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Drug monitoring of etoposide (VP16-213)

I. A combined method of liquid chromatography and mass spectrometry

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Summary. Drug monitoring is performed by means of sample extraction, sample purification by high-performance liquid chromatography (HPLC), and sample detection by time-of-flight mass spectrometry. This mass spectrometry utilizing ^{252}Cf fission fragment-induced ionization and desorption of nonvolatile compounds is suitable as a universal, nondestructive detector in HPLC. Liquid chromatography and mass spectrometry are combined, so that mass analysis can be operated online and offline to the fractional sampling of the effluent and the samples can still be recovered.

As an alternative to HPLC separation, samples can be purified by thin-layer chromatography (TLC), resulting an offline TLC + MS combination. Preliminary pharmacokinetic data for etoposide (VP16-213) together with calibration data are presented, and are discussed with reference to the sensitivity and detection limit of the new experimental method.

Introduction

The epipodophyllotoxine derivatives etoposide (VP16-213) and teniposide (VM26) are antitumor agents, both of which are active against a number of human malignant diseases [9]. The pharmacokinetics of VP16-213 have been studied with the homologous compound VM26 as an internal standard [6, 7], or the hydroxy acid of VM26 [5]. All measurements were based on HPLC, but different research groups have utilized different detection methods, i.e., UV absorption [4, 6, 7, 13] and electrochemical detection [5, 8].

The preliminary results for the pharmacokinetic parameters (e.g., peak plasma levels, elimination half-lives, distribution volumes, and system clearances) are not in total agreement [1], which indicates for interindividual deviations or systematic errors in the analyses. The capabilities of HPLC in the quantitative analysis of pharmaceutical preparations are generally recognized [14]. However, lack of specificity is common, owing to the regular use of UV and fluorescence detection. It seemed to us that the combination of liquid chromatography/mass spectrometry (LC/MS) would have a specificity and versatility comparable to the established combination of gas chromatography/mass spectrometry (GC/MS).

Thus we developed and utilized a mass spectrometric method of detection, which is described here and discussed in terms of sensitivity and detection limit. Its applicability in clinical routine analysis is demonstrated. A pharmacokinetic study will be presented in a second part (in preparation).

Materials and methods

Concerning the HPLC/MS method the logical relationship of all components is displayed in a schematic flow diagram (Fig. 1) emphasizing the central part of any combined HPLC/MS system: the interface. It links the liquid chromatograph to the mass spectrometer, transporting all nonvolatile compounds from the liquid phase to the high-vacuum stage. Besides the liquid chromatograph all parts were designed and assembled at our laboratory [2, 10].

The high-performance liquid chromatography (HPLC). The modular liquid chromatograph is equipped with a constant flow pump Kontron 410 and a variable wavelength UV absorption detector Kontron UVIKON 720 LC. The HPLC is run in the reversed-phase mode with isocratic elution. Commerical solvents (methanol, acetonitrile) and double-distilled water are utilized without further purification, but they are degassed and filtered after mixing. A radially compressed cartridge RCSS Radial-PAK C_{18} (10 μm) mounted together with an RCSS Guard-PAK C_{18} in a Waters RCM 100 module is used as a column. Samples are loaded via a Rheodyne 7125 loop injector (50 μl).

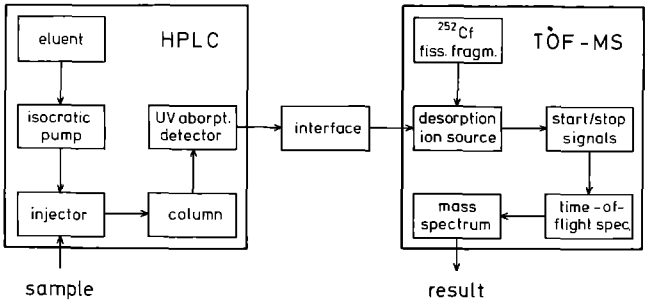

Fig. 1. Component diagram of combined HPLC/MS analysis

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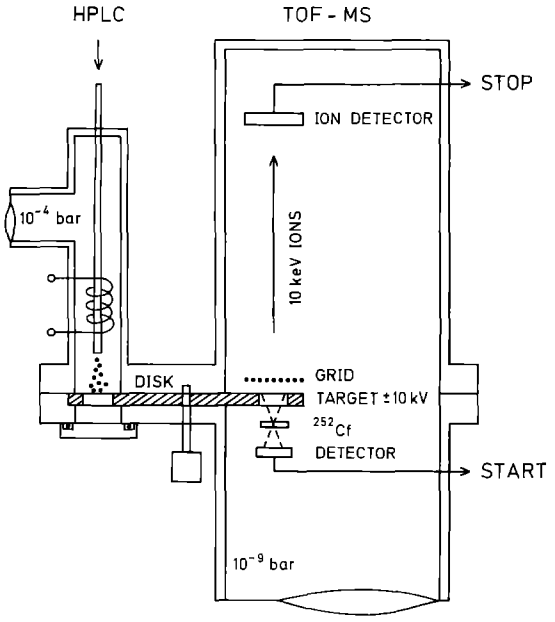

Fig. 2. Vertical section through the time-of-flight mass spectrometer with a disk interface rotating 12 discrete target foils, which are prepared by a vacuum-spray method (HPLC/MS). Offline prepared samples by TLC are introduced via a vacuum lock

The HPLC/MS interface. The HPLC effluent emerges directly into a sampling chamber (Fig. 2), which is evacuated by a rough vacuum pump with high pumping speed (40 l/s). The tip of the stainless steel capillary (i.d. 0.23 mm) is heated (approx. 30 W) for proper nebulization of the effluent [11]. Thus, the expanding aerosol is collected as a thin layer on a target foil, where it freezes instantly. Since the mobile phase vaporizes continuously, all nonvolatile compounds are collected in a freeze-drying process. The flow rate (≤ 1 ml/min) for this vacuum-spray method is limited, depending on the vapor pressure of the mobile phase.

A PTFE disk sandwiched between two main flanges spans the pressure difference between the sampling chamber (approx. 10^{-4} bar) and the ion source of the mass spectrometer (approx. 10^{-9} bar), which is evacuated by a turbo molecular pump (250 l/s). Furthermore, this disk serves as a sample carousel, as it rotates 12 discrete target foils in steps of 30°. Thus each sample analysis results up to 12 fractions and a corresponding number of mass spectra [10], which is sufficient for most HPLC/MS applications. The insulating interface disk transports the target foils successively into the ion source of the mass spectrometer.

Thin-layer chromatography (TLC). As an alternative to the separation of plasma samples by HPLC, a TLC step is introduced into the process at this point. In comparison with HPLC this mode of separation has the disadvantage that it requires manual handling. However, on the whole the TLC method is less troublesome and time-consuming, while yielding quantitative results equal to those of the HPLC method.

The TLC procedure and target preparation utilized here have been described elsewhere [3]. Each target foil is transferred through a vacuum lock into the mass spectrometer, resulting an offline TLC + MS combination [10].

Time-of-flight mass spectrometry. Decaying by spontaneous fission a ^{252}Cf source emits fission fragments with isotropic distribution. Due to momentum conservation each fission event produces two antiparallel fragments. About 1000 fragments per second are emitted into the solid angle of the target (upward cone in Fig. 2). These events of interest are recorded by detection of the coincident fragments (downward cone) using a digital clock (1 GHz). When a fission fragment passes the target it causes a sudden electronic perturbation leading to the presence of molecular and fragment ions of the compound on the target surface. These so-called secondary ions are usually singly charged ($z = \pm 1$ e).

The bombarded target is set at a high voltage potential ($U = \pm 10$ kV). Depending on its polarity either cations or anions are desorbed from the target surface and accelerated to a defined kinetic energy ($T = 10$ keV). Beyond the grounded grid (Fig. 2) these ions fly to an ion detector with a constant energy T (actually the ratio $T/z = U$ is constant) and a velocity v, which is typical for the ion mass m (or the ratio m/z): $T = zU = mv^2/2$.

The time-of-flight t (typically 1–10 µs for a 50-cm flight path) is recorded for all detected ions and stored event by event in a multi channel analyzer. Within a few hundred seconds a time-of-flight spectrum is acquired, a distribution of several thousand events in 12 288 channels (1 ns each). A discrete mass-to-charge ratio, m/z, is assigned to each line in the time-of-flight spectrum, with a calibration constant c: $m/z = 2U/v^2 = ct^2$.

For presentation the data are transferred to m/z distributions as usual in mass spectrometry.

As this mass analysis with fission-fragment-induced ionization is nondestructive for nonvolatile high-molecular compounds, it can be repeated for results on positive and negative ions from the same target, and the target (i.e., collected sample) can still be recovered.

Results and discussion

This section describes the drug monitoring of etoposide (VP16-213) in plasma with the homologous compound teniposide (VM26) as an internal standard [7]. The steps of the complete analysis are illustrated in a flow chart (Fig. 3).

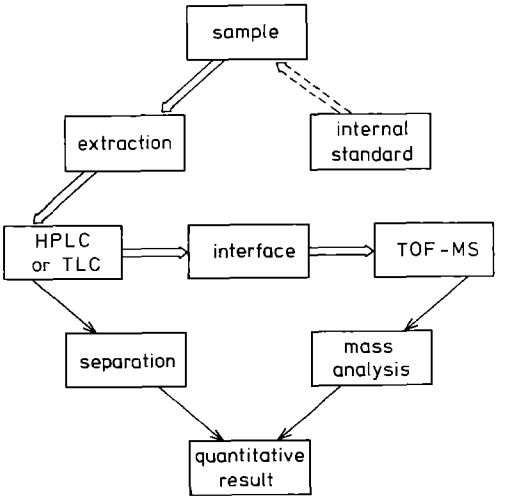

Fig. 3. Flow chart of the quantitative LC/MS analysis. The flow of the substance is indicated by double arrows

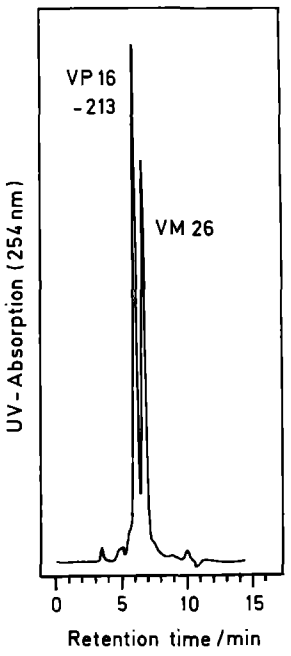

Fig. 4. UV absorption profile from the two compounds separated by HPLC. A better separation is not intended, as a single fraction is sampled from 5.5 to 7.5 min retention time

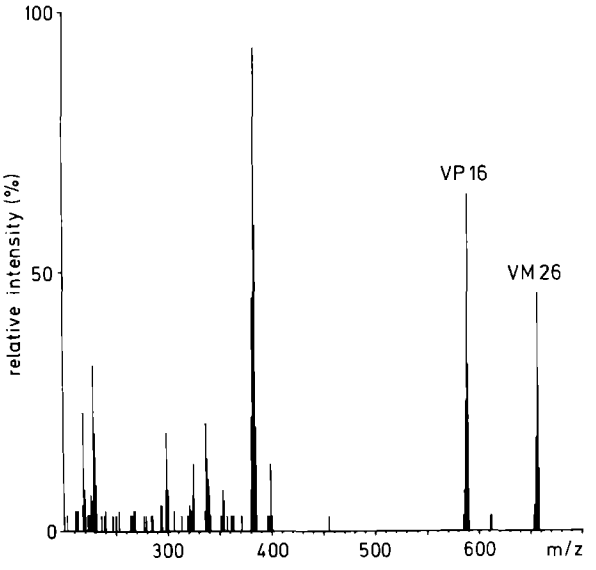

Fig. 5. Mass spectrum of a 1.25-ml serum sample, taken 2 h after a 250 mg etoposide infusion spiked with 10 µg teniposide, extracted, and analyzed by HPLC/MS

Sample preparation

Usually, eight to ten blood samples (3–5 ml) are gathered from a patient during 24 h after the infusion of etoposide (VP16-213). Half the samples are taken during the first 2 or 3 h for registration of the fast decline (component α). Also, all urine is collected during this 24 h period. Each sample is centrifuged and the plasma is frozen until examination. Teniposide (VM26) is added as an internal standard (10 µg/ml) and a 10-min shaking phase follows to equilibrate the distribution of the internal standard before the extraction procedure. The 1-ml plasma or urine samples are extracted with 1 ml chloroform at pH 4.5 [7]. Before the organic phase is separated the sample is centrifuged. This separation is cleaner when the sequence: centrifugation, freezing, melting, centrifugation, siphoning off is used. These steps have been tested with ^{3}H-labeled etoposide, yielding a recovery of 97.2% ± 0.5% in the organic phase. The extract is evaporated by freeze-drying and redissolved by sonication.

In TLC separation the samples are redissolved in 10 µl acetone and applied to the starting line [3].

With HPLC separation each sample is redissolved in 100 µl solvent of the mobile phase, which is a mixture of methanol, acetonitrile, and water (2 : 1 : 1). A 50 µl portion is loaded onto the loop injector. The chromatogram of the UV detector indicates as a first result the concentration ratio of VP16-213 to VM26 (Fig. 4). The UV absorption signals are only used for the sampling of the two compounds, i.e., to fractionate properly.

Sample analysis: Calibration and detection limit

A mass spectrometer is used as detector in drug monitoring to achieve specific and quantitative results. Production of a molecular ion peak from the nonvolatile high-molecular drug requires a "soft ionization method" [12]. In our study we used the fission fragment-induced ionization method explained above.

In routine analyses a single fraction of approx. 1 ml is collected from each chromatogram (HPLC or TLC), containing the two compounds of interest (drug and standard) only. Thus, 12 different samples can be collected and analyzed by the mass spectrometer in one series.

The two peak areas according to the drug and the standard (Fig. 5) are used to calculate an intensity ratio by computer analysis. For quantitative results a calibration curve is produced for which all steps of sample preparation and analysis are used: VP16-213 (1–100 µg) and VM26 (10 µg) are added to 1 ml plasma and mixed. The resulting intensity ratios are plotted against the drug concentration (Fig. 6). Within the linear range from 1–100 µg

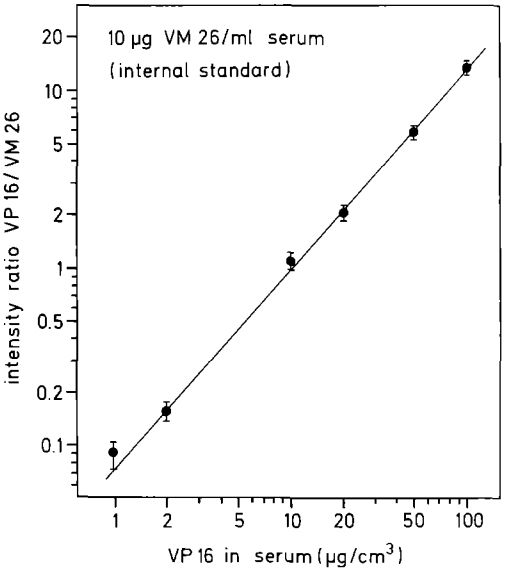

Fig. 6. Calibration curve for etoposide in serum using 10 µg/ml teniposide as internal standard analyzed by HPLC/MS. The correlation coefficient is 0.993

drug per ml plasma the slope (i.e., the sensitivity) is near 1. The detection limit for this drug is smaller than 1 μg/ml. For the TLC + MS combination the calibration curve is similar, whereas the detection limit is smaller than 0.1 μg/ml [3].

Preliminary pharmacokinetic results

The applicability of the method described is demonstrated by analyzing plasma and urine samples from patients suffering from non-small-cell carcinoma of the lung.

Patient A received 200 mg etoposide over 20 min IV infusion. The plasma concentration based on nine samples within 24 h is plotted against time (Fig. 7). Its decline, starting from the maximum value of 45 μg/ml plasma, fits a two-compartment model. The slopes of the straight lines in the semi-log plot express the elimination half-lives $t_{1/2}\alpha$ = 50 min and $t_{1/2}\beta$ = 220 min.

Patient B was treated on 3 successive days with a daily dose of 200 mg etoposide by IV infusion. The plasma concentration curve (Fig. 8) reveals the same biphasic decline for each day, without a cumulation effect.

Recovery of unchanged drug in the 24-h urine specimen is listed in Table 1. The overall recovery within 4 days amounts to 30% of the dose administered. With repeated

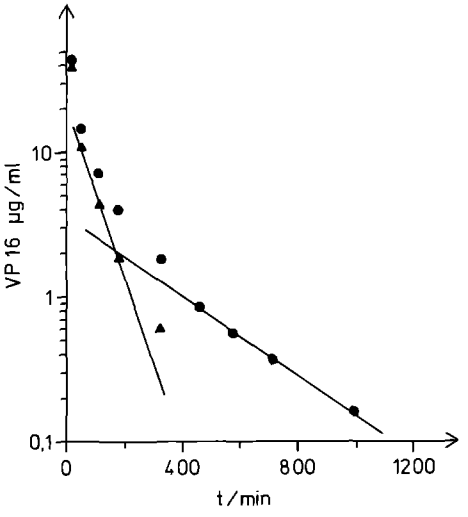

Fig. 7. Elimination profile after a 20-min infusion of 200 mg etoposide

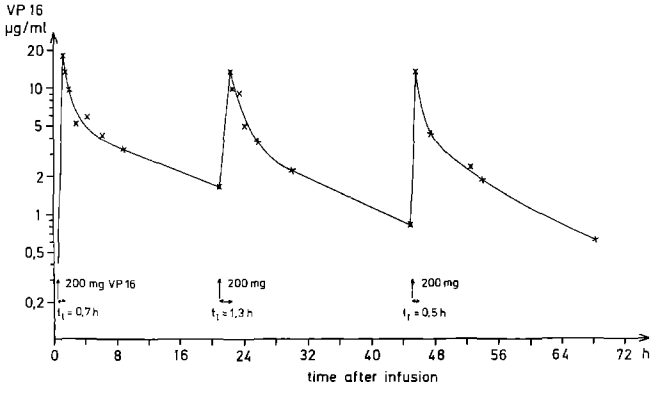

Fig. 8. Elimination profile of etoposide over 3 days. The daily dose and infusion times are indicated

Table 1. Daily excretion of unchanged drug in 24-h urine specimen. The daily dose of 180 mg etoposide was given on 3 consecutive days (cf. Fig. 8)

Day	1	2	3	4
Excretion (mg/day)	21.0	45.5	83.6	4.8

administration the excretion in urine increases from day to day. Together with the low excretion after the treatment period this observation indicates a third (deep) compartment with an elimination half-life $t_{1/2}\gamma$ = 1–2 days.

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