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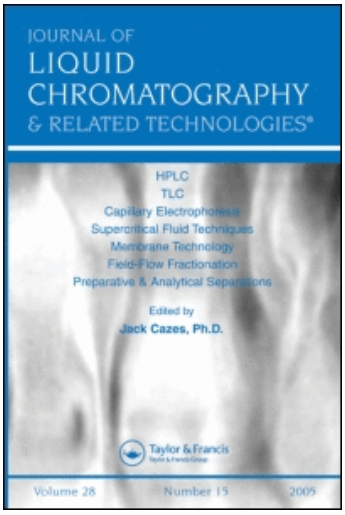

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A New Rapid and Economical High Performance Liquid Chromatographic Assay with Electrochemical Detection for the Determination of Etoposide (VP-16) in Human Plasma Samples

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A NEW RAPID AND ECONOMICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH ELECTROCHEMICAL DETECTION FOR THE DETERMINATION OF ETOPOSIDE (VP-16) IN HUMAN PLASMA SAMPLES

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ABSTRACT

A high performance liquid chromatographic assay with electrochemical detection has been developed for the determination of etoposide in human plasma samples. 2-acetamidophenol was used as internal standard and extraction was performed with ethyl ether-dichloromethane (2:1) in a single step. Analyses were carried out on a Nova Pak C_8 column using methanol-75 mM acetate buffer (45:55) as mobile phase. The effluent from the column was monitorized at +800 mV against Ag/AgCl and the resulting current was registered. The assay was linear within the range of 0.02-4 μg/mL, coefficients of variation being lower than 10%. Detection limit was 5 ng/mL. The

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method is rapid and economical. Therefore, it is suitable for routine monitoring of etoposide pharmacokinetics after administration of low oral doses according to recent therapeutic schemes.

INTRODUCTION

Etoposide (VP-16) is an antitumor agent derived from epipodophyllotoxin which is widely used in the treatment of several malignancies.[1,2] Although a number of methods have been described for the quantitation of etoposide concentrations in human fluids by high performance liquid chromatography (HPLC), they present some disadvantages. Procedures using spectrophotometric[3-6] or fluorescence[7] detection exhibit low sensitivity. Therefore, plasma concentrations can be below detection limits, specially when etoposide is administered according to recently reported regimens consisting of low oral doses.[8,9] Electrochemical detection has shown to be an adequate alternative to improve sensitivity. Notwithstanding, the available methodologies require a time-consuming extraction procedure[10] long run times,[11,12] or expensive on-line equipment.[13] Moreover, the reagents used in these methods are expensive. Hence, such procedures do not appear to be suitable for routine monitoring of etoposide pharmacokinetics in clinical practice.

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The purpose of the present work was to develop an improved HPLC assay with electrochemical detection for the quantitation of etoposide plasma concentrations in patients receiving low oral doses. The procedure appears to be simple, specific and sensitive, as well as economical. Hence it can be used in routine monitoring of etoposide clinical pharmacokinetics.

EXPERIMENTAL

Materials

Etoposide was kindly provided by Bristol-Myers-Squibb de México (Mexico City). 2-acetamidophenol, which was used as internal standard, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, chromatographic grade, was obtained from Merck (Darmstadt, Germany). High quality deionized water, employed for solution preparation, was obtained using a Milli Q Reagent Water System (Continental Water Systems, El Paso, TX, USA). All other reagents were of analytical grade.

Solutions

Stock solutions (1 mg/mL) of etoposide and of the internal standard were prepared in methanol. Standard etoposide solutions, used for the preparation of calibration curves ranging from 0.1 to 20 μg/mL, were prepared by appropriate dilution with methanol. A standard solution of 2-acetamidophenol, at a fixed concentration of 0.5 μg/mL, was prepared by dilution of the stock solution with deionized water. Standard solutions were stored at -20°C.

Sample Preparation

1 mL plasma samples (unknown samples, drug free plasma or plasma samples containing known etoposide amounts) were placed in 15 mL conical glass tubes and spiked with 0.1 μg (200 μL of a 0.5 μg/mL solution) of 2-acetamidophenol, the internal standard. After addition of 4 mL of a 0.5 M NaH_2PO_4, pH 7.2, solution, samples were extracted with 5 mL of a mixture of ethyl ether and dichloromethane (2:1 v/v) by vortexing at maximal speed for 1 min. The two layers were separated by centrifugation at 4500 RPM for 5 min, the upper organic layer was transferred to a clean tube and the solvent was evaporated to dryness at 45°C under a gentle nitrogen stream. The dry residue was redissolved in 200 μl of mobile phase (see below) and 100 μl aliquots were injected into the chromatographic system.

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Chromatographic Conditions

The chromatographic system consisted of a M-45 solvent delivery system (Waters Assoc., Milford, MA, USA), a 100-μL loop injector (Rheodyne, Cotati, CA, USA), an electrochemical transducer coupled to a LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) and a Servogor 120 recorder (Norma Goerz Instruments GmbH, Neudorf, Austria). Analyses were carried out on a 150 x 3.9 mm I.D. Nova-Pak C_8 column of 4 μm particle size (Waters). To prolong the life of the analytical column, a precolumn (40 x 4 mm I.D.) containing 37-50 μm Corasil C_{18} (Waters) was incorporated into the system. Column elution was carried out at room temperature using a mixture of methanol with 75 mM acetate buffer pH 3.8 (45:55 v/v) as mobile phase at a fixed flow rate of 1 mL/min. Detection was performed using a glassy carbon working electrode maintained at +800 mV against Ag/AgCl and the resulting current was recorded.

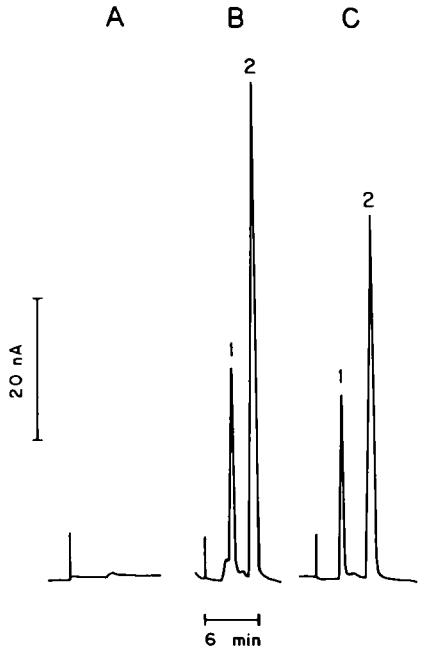

Figure 1. Chromatograms of human plasma extracts. A: Blank plasma. B: Plasma spiked with 2 μg/mL of etoposide (2) and 0.1 μg/mL of internal standard (1). C: Plasma sample drawn from a pacient 1 h after a 50 mg oral etoposide dose, spiked with 0.1 μg/mL of internal standard.

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Calibration

The assay was calibrated by addition of known amounts of etoposide and of the internal standard to drug-free plasma samples. Samples used for calibration contained etoposide concentrations ranging from 0.02 to 4 μg/mL. The internal standard was used at a fixed concentration of 0.1 μg/mL. Calibration curves were constructed by plotting the peak-height ratio of etoposide to the internal standard (y-axis) as a function of the actual etoposide concentration in the sample (x-axis). The accuracy and precision of the method were evaluated by adding known drug amounts to replicate plasma samples over the concentration range used for calibration.

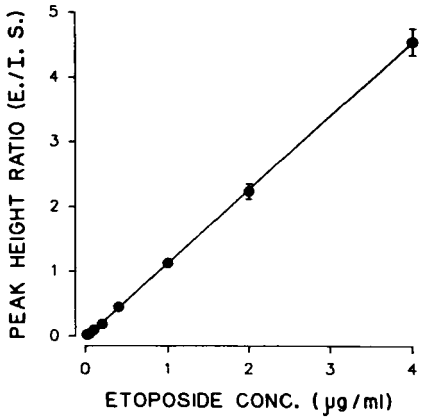

Figure 2. Calibration curves of etoposide in human plasma established in the range of 0.02 to 4 µg/mL. Data are expressed as mean ± S.E.M. of at least six determinations.

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RESULTS

Typical chromatograms of extracted plasma samples are shown in Fig. 1. Retention times for the internal standard and etoposide were 3.0 and 5.6 min respectively. No interfering peaks occurred at these times. Any endogenous contaminants remaining in the extracts, which exhibited a response at the voltage level used in the assay, eluted before the internal standard. Therefore, samples could be injected immediately after elution of etoposide. The recoveries of etoposide and of the internal standard were assessed by comparison of peak heights from plasma extracts with those from standard solutions. Recoveries were similar for both compounds, being higher than 90%.

A linear relationship (r = 0.9999) was found when the peak height ratio of etoposide to that of the internal standard was plotted on the ordinate against etoposide plasma concentration in the abscissa (Fig. 2). The equation obtained by least-squares was $y = 1.1405x - 0.0160$. The precision and accuracy of the assay are shown in Table 1. It can be seen that a good accuracy was achieved, while the intra-assay coefficient of variation was always lower than 10%. Day-to-day precision of the assay was evaluated over a period of five weeks (n=6). Inter-assay coefficients of variation were 7.48, 5.07 and 9.71% for 0.04, 0.2 and 2 µg/mL respectively. The detection limit, defined as the etoposide plasma concentration producing a signal-to-noise ratio of 3, was 5 ng/mL

Table 1

Accuracy and Precision of the High Performance Liquid Chromatographic Assay for Determination of Etoposide Plasma Levels

Theoretical Concentration (μg/mL)	Measured Concentration (μg/mL; mean ± S.E.M.) (n=6)	Accuracy (%)	C.V. (%)
0.02	0.022 ± 0.002	106.40	5.21
0.04	0.043 ± 0.001	107.62	5.90
0.1	0.100 ± 0.003	100.40	8.12
0.2	0.194 ± 0.004	97.02	6.78
0.4	0.408 ± 0.016	101.98	9.59
1.0	1.009 ± 0.027	100.87	7.01
2.0	1.984 ± 0.078	99.20	9.61
4.0	4.064 ± 0.128	101.61	7.04

S.E.M. : Standard error of mean
C.V.: Intra-assay coefficient of variation

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The application of the method for clinical pharmacokinetic studies was evaluated. Blood samples were obtained at selected times over a period of 24 h from a patient receiving a 50 mg oral etoposide as solution (Vepesid™, Bristol-Myers-Squibb de México, Mexico City). Plasma was obtained and the etoposide concentration was determined by the procedure described. The observed plasma concentration against time curve is shown in Fig. 3. The maximal etoposide plasma concentration was 3.1 μg/mL, being reached at 1.5 h. The terminal half-life was 6.1 h and the area under the curve amounted to 23.2 μg h/mL.

DISCUSSION

Several assays for the determination of etoposide plasma concentrations using HPLC coupled to electrochemical detection have been reported.[10-13] Although these procedures have shown to be reliable and sensitive, they are not suitable for routine monitoring in clinical practice. The procedure described by Duncan and coworkers[10] includes a back extraction step, which importantly prolongs the sample preparation time and does not use an internal standard.

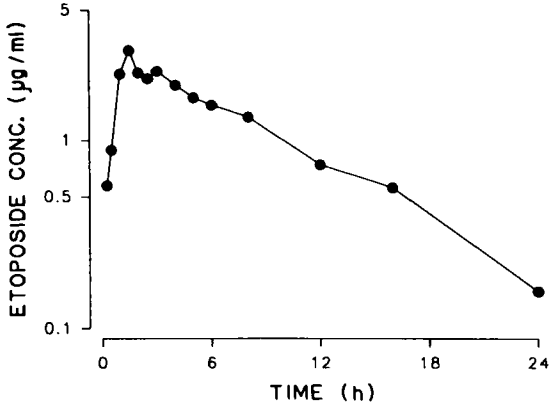

Figure 3. Time-course of etoposide plasma levels in a pacient after a single oral dose of 50 mg in solution.

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Eisenberg and colleagues[11] reported a procedure requiring of a total run time of 19 min in order to elute all interfering compounds from the column before analysis of the following sample, and therefore it is not practical for pharmacokinetic purposes. The method of Littlewood and coworkers[12] appears to be superior, as the total run can be achieved in 9 min. This time, however, is 3 min longer than the one needed with our procedure which only requires of 6 min.

The cost of routine pharmacokinetic analysis is of great relevance, as it can significantly increase the price of treatments with drugs, such as etoposide, which require frequent monitoring in order to ensure an adequate therapeutic response. Van Opstal and colleagues[13] reported an assay in which etoposide analysis can be performed by direct injection into the chromatographic system without prior extraction. This procedure, although it is practical, requires an on-line sample preparation system which is expensive and not universally available. In the procedures described for etoposide determination in plasma using HPLC with electrochemical detection, in which an extraction step is included,[10-12] the solvent used is dichloroethane. This solvent is expensive, its cost being about three times higher than the ethyl ether-dichloromethane mixture employed in the present method. Furthermore, most procedures use teniposide, another antitumor agent, as internal standard.[11-13] The commercial price of teniposide is considerably higher than that of 2-acetamidophenol.

The procedure here presented fulfills the precision, accuracy and sensitivity requirements for the determination of etoposide clinical pharmacokinetics after administration of low oral doses. The procedure presents the advantage of a short run time, allowing the analysis of a considerable number of samples per working day. Furthermore, the procedure appears to be more economical than those previously reported. Hence, the present method appears to be a suitable alternative for the monitoring of etoposide pharmacokinetics in routine clinical practice.

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