



Quantitative Determination of Etoposide in Human Plasma Using LC/MS/MS

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Overview

PURPOSE

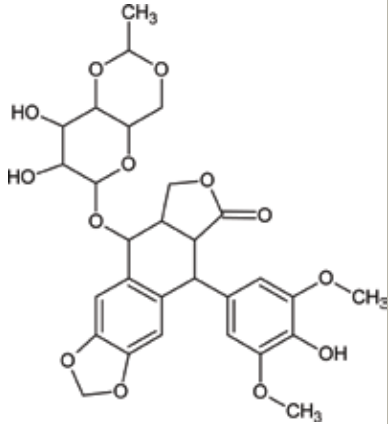
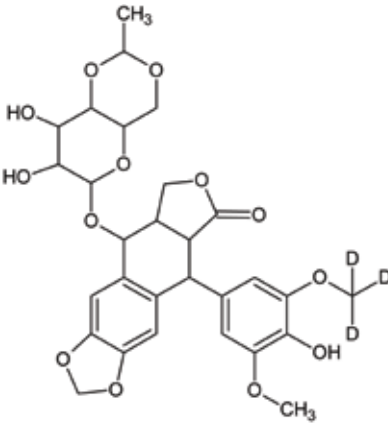
To validate an LC/MS/MS method for the determination of etoposide, an anti-cancer agent, in human plasma.

METHOD SUMMARY

Etoposide was extracted from 100 μ L aliquots of plasma treated with deuterated etoposide as the internal standard using an ether based liquid-liquid extraction (LLE). Following evaporation and reconstitution of the organic supernatant, HPLC separation was achieved on an Agilent Zorbax SB-C18 (50 x 2.1mm, 5 micron) column. A mobile phase gradient operating at a flow of 300 μ L/min increased from 25 to 90 %B over 2 minutes where mobile phase A was 10 mM ammonium acetate (aq) acidified with 0.1% formic acid and mobile phase B was acetonitrile:methanol:formic acid (50:50:0.1, v:v:v). An LLOQ of 1.00 ng/mL was achieved using multiple reaction monitoring of the etoposide-ammonium adduct ion on an AB Sciex API 4000™ operating in positive ESI.



Overview (continued)

Etoposide	Etoposide-D3
Molecular Formula: $C_{29}H_{32}O_{13}$	Molecular Formula: $C_{29}H_{29}D_3O_{13}$
Molecular Mass: 588.86	Molecular Mass: 591.58
Monoisotopic Mass: 588.18	Monoisotopic Mass: 588.18
MRM transition (NH_4^+ adduct): 606.0/229.0	MRM transition (NH_4^+ adduct): 609.0/229.0
Structure: 	Structure: 

Methodology

SAMPLE PREPARATION

1. Thaw the samples at room temperature
2. Aliquot 100 μ L of each sample into 96-well plate
3. Add 50.0 μ L working internal standard [100 ng/mL of etoposide-d3 in 50:50 ACN:H₂O]
4. Add 50.0 μ L of pH 4.5 buffer (aq)
5. Vortex samples to mix thoroughly

LIQUID - LIQUID EXTRACTION

1. Add 800 μ L MTBE
2. Use Tomtec to mix samples thoroughly
3. Centrifuge to separate aqueous and organic layers
4. Use Tomtec to transfer 600 μ L of the MTBE supernatant to a new 96-well plate
5. Evaporate to dryness
6. Reconstitute with 200 μ L mobile phase



HPLC Conditions

Autosampler: CTC PAL

Sample Loop	Injection Volume	Needle Wash 1	Needle Wash 2
20-50 μ L	210-20 μ L	5% NH ₄ OH in MeOH	25:75 MeOH:H ₂ O

Column: Zorbax SB-C18, 5 μ m, 50 x 2.1 mm (Agilent Technologies)

Pump: Shimadzu LC-20AD pumps and controller

Binary HPLC gradient:	(initial conditions: 25% B, 0.300 mL/min)			
	Time	Module	Events	Parameter
	0.30	Pumps	Pump B Conc.	25
	2.00	Pumps	Pump B Conc.	90
	2.00	Pumps	Total Flow	0.300
	2.10	Pumps	Pump B Conc.	100
	2.10	Pumps	Total Flow	0.600
	3.50	Pumps	Total Flow	0.600
	3.50	Pumps	Pump B Conc.	100
	3.51	Pumps	Total Flow	0.300
	3.51	Pumps	Pump B Conc.	25
	4.50	Controller	Stop	



MS Conditions

Platform: AB Sciex API 4000™

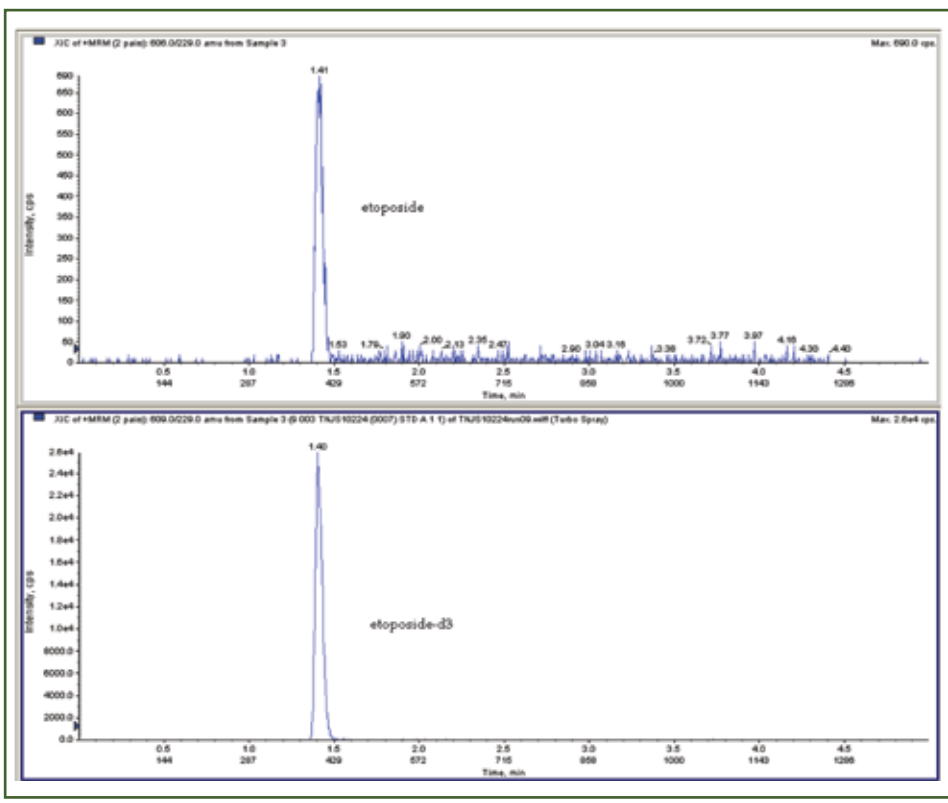
Instrument Parameters:

Ionization	Source Temperature	CAD	Curtain Gas	GS1	GS2
ESI+	500°C	10	20	65	65

dwll	DP	EP	CE	CXP	IS
100 msec	50 V	10 V	30 V	20 V	5000

Example chromatogram: LLOQ with internal standard
(1.00/100 ng/mL etoposide/etoposide-d3)

Example Regression: Linear 1/x2 (1.00 – 500 ng/mL etoposide)





Results

A linear range of 1-500 ng/mL was established and interday and intraday accuracy ($\leq 7\%$) and precision ($< 11\%$) was well demonstrated. Lack of MRM response at the analyte and internal standard retention times in six lots of matrix verified method specificity. A clean, efficient extraction is illustrated by recoveries greater than 80% across the linear range and a matrix factor of 1.07. Stability investigations were also performed satisfactorily, including short- and long-term stability in plasma, freeze-thaw stability in plasma, whole blood stability and processed extract stability.

GLP Validated Assay Performance Summary - Etoposide

Method	LC/MS/MS
Matrix	Human Plasma
Anti-coagulant	K ₂ EDTA
Internal Standard	Etoposide-d ³
Aliquot Volume	100 μ L
LLOQ / ULOQ	1.00 ng/mL / 500 ng/mL
Regression, Weighting:	Linear 1/x ²
LLOQ QC	
Inter-Assay Accuracy (%Bias):	-1.1 %
Inter-Assay Precision (%CV):	4.9 %
Low, Low-Medium, Medium, and High QC	
Inter-Assay Accuracy (%Bias):	-0.5 to 7.0 %
Inter-Assay Precision (%CV):	1.3 to 10.5 %
Ability to Dilute:	2,500 ng/mL (DF = 10) 20,000 ng/mL (DF = 100)
Analyte Stability in Solution:	24 hours at room temperature : 98 days at -20 °C nominal
Freeze-Thaw Matrix Stability:	4 cycles from -70°C and -20°C nominal to room temperature
Bench-Top Matrix Stability:	24 hours at room temperature
Long-Term Matrix Stability:	99 days at -20°C nominal 99 days at -70°C nominal
Reinjection Reproducibility:	88 hours, 30 minutes at room temperature
Processed Extract Stability:	79 hours at room temperature
Whole Blood Stability:	2 hours at room temperature 2 hours at 1-8 °C
Hemolysis:	No effect



Conclusion

A validated method for the determination of etoposide in human plasma has been established. This method has been used for the analysis of clinical samples.