniposide, internal standard (1.01 μg/ml). Chromatographic conditions and abbreviations as in Fig. 2.

confidence levels previously obtained using 1 ml of plasma (Table I) were slightly wider (518 ± 50 ng/ml, $n = 5$, $S_x/\bar{x} = 3.1\%$) because baseline noise was becoming significant. Thus it is feasible to perform the assay on patient plasma samples of only 100 μl to the 500 ng/ml level.

A problem with this fluorescence assay is that fluorescing substances are detected at long retention times. These peaks can interfere with subsequent injections. At concentrations of 500 ng of etoposide per ml plasma, samples can be safely overlapped to avoid these relatively small interferences so that three samples can be run in 90 min. However, it is suggested that for routine clinical use column switching technology [3] should be implemented to allow samples to be run every 10—15 min. In any case, since the internal standard is so similar to the drug being measured in its extraction, chromatographic and fluorescence behavior, it is expected that instrumental variations in analyses which take some time to complete, will be minimal. This is supported by consistent peak height ratios for pure samples injected and observed in day-to-day operations. For this reason, it is expected that frequent recalibration of the instrument will not be necessary.

The interferences at long retention times can also be minimized by operating at 235 nm for excitation, but some sensitivity is lost with a comparable detection limit for etoposide from plasma being about 150 ng/ml. Similar linearity over a comparable analysis range was obtained as with analysis at 215 nm.

CONCLUSION

An improved reversed-phase HPLC assay for the antineoplastic drugs etoposide (VP 16-213) and teniposide (VM 26) has been developed for clinical analysis of plasma levels of these drugs. Fluorescence detection of these compounds increases the sensitivity and selectivity of analysis compared to UV detection. The improved assay is useful for the analysis of these drugs to about 50 ng/ml (85 pmol/ml) of plasma and will allow analysis of plasma levels to about 48 h even with administration of low drug dosages.

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