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Simultaneous determination of etoposide and a piperine analogue (PA-1) by UPLC–qTOF-MS: Evidence that PA-1 enhances the oral bioavailability of etoposide in mice

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ABSTRACT

In the present investigation, a UPLC–qTOF-MS/MS method has been developed for the simultaneous determination of etoposide and a piperine analogue, namely, 4-ethyl 5-(3,4-methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide (PA-1). The analytes were separated on a reverse phase C18 column using methanol–water (72:28, v/v) mobile phase with a flow rate of 250 μL/min. The qTOF-MS was operated under multiple reaction monitoring mode using electro-spray ionization (ESI) technique with positive ion polarity. The major product ions for etoposide and PA-1 were at *m/z* 185.1350 and 164.1581, respectively. The recovery of the analytes from mouse plasma was optimized using solid phase extraction technique. The total run time was 6 min and the elution of etoposide and PA-1 occurred at 1.24 and 2.84 min, respectively. The calibration curves of etoposide as well as PA-1 were linear over the concentration range of 2–1000 ng/mL (r^2, 0.9829), and 1–1000 ng/mL (r^2, 0.9989), respectively. For etoposide intra-assay and inter-assay accuracy in terms of % bias was in between −7.65 to +6.26, and −7.83 to +5.99, respectively. For PA-1 intra-assay and inter-assay accuracy in terms of % bias was in between −7.01 to +9.10, and −7.36 to +6.71, respectively. The lower limit of quantitation for etoposide and PA-1 were 2.0 and 1.0 ng/mL, respectively. Analytes were stable under various conditions (in autosampler, during freeze–thaw, at room temperature, and under deep-freeze conditions). The method was used for a pharmacokinetic study which showed that PA-1 enhanced the oral bioavailability of etoposide in mice by 2.32-fold.

1. Introduction

In several studies piperine (1-piperoyl piperidine) an alkaloid from peppers (*Piper* sp.) has been shown to be effective as a bioavailability enhancer of several drugs and other pharmacologically active substances (phenytoin, propranolol, theophylline, nevirapine, beta-lactam antibiotics, curcumin, coenzyme Q10, and epigallocatechin) in animals and human volunteers [1–7]. Recently a novel series of substituted piperine analogues have been synthesized in our laboratory [8], for the purpose of identifying potential leads as bioenhancer(s) for anti-cancer agents, majority of which exhibit low/variable oral bioavailability. In this programme piperine along with its 50 other derivatives, that have been screened so far, one piperine analogue, namely, 4-ethyl 5-(3,4-methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide (PA-1) was found to significantly modulate the plasma levels of etoposide in mice.

Etoposide is an antitumor agent currently in use for the treatment of small cell lung cancer, testicular cancer and lymphomas. However etoposide has shortcomings of low and variable bioavailability after oral dosing [9,10]. Currently different pharmacological approaches are being explored in order to demonstrate any potentially useful improvement in rate and extent of etoposide absorption into the systemic circulation in the clinical setting of oral therapy [11–17].

This paper presents (i) a method for the simultaneous determination of both the analytes etoposide and PA-1, by UPLC–qTOF-MS, and (ii) a pharmacokinetic study to show that oral bioavailability of etoposide is enhanced by PA-1 in mice.

Abbreviations: UPLC–qTOF-MS/MS, ultra performance liquid chromatography–quadrupole time of flight mass spectrometer; PA-1, 4-ethyl 5-(3,4-methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide.

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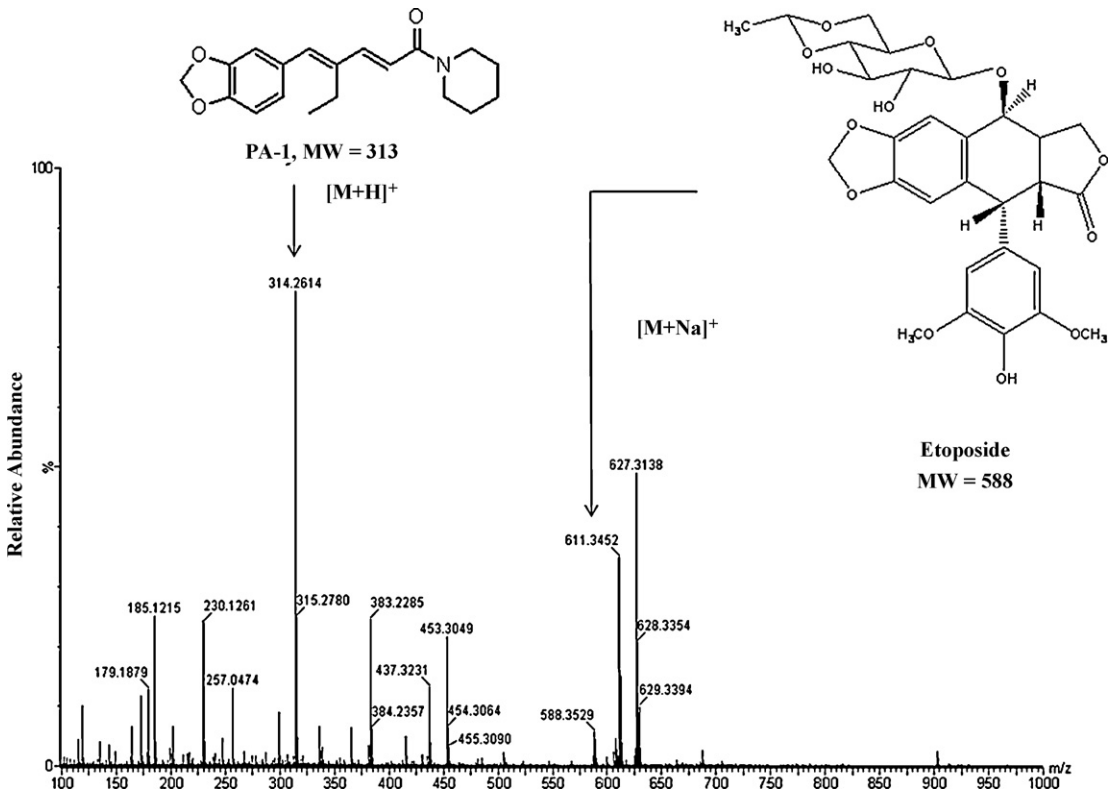

Fig. 1. The mass spectrum of etoposide and PA-1 in combination showing the molecular ion peaks, chemical structures and molecular weights.

2. Experimental

2.1. Chemicals

Etoposide was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade solvents were procured from Rankem (RFCL Ltd., Mumbai, India). The HPLC grade water was obtained from a Water Purification System (Synergy UV, Millipore, USA).

2.2. Synthesis of PA-1

A 96% pure PA-1 ($C_{19}H_{23}NO_3$; MW, 313; melting point, 112 °C) was prepared in a multi-step reaction sequence starting from piperonal as described in detail earlier [8]. Chemical structure of PA-1 (4-ethyl 5-(3,4-methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide (Fig. 1) was confirmed by various spectral data [8].

2.3. Instrumentation

A UPLC–qTOF-MS system (Acquity UPLC; Synapt, Waters, USA, equipped with MassLynx acquisition software, version 4.1) was used under the following conditions: column, C-18, 50 mm × 2.1 mm; particle size, 1.7 μm (Acquity, BEH); flow rate, 250 μL/min; mobile phase, methanol: water (72:28, v/v), injection volume, 5 μL. The analyte infusion experiments were performed using an in-built syringe pump. A mass spectrometer with ESI interface was used for MS/MS analysis. ESI parameters were as follows: capillary voltage, 3.0 kV (positive ion polarity; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow, 50 L/h; and desolvation gas flow, 750 L/h. The multiple reaction monitoring (MRM) mode was used to monitor the transition of etoposide molecule *m*/*z* 611.3457 [M+Na] to 185.1350, and of PA-1 *m*/*z* 314.2614 $[M+H]^+$ to the product ion 164.1581.

2.4. Preparation of reference, standard and quality control solutions

Reference solutions of etoposide (stock I) and PA-1 (stock II) were prepared by weighing 100 mg of each compound. The quantities were transferred to 50 mL volumetric flasks, dissolved and diluted suitably with HPLC grade methanol. Both the reference solutions (2 mg/mL) were covered with aluminium foil and sealed with paraffin film to avoid photodegradation and loss due to evaporation. Stock I and stock II were mixed together, and diluted suitably with methanol. A 50-μL of this solution was used to spike blank mouse plasma samples (450 μL) to achieve 8 calibration standards (CAL STD) containing etoposide and PA-1 in combination. CAL STD-1: etoposide, 2 ng/mL + PA-1, 1 ng/mL; CAL STD-2: 5 ng/mL each; CAL STD-3: 10 ng/mL each; CAL STD-4: 50 ng/mL each; CAL STD-5: 100 ng/mL each; CAL STD-6: 200 ng/mL each; CAL STD-7: 500 ng/mL each; and CAL STD-8: 1000 ng/mL each. Three quality control (QC) standards (LQC: 3 ng/mL; MQC: 450 ng/mL; HQC 950 ng/mL each of etoposide and PA-1) were prepared and used to spike blank mouse plasma.

2.5. Method validation procedures

The analytical method was validated to meet the acceptance criteria as per guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The specificity of the method was established by comparing blank plasma samples with those spiked with the analytes to find out interference from endogenous components. The CAL STD solutions were utilized for establishment of linearity and range (linear least-squares regression with a weighting index of $1/x$). The precision and accuracy parameters were ascertained in LLOQ, LQC, MQC, and HQC samples (7 replicates each in 3 sets) on the same day and on 3 consecu-

tive days. The intra-assay and inter-assay accuracy (% bias) of the method was determined from mean measured concentrations and nominal concentrations as follows: % bias = [(mean measured conc. – nominal conc.)/nominal conc.] × 100. The intra-assay and inter-assay precision (% relative standard deviation or RSD) of the method was calculated from mean measured concentrations as follows: % RSD = (SD of mean measured conc./mean measured conc.) × 100. The stability of analytes in plasma was investigated under following conditions: (a) 1 month storage at deep freeze (−80 °C); (b) 3 consecutive freeze–thaw cycles from −20 °C to room temperature; (c) 24 h storage at room temperature; and (d) short-term stability (of processed samples) at 10 °C for 24 h in autosampler. After specified storage conditions, samples were processed and analyzed. The matrix effect was investigated by post-extraction spike method. Peak area (*A*) of the analyte in spiked blank plasma with a known concentration (MQC) was compared with the corresponding peak area (*B*) obtained by direct injection of standard in the mobile phase. The ratio ($A/B \times 100$) is defined as the matrix effect [18].

2.6. Experimental animals

Swiss mice (25–30 g) were obtained from the Animal House of this Institute, and kept in regulated environmental conditions (temperature: 25 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light cycle). Animals were fed on standard pelleted diet (Ashirwad Industries, Chandigarh, India) and water was provided *ad libitum*. Animal experiments were approved by Institutional Ethics Committee. Animals were fasted overnight before the experiment and segregated into 5 groups. Group I animals were administered with etoposide (20 mg/kg, p.o.). Group II (A–F) animals were given etoposide (20 mg/kg, p.o.) along with varying doses of PA-1 (1.25, 2.5, 5, 10, 20 and 40 mg/kg, p.o.). Group III animals were given PA-1 (20 mg/kg, p.o.). Group IV animals received etoposide (20 mg/kg, p.o.) in combination with PA-1 (20 mg/kg, p.o.) (pre-mixed). Group V animals were given etoposide (20 mg/kg) intravenously through caudal vein. Test compounds were suspended/solubilized in 2% sodium carboxymethyl cellulose (for oral administration), or in 2% propylene glycol in normal saline (for intravenous administration). From mice in group I, III, IV, and V blood samples were collected in pre-heparinized glass tubes at different time intervals post-dosing (0, 0.083, 0.166, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16 h). From animals in Group II, blood samples were collected at 0.5 h post-dosing. Blood samples were centrifuged (5000 rpm; 10 min at 20 °C) to separate the plasma.

2.7. Sample preparation

The etoposide and PA-1 were recovered simultaneously from plasma using solid phase extraction (SPE) technique involving semi-automated vacuum chamber and vacuum pump (Supelco, USA). The various steps involved in the recovery procedure were: (a) conditioning of SPE cartridge (C18, 3 mL capacity, 100 mg bed, Samprep-Ranbaxy, Mumbai, India) with 1.0 mL methanol, followed by 1.0 mL water, (b) loading of diluted (1:4, v/v) plasma samples (1.0 mL) onto cartridge and drying under positive pressure, and (c) samples were washed with 2 mL of water followed by elution with 2 mL of methanol. The eluants were carefully collected in 2.0 mL capacity glass vials for direct analysis in UPLC–qTOF-MS system.

2.8. Pharmacokinetics

Concentration–time curves were established for each analyte from the treated mice (groups I, III, IV and V), and used for the determination of pharmacokinetic parameters such as peak plasma concentration (C_{max}), peak time (T_{max}), extent of absorption (AUC), half-life ($t_{1/2}$), clearance (Cl), and volume of distribution (V_d) by a non-compartmental analysis using PK Solutions Version 2.0; Summit Research Services, USA.

3. Results

3.1. UPLC–qTOF-MS/MS analysis

Optimum chromatographic separation of etoposide and PA-1, was achieved by methanol:water (72:28, v/v), with a flow rate of 250 μL/min. Both analytes (2 ng/mL) were added simultaneously in the samples and the resulting chromatograms showed a retention time of 1.24 min for etoposide, and 2.84 min for PA-1 (Fig. 2). A full scan in positive ion mode was used for both the analytes. During direct infusion, the mass spectra of etoposide and PA-1 showed precursor ion peaks at *m*/*z* 611.3457 as $[M+Na]^+$ and *m*/*z* 314.2614 as $[M+H]^+$, respectively (Fig. 1). $[M+Na]^+$ 611.3457 ion was fragmented to *m*/*z* 239.1666, 229.1414 and 185.1350 (main product ion) (Fig. 3A). $[M+H]^+$ 314.2614 ion was fragmented to *m*/*z* 179.1878, 164.1581 (main product ion) and 135.0897 (Fig. 3B). Quantification was done on the basis of main product ions. Identical cone voltage of 40 V was used for monitoring the precursor ions. The collision energies of 80 and 33 V were found to be optimum for *m*/*z* 185.1350 (etoposide), and *m*/*z* 164.1581 (PA-1).

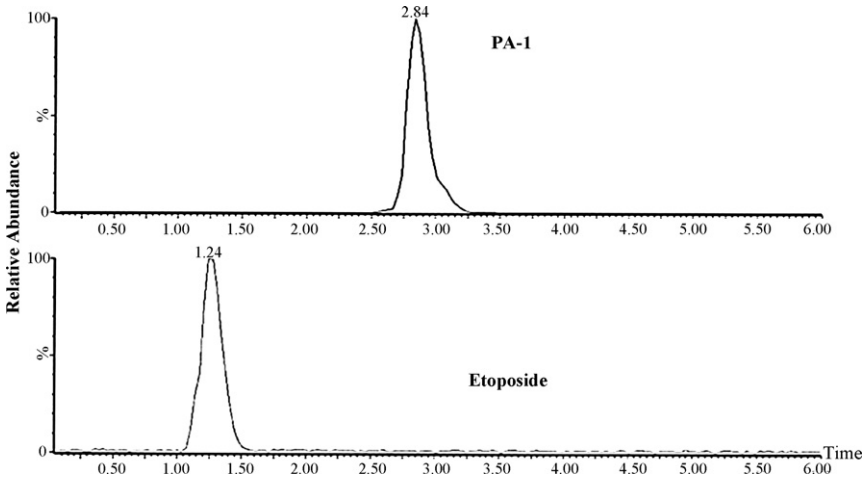

Fig. 2. Chromatograms showing standard etoposide and PA-1.

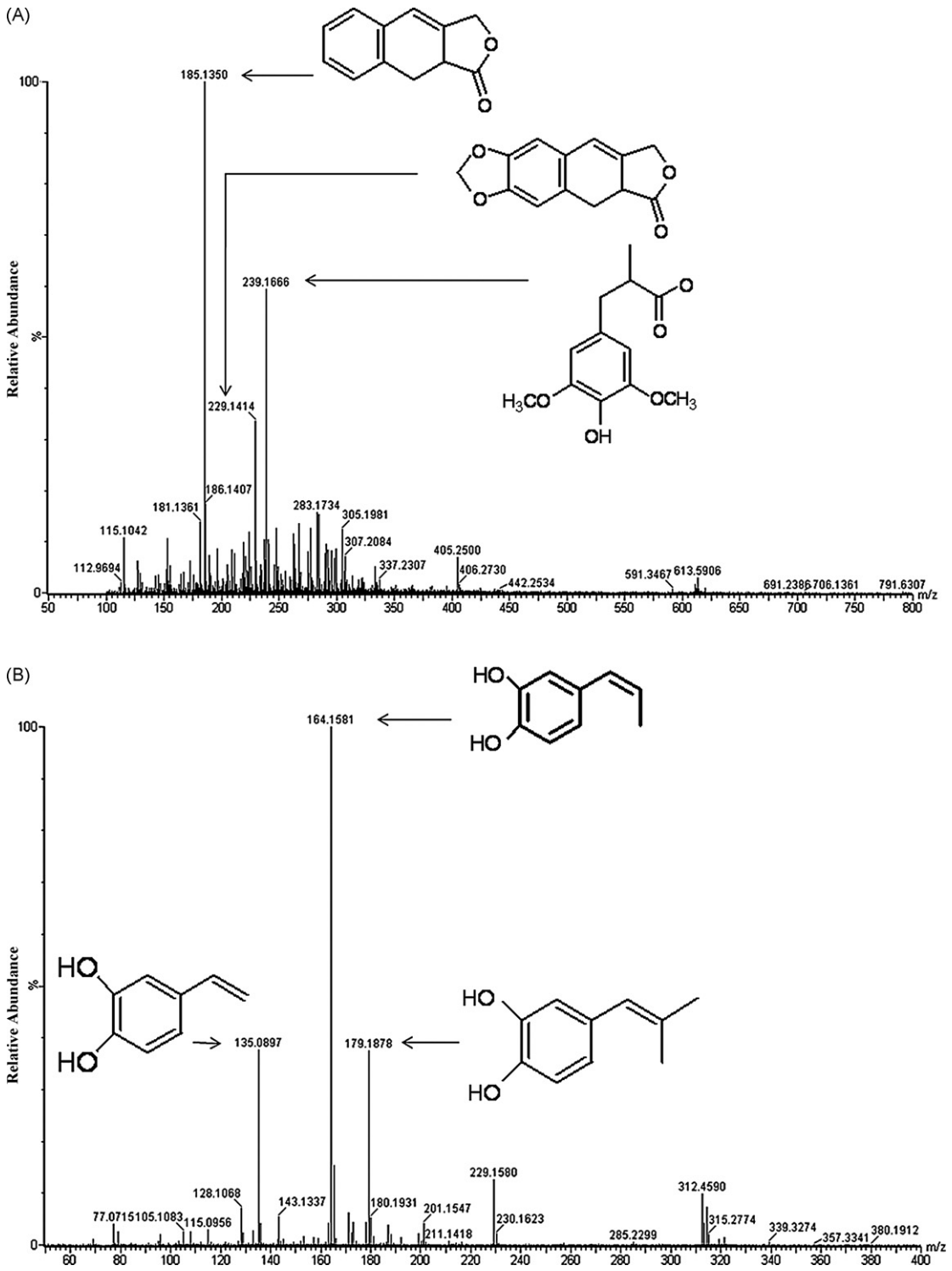

Fig. 3. (A) Product ion spectra of etoposide showing fragmentation transitions. (B) Product ion spectra of PA-1 showing fragmentation transitions.

3.2. Method validation

3.2.1. Specificity

The method was found to be specific: extracted blank plasma (Fig. 4) when compared with plasma samples spiked with etoposide and PA-1 (2 ng/mL each) (Fig. 2) did not show any interference at the respective retention times of each analyte.

3.2.2. Linearity and range

The calibration curves of etoposide were linear over the concentration range of 2–1000 ng/mL (r^2, 0.9829). The calibration curves of PA-1 were linear over the concentration range of 1–1000 ng/mL (r^2, 0.9989).

3.2.3. Accuracy and precision

The combined recovery of etoposide and PA-1 was carried out in LLOQ, LQC, MQC and HQC samples. The recovery (mean ± SE) of etoposide was 95.85 ± 2.98% (from LLOQ), 96 ± 2.97% (from LQC), 96.70 ± 2.20% (from MQC), and 97.16 ± 2.12% (from HQC). The recovery of PA-1 was 98.41 ± 1.21% (from LLOQ), 97 ± 1.94% (from LQC), and 98.70 ± 1.21% (from MQC), and 98.7 ± 1.16% (from HQC).

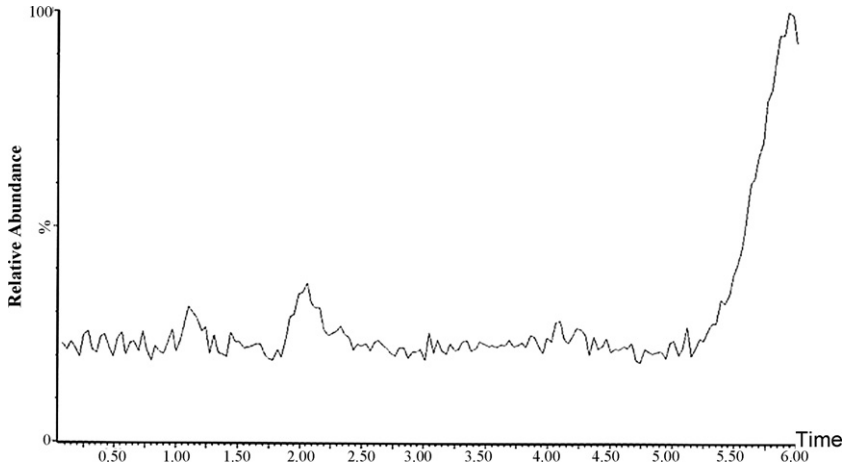

Fig. 4. Chromatogram of extracted plasma (blank).

Table 1
Accuracy (% bias) data.

Compound	Nominal conc. (ng/mL)	Intra-assay			Inter-assay		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Etoposide	2	+6.01	−7.65	−7.55	+5.87	−7.48	−7.63
	3	−6.63	+4.36	+6.26	−7.83	+5.99	+7.35
	450	−4.69	−5.40	+3.03	−6.61	−7.24	+4.21
	950	+4.71	−5.92	−6.17	+4.27	−6.02	−5.81
PA-1	1	−7.01	−4.00	+9.1	−7.36	−4.16	+6.71
	3	−4.16	−4.10	−3.33	−5.28	−5.66	−2.88
	450	+2.60	−2.71	−1.33	+3.93	−3.34	−3.81
	950	−2.99	+2.12	+3.77	−3.54	+2.60	+2.88

The intra-assay accuracy in terms of % bias was in the range of −7.65 to +6.26 for etoposide, and in the range of −7.01 to +9.1 for PA-1. Inter-assay accuracy was −7.83 to +5.99 for etoposide, and −7.36 to +6.71 for PA-1 (Table 1). Intra-assay precision (% RSD) was in the range of 1.21–6.95, for etoposide, and, in the range of 2.32–10.7, for PA-1. The inter-assay precision was 1.82–7.56 for etoposide, and 2.91–9.90 for PA-1 (Table 2). The accuracy and precision of the method were within the acceptable limits of ±15%.

3.2.4. Lower limit of quantitation (LLOQ)

The LLOQ for etoposide and PA-1 were 2.0 and 1.0 ng/mL, respectively.

3.2.5. Stability

The stability of the analytes in plasma was investigated in LQC and HQC samples. The recovery of the analytes relative to that at time zero is summarized in Table 3. After 1 month (storage stability) the recovery of etoposide was 97.5% (LQC), and 95.8% (HQC). The recovery for PA-1 was 96.2% (from LQC), and 96.8% (HQC). After 1, 2 and 3 cycles of freeze–thaw the recovery of etoposide was in the range of 98.6–99.3% (LQC) and 96.9–99.6% (HQC). The recovery for PA-1 was in the range of 96.5–98.9% (LQC) and 96.9–97.8% (HQC). After 24 h (stability at room temp.) the recovery of etoposide was 98.2% (LQC), and 97.8% (HQC). The recovery for PA-1 was 98.6 (LQC), and 95.3% (HQC). The recovery (short-term autosampler stability) of etoposide was 98.9% (LQC) and 97.6% (HQC). For PA-1 the recovery was 97.5% (LQC) and 95.8% (HQC).

3.2.6. Matrix effect

The matrix effect ($A/B \times 100$) for etoposide was 95.84% (% RSD: 2.91; $n = 5$), and for PA-1 it was 97.21% (% RSD: 3.15; $n = 5$). Percent RSD < 5 suggested that the method was free from matrix effect.

3.3. Dose-dependent effect of PA-1

After oral administration of 20 mg/kg of etoposide a concentration of 639.1 ± 23.8 ng/mL of the drug was observed in the plasma of mice at 0.5 h (T_{max}). In further experiments etoposide was admin-

Table 2
Precision (% RSD) data.

Compound	Nominal conc. (ng/mL)	Intra-assay			Inter-assay		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Etoposide	2	6.95	5.42	5.67	7.56	5.77	6.00
	3	3.59	4.57	2.48	2.75	4.64	2.72
	450	2.71	2.32	3.60	2.91	3.02	5.07
	950	1.63	1.21	2.36	2.01	1.82	2.12
PA-1	1	8.10	9.70	10.7	9.25	9.90	9.88
	3	8.11	8.20	7.1	7.33	8.01	7.99
	450	2.72	2.32	3.50	2.91	3.02	5.07
	950	2.37	2.50	4.49	4.72	4.53	4.77

Table 3
Stability data.

Condition	Etoposide		PA-1	
	LQC	HQC	LQC	HQC
Recovery (ng) after storage (−80 °C)				
0 month	2.88 ± 0.014	923.1 ± 6.07	2.91 ± 0.013	937.7 ± 2.18
1 month	2.81 ± 0.02 (97.5%)	884.6 ± 7.80 (95.8%)	2.80 ± 0.02 (96.2%)	908.6 ± 7.96 (96.8%)
Recovery (ng) after freeze–thaw cycles				
Cycle 0	2.88 ± 0.014	923.1 ± 6.07	2.91 ± 0.013	937.7 ± 2.18
Cycle 1	2.86 ± 0.013 (99.3%)	919.7 ± 4.12 (99.6%)	2.88 ± 0.012 (98.9%)	917.3 ± 3.14 (97.8%)
Cycle 2	2.84 ± 0.012 (98.6%)	908.4 ± 5.87 (98.4%)	2.81 ± 0.013 (96.5%)	909.4 ± 5.32 (96.9%)
Cycle 3	2.84 ± 0.015 (98.6%)	894.5 ± 7.08 (96.9%)	2.84 ± 0.016 (97.5%)	909.1 ± 5.71 (96.9%)
Recovery (ng) after storage at room temp.				
0 h	2.88 ± 0.014	923.1 ± 6.07	2.91 ± 0.013	937.7 ± 2.18
24 h	2.83 ± 0.016 (98.2%)	903.5 ± 4.57 (97.8%)	2.87 ± 0.015 (98.6%)	893.9 ± 7.36 (95.3%)
Recovery (ng) after storage in autosampler				
0 h	2.88 ± 0.014	923.1 ± 6.07	2.91 ± 0.013	937.7 ± 2.18
24 h	2.85 ± 0.015 (98.9%)	901.0 ± 8.66 (97.6%)	2.84 ± 0.016 (97.5%)	898.5 ± 8.15 (95.8%)

Values (mean ± SE) are derived from 7 replicates. Figures in parenthesis represent percentage analyte concentration relative to time zero. Theoretical concentrations: LQC, 3 ng; HQC, 950 ng.

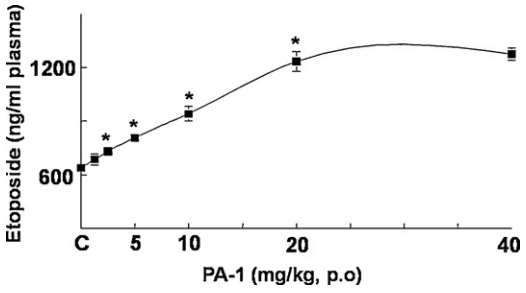

Fig. 5. Dose-dependent effect of PA-1 on etoposide levels at 0.5 h (C_{max}) post-dosing. Each data point is mean ± SE (n = 6) from plasma of mice treated with etoposide (20 mg/kg, p.o.) in absence (*C*), and presence of varying doses of PA-1 (1.25–40 mg/kg, p.o.). For details refer Section 2.6. *P < 0.001 vs. *C* (Dunnet's test).

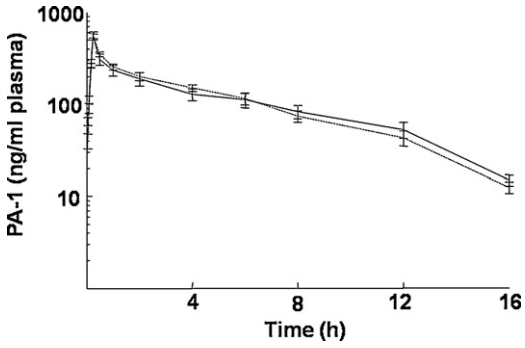

Fig. 7. Plasma conc. vs. time curve of PA-1 (20 mg/kg, p.o.) (solid lines) and PA-1 in presence of etoposide (20 mg/kg, p.o.) (dotted lines). Each time point is mean ± SE (n = 6). For details refer Section 2.6.

istered along with varying doses of PA-1. Results are shown in Fig. 5. Etoposide levels in plasma increased with the increasing doses of PA-1, showing a maximum of 93.2% increase in the C_{max} with 20 mg/kg of PA-1. In subsequent experiments this dose of PA-1 (20 mg/kg) was used in combination with etoposide (20 mg/kg).

3.4. Pharmacokinetics

Concentration vs. time profiles are depicted in Fig. 6 (for etoposide alone and in presence of PA-1), in Fig. 7 (for PA-1 alone and in presence of etoposide), and in Fig. 8 (for etoposide, i.v.). The results showed that PA-1 increased the AUC and $t_{1/2}$ of etoposide, whereas V_d and Cl were decreased. The relative bioavailability (RB) as well as the absolute bioavailability (AB) of etoposide was enhanced by 2.32-fold in presence of PA-1 (Table 4A). Etoposide did not influence the pharmacokinetic profile of PA-1 (Table 4B).

4. Discussion

UPLC with qTof-MS offers better quality data in terms of increased detection limits, and chromatographic resolution with greater sensitivity. In the present investigation a method for the simultaneous determination of etoposide and PA-1 by UPLC–qTOF-MS/MS has been optimized. Etoposide and PA-1 have been quantified on the basis of their major fragments. The major product ions observed in the positive ion ESI spectra for etoposide were at m/z 239.1666 [$M^+ - C_{17}H_{17}O_8$], 229.1414

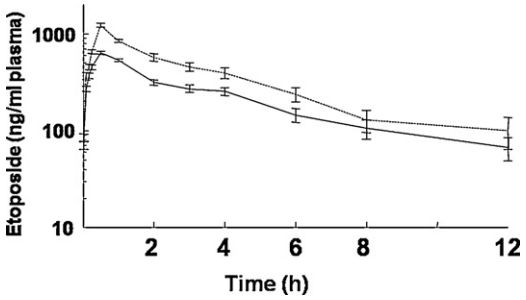

Fig. 6. Plasma conc. vs. time curves of etoposide (20 mg/kg, p.o.) (solid lines) and etoposide (20 mg/kg) in presence of PA-1 (20 mg/kg, p.o.) (dotted lines). Each time point is mean ± SE (n = 6). For details refer Section 2.6.

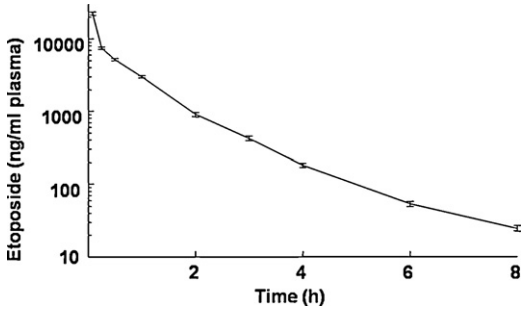

Fig. 8. Plasma conc. vs. time curve of etoposide (20 mg/kg, i.v.). Each time point is mean ± SE (n = 5). For details refer Section 2.6.

Table 4A
Pharmacokinetic parameters (etoposide).

Parameter	Etoposide (*A*) (20 mg/kg, p.o.)		Etoposide (*B*) (20 mg/kg, i.v.)
	Control	In presence of PA-1 (20 mg/kg, p.o.)	
$AUC_{0-\infty}$ (ng h/mL)	3142.84 ± 196.40	7293.55 ± 224.96*	11562.1 ± 527.65
C_{max} (ng/mL)	639.17 ± 23.83	1235.0 ± 56.59*	
T_{max} (h)	0.50	0.50	
$t_{1/2}$ (h)	6.61 ± 0.88	13.57 ± 1.62**	1.94 ± 0.29
Cl (mL/h/kg)	6732.67 ± 590.82	4073.1 ± 294.66**	1744.33 ± 79.85
V_d (mL/kg)	63557.94 ± 3192.04	46654.36 ± 2878.36**	4883.18 ± 294.42
RB (%)	100.0	232.06	
AB (%)	27.18	63.08	

A = values derived from Fig. 6; *B* = values derived from Fig. 8 (PK Solutions version 2.0; Summit Research Services, USA). RB (relative bioavailability) = $(AUC_{etoposide+PA\text{-}1}/AUC_{etoposide}) \times 100$. AB (absolute bioavailability) = $(AUC_{oral}/AUC_{i.v.}) \times (Dose_{i.v.}/Dose_{oral}) \times 100$.

* $p < 0.001$ vs. control (SPSS Data Editor Version 12.0).

** $p < 0.01$ vs. control (SPSS Data Editor Version 12.0).

Table 4B
Pharmacokinetic parameters (PA-1).

Parameter	PA-1 (20 mg/kg)	
	Control	In presence of etoposide (20 mg/kg, p.o.)
$AUC_{0-\infty}$ (ng h/mL)	1727.15 ± 90.12	1743.17 ± 78.44
C_{max} (ng/mL)	544.66 ± 21.73	567.66 ± 26.01
T_{max} (h)	0.25 ± 0.00	0.25 ± 0.00
$t_{1/2}$ (h)	2.47 ± 0.18	2.34 ± 0.13
Cl (mL/h/kg)	12968.69 ± 544.39	11930.68 ± 513.55
V_d (mL/kg)	49496.90 ± 2474.74	41133.22 ± 2056.90

Values are derived from Fig. 7 using a software (PK Solutions version 2.0; Summit Research Services, USA).

$[M^+-C_{16}H_{23}O_9]$, and 185.1350 $[M^+-C_{17}H_{23}O_{11}]$. For PA-1 the major product ions observed in the positive ion ESI spectra were at *m/z* 179.1878 $[M^+-C_8H_8O_1N_1]$, 164.1581 $[M^+-C_9H_{11}O_1N_1]$ and 135.0897 $[M^+-C_{11}H_{16}O_1N_1]$. The method was validated in terms of specificity, accuracy, precision, sensitivity and stability of the analytes, and utilized for the simultaneous determination of etoposide and PA-1, in plasma (mice). After oral administration etoposide could be quantified only up to 12 h of sampling time, while PA-1 could be quantified only up to 16 h of the sampling time. After i.v. administration etoposide could be quantified up to 8 h of the sampling time.

A pharmacokinetic study revealed that PA-1 enhanced the C_{max} and AUC of etoposide. Assessment of bioavailability from plasma concentration–time data usually involves determining the maximum (peak) plasma drug concentrations (C_{max}) and the area under the plasma concentration–time curve (AUC). The plasma drug concentration increases with the rate of absorption; therefore the most widely used general index of absorption is C_{max}. AUC is another reliable measure of bioavailability. It represents the total amount of unchanged drug that reaches systemic circulation [19]. A comparative profile as revealed in Fig. 6 and Table 4A showed that PA-1 caused an enhancement in both the extent and the absorption rate profile of etoposide. In PA-1 treated group V_d decreased with a concomitant decrease in Cl. These changes along with a longer $t_{1/2}$ in PA-1 treated group compared to untreated group, suggested, that an increase in the overall rate of drug elimination, is slowed down during the terminal phase, thus resulting in the observed enhancement of etoposide bioavailability.

Considerable evidence is accumulating to suggest that cytochrome P450 dependent biotransformation and p-glycoprotein (P-gp) mediated efflux are the major regulators of oral drug bioavailability. The main enzymes involved in the metabolism of etoposide are CYP 3A4 and CYP 1A2. Etoposide is also reported to be a substrate for P-gp [20]. The incomplete and poor oral bioavailability of etoposide has also been attributed to its poor absorption owing to its physicochemical characteristics [21]. Using various *in vitro* and animal-derived models further studies are in progress to ascertain a possible interaction of PA-1 with these biochemical determinants of oral bioavailability to broaden the mode of action profile of PA-1.

5. Conclusion

A specific, accurate and precise UPLC–qTOF-MS/MS method for the determination of etoposide and PA-1, both individually and simultaneously was optimized. The results showed that a piperine analogue, PA-1, enhanced the bioavailability of orally administered etoposide in mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.01.048.

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