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Journal of Chromatography B, 863 (2008) 223–228
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of ecabet in human plasma by high-performance liquid chromatography–tandem mass spectrometry

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Received 2 October 2007; accepted 4 January 2008
Available online 16 January 2008

Abstract

This paper describes a simple, robust and cost-effective assay for the determination of ecabet in human plasma. After a simple step of protein precipitation using methanol, plasma samples were analyzed by reverse phase high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) with valsartan as the internal standard (I.S.). Ecabet and the I.S. valsartan were separated on a Venusil MP C18 analytical column using methanol–10 mM ammonium acetate (75:25, v/v, pH 3.0) as mobile phase at a flow rate of 1.0 mL/min. Ecabet and I.S. were eluted at 0.91 and 0.92 min, respectively, ionized in negative mode, and then detected by multiple reaction monitoring (MRM) essay. The MRM transitions of m/z 379.1 → m/z 277.1 and m/z 434.3 → m/z 350.1 were used to quantify ecabet and I.S., respectively. The assay was linear over the concentration range of 10–6000 ng/mL and was successfully applied to a pharmacokinetic study in healthy volunteers.
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Keywords: Ecabet; LC–MS/MS

1. Introduction

Ecabet, (1R,4aS,10aR)-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methyl-ethyl)-6-sulpho-1-phenanthrenecarboxylic acid, derived from a component in pine resin and used to treat gastric diseases in ancient China [1], is a novel locally acting anti-ulcer drug [2–6]. Oral administration of ecabet reduced pepsin enzymatic activity [2,7,8] and urease enzymatic activity of *Helicobacter pylori* [5,6], inhibited the synthesis of IL-8 by *H. pylori* [9,10], increased gastric pH [3,11,12], and modulated capsaicin-sensitive sensory nerves [11]. Moreover, ecabet was associated with enhanced production of several gastric mucosal defensive factors, including PGE_2, PGI_2, and mucin [2,3,12,13]. It is currently used in clinical practice as an oral treatment for gastric ulcer and gastritis in Japan and China, under the commercial name of Gastrom (TA-2711, ecabet monosodium) [14]. It can also be used in combination dosage with other drugs, such as cimetidine [15,16], rebamipide [17], and some proton pump inhibitors and antibiotics [18–20].

Some recent studies indicate that ecabet also shows efficacy on functional dyspepsia [21], ulcerative colitis (UC) [22,23], esophageal lesions induced by the reflux of gastric juice [24], gastric adaptive relaxation, and intragastric pressure [25]. An enema formulation of ecabet (TA-2711E) for the treatment of UC has been evaluated in phase III trials in Japan and phase II studies in both Europe and the US [26].

In order to study the pharmacokinetics of ecabet used in different formulations or dosed to different populations, it is necessary to establish a method for the determination of ecabet concentration in plasma. Metabolic fate of ecabet (disposition, metabolism, and protein binding in rats and dogs, and distribution in the rat stomach) has been investigated by radioactive label [27,28], but such methods suffer from low specificity and are ethically undesirable.

In the last decade, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been widely employed for the bioassay of drugs because of its excellent specificity, speed, and sensitivity [29,30]. This paper describes a highly simple, robust and cost-effective assay for the deter-

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doi:10.1016/j.jchromb.2008.01.013

mination of ecabet in human plasma by LC–MS/MS for the first time. The essay has been successfully applied to a pharmacokinetic study of ecabet in healthy volunteers after a single oral administration of an ecabet disodium tablet containing 1 g ecabet.

2. Experimental

2.1. Chemicals and reagents

Ecabet disodium (purity > 99%) was provided by Aikang Pharmaceutical Co. (Hangzhou, China). Valsartan (purity > 99%) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Structures of ecabet and I.S. (valsartan) are shown in Fig. 1. Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water was prepared from deionized water. All other chemicals and solvents (analytical grade) were used without further purification. Blank (drug free) human plasma was obtained from Changchun Blood Donor Service (Changchun, China).

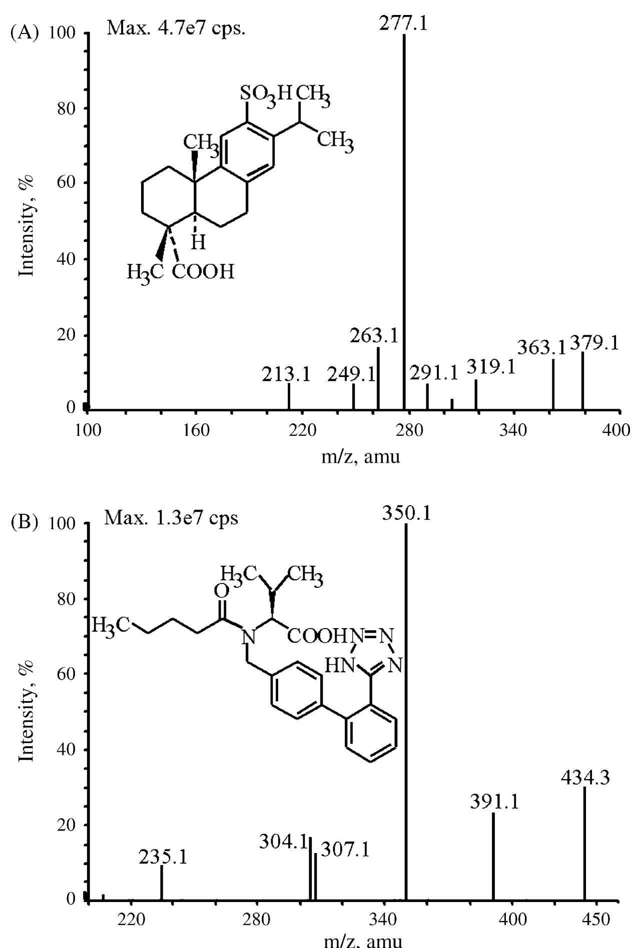

Fig. 1. Structures and full-scan product ion spectra of $[M-H]^-$ for (A) ecabet and (B) valsartan.

2.2. Instrumentation

The LC experiment utilized an Agilent 1100 series (Agilent, Palo Alto, CA, USA) HPLC system, consisting of a pump, an autosampler, and a column oven. The LC system was coupled with an API4000 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada), equipped with an electrospray ionization (ESI) source. Analyst software (Applied Biosystems/MDS SCIEX, version 1.3) installed on a *DELL* computer was used for data acquisition and processing.

2.3. LC–MS/MS conditions

A C_{18} analytical column (Venusil MP, 50 mm × 4.6 mm i.d., 5 μm, Agela, USA) was used in this study. An isocratic mobile phase consisting of methanol and 10 mM ammonium acetate (75:25, v/v) adjusted to pH 3.0 using formic acid was used at a flow rate of 1.0 mL/min. A 20 μL sample was injected onto the column and the eluant was split with approximately 1:1 ratio so that the flow of ~0.5 mL/min was sampled by the ESI source. The column temperature was maintained at 35 °C.

All measurements were carried out with the mass spectrometer operated under the negative ESI mode. The multiple reaction monitoring (MRM) transitions were *m/z* 379.1 → 277.1 and *m/z* 379.1 → 263.1 for ecabet, and *m/z* 434.3 → 350.1 for valsartan. Other parameters were as follows: collision gas, curtain gas, gas 1 and gas 2 (nitrogen) 5, 10, 55 and 45 p.s.i., respectively; dwell time 200 ms; IonSpray voltage −3300 V; source temperature 550 °C; declustering potential (DP) −130 V for ecabet and −50 V for valsartan; collision energy (CE) −60 eV (*m/z* 379.1 → 277.1) and −65 eV (*m/z* 379.1 → 263.1) for ecabet and −10 eV for valsartan. Unit resolution was used for both Q1 and Q3 mass detection.

2.4. Preparation of calibration standard samples and quality control samples

The standard stock solution (400 μg/mL) of ecabet and I.S. stock solution (400 μg/mL) of valsartan were prepared by dissolving ecabet disodium (11.2 mg) and valsartan (10.0 mg) in 2 mL methanol:water (50:50, v/v) followed by dilution to 25 mL with methanol:water (50:50, v/v), respectively. Low, medium and high quality control (QC) solutions (20, 600, and 4800 ng/mL) were prepared with water. I.S. working solution (1 μg/mL) was prepared by diluting the I.S. stock solution with methanol:water (50:50, v/v). All solutions were stored at 4 °C.

A spiked calibration standard sample (6000 ng/mL) was prepared by pipetting 750 μL of the standard stock solution (400 μg/mL) into a 50 mL volumetric flask and making it to volume with blank plasma. Other spiked calibration standard samples (10, 20, 60, 200, 600, and 2000 ng/mL) were prepared by dilution of the 6000 ng/mL calibration standard sample with blank plasma. QC samples (20, 600, and 4800 ng/mL) were prepared in a similar way from an independently prepared standard stock solution. All plasma samples were prepared in bulk, ali-

quoted, and frozen at −20 °C, with aliquots thawed and analyzed with each run.

2.5. Sample preparation

Frozen plasma samples were thawed at room temperature and vortex-mixed briefly. Aliquots of plasma (50 μL) were transferred to microcentrifuge tubes followed by additions of I.S. working solution (100 μL), and aliquots of methanol (700 μL) were added to precipitate the proteins. Tubes were vortex-mixed for 30 s and centrifuged at 10,000 × *g* for 10 min. Supernatants (500 μL) were transferred to new clean tubes followed by additions of acetic acid (20 μL). The mixtures were vortex-mixed for 15 s, and then transferred to the autosampler for LC–MS/MS analysis.

2.6. Assay validation

Based on the FDA guidelines for the validation of bioanalytical methods [31], the method reported here was fully validated for its specificity and matrix effects, linearity and sensitivity, extraction recovery, and accuracy and precision. The analyte stability was also evaluated.

To determine the specificity of the assay, six replicates of the pooled blank human plasma were analyzed to investigate the potential interferences around the chromatography peak region for analyte and I.S. Matrix effects on analyte and I.S. were calculated by comparing the peak areas of analyte and I.S. in QC samples with those of analyte and I.S. in QC solutions, respectively, following the same extraction procedure.

Linear regression of calibration curves based on peak area ratios of analyte to I.S. obtained from LC–MS/MS was weighted according to $1/x^2$ (x = concentration). The lower limit of quantitation (LLOQ) was defined as the concentration below which the relative error (R.E.) without ± 15% or the inter-day relative standard deviation (R.S.D.) exceeded 15%, and the limit of detection (LOD) was defined as the concentration with S/N of 3.

The assays were performed on three separate days, and on each day six replicates of analyte at each concentration level were analyzed together with an independently prepared calibration curve. Intra- and inter-day precisions calculated as R.S.D. (%) were required to be below 15%, and accuracy as R.E. (%) to be within ±15%.

The extraction recoveries of analyte and I.S. were evaluated by comparing the peak areas of analyte and I.S. in extracted QC samples with those of analyte and I.S. reconstituted in blank plasma extracts at the corresponding concentrations.

The long-term stability, freeze-thaw stability, post-processing stability were evaluated using QC samples after storage at −20 °C for 6 weeks, after three freeze/thaw cycles, and after storage in reconstitution solutions in the autosampler at room temperature for 24 h, respectively.

2.7. Clinical pharmacokinetic study in healthy volunteers

Each of the 10 healthy volunteers was given a single oral administration of an ecabet disodium tablet (containing 1 g ecabet) with 250 mL water. Blood samples (1 mL) were then collected into heparinized tubes by venepuncture before and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, and 36 h after the oral dosing. After centrifugation (3000 × *g* for 10 min at 4 °C), plasma samples were collected and stored immediately at −20 °C until analysis. All samples were analyzed within 6 weeks. Pharmacokinetic parameters were calculated using Topfit 2.0.

3. Results and discussion

3.1. MS conditions

The ESI response of analyte was evaluated by recording the full-scan mass spectra in both positive and negative ionization modes with a syringe pump for sample injection. Ecabet was ionized in both positive and negative modes, but it had much higher ionization efficiency in the negative ionization mode. Therefore, the negative ionization mode was chosen for LC–MS/MS analysis and the product ion mass spectra of both the analyte and the I.S. are shown in Fig. 1. The product ion at *m/z* 277.1 was the most abundant fragment ion for the deprotonated molecular ion $[M-H]^-$ of ecabet (Fig. 1(A)), thus the transition *m/z* 379.1 → 277.1 was selected for the quantification of ecabet. The transition from *m/z* 379.1 to the less abundant *m/z* 263.1 product ion was also monitored as the qualifier transition to minimize the possibility of false positive detection in case of interference to the quantifier transition. The MS/MS settings were tuned to maximize the response of each of the precursor/product ion combinations.

3.2. Chromatographic conditions

A number of C_{18} columns (Zorbax extend C_{18}, Nucleosil C_{18}, Hypersil C_{18}, and Venusil MP C_{18}) were evaluated and the Venusil MP C_{18} (50 mm × 4.6 mm i.d., 5 μm) gave the best chromatography with a flow rate of 1.0 mL/min. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of ecabet with short run time. Ecabet has sulphonic and carboxylic groups which makes it a very acidic compound. As the pK_a of the carboxylic group is 5.1, ecabet exists for 88%, 50%, and 0% in the nonionized form at pH 4.0, 5.0, and 8.0, respectively [6]. The chromatography for ecabet was expected to be highly sensitive to the pH of the mobile phase, therefore a series of experiments was carried out to choose the optimum pH value. When the pH of mobile phase was equal or more than 8.0, a single chromatographic peak corresponding to the completely ionized form of ecabet was detected at approximately 0.47 min. However, this elution time falls into the column dead time and the ESI response of the analyte readily suffers from the interference of the co-eluting endogenous substances. When the pH of mobile phase was equal or less than 3.0, a single chromatographic peak corresponding to the completely nonionized form of ecabet was detected. The mobile phase pH was eventually chosen at 3.0 with the consideration that the column lifetime could be shortened when pH was less than 3.0. The analyte was eluted at approximately 0.91 min with

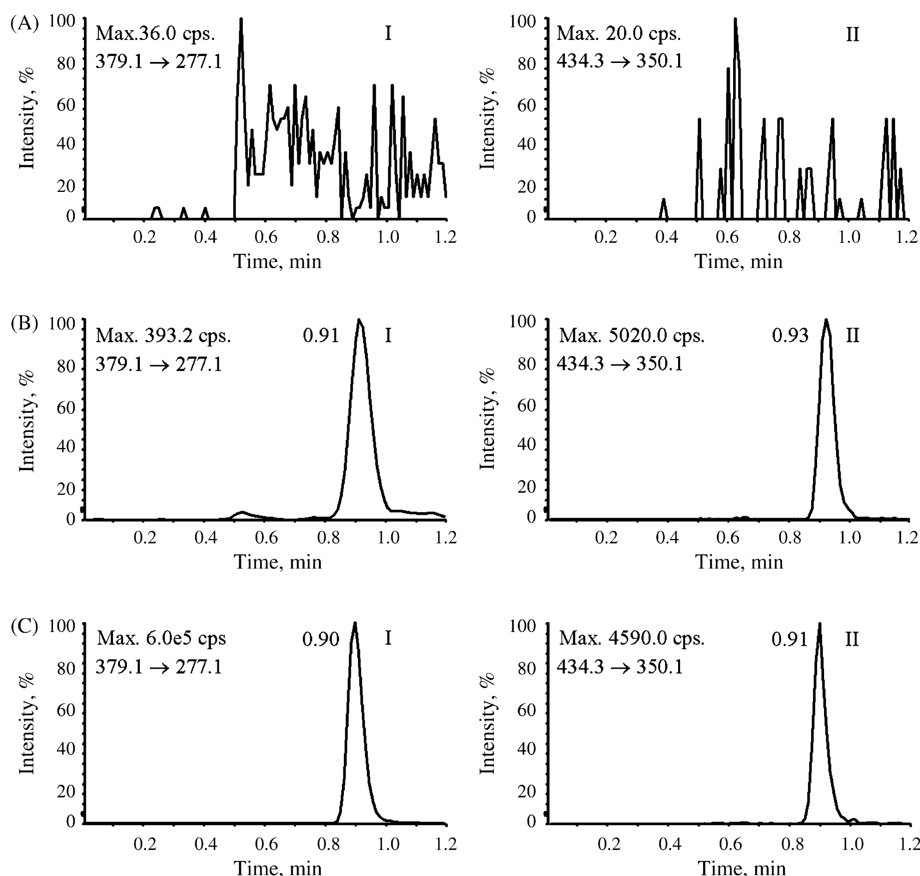

Fig. 2. Representative MRM chromatograms of (A) blank plasma; (B) plasma spiked with ecabet at the lower limit of quantitation (10 ng/mL) and (C) plasma sample from a healthy volunteer 1 h after a single oral administration of an ecabet disodium tablet containing 1 g ecabet. Peak I, ecabet; Peak II, valsartan.

improved peak shape and minimized matrix effect. Hydrophilic impurities were diverted to waste for 0.5 min after an injection using a ten-way switching valve. The total run time is 1.2 min per sample, thus allowed a high sample throughput (270–330 samples per day).

3.3. Sample preparation

In the terms of sample preparation, protein precipitation was chosen because the extraction recovery was high, and the simple preparation procedure can save considerable time and simplify the operating process. Different volumes of methanol were evaluated for the efficiency of protein precipitation; it was found that 4.5 times volume of the plasma could precipitate the plasma proteins completely. Peak shape was improved by using acetic acid to adjust the pH of supernatant to 3.0 after protein precipitation. The analyte was stable under these conditions.

3.4. Method validation

3.4.1. Specificity and matrix effects

Fig. 2 shows the typical MRM chromatograms (with the transition *m/z* 379.1 → 277.1 used for quantification) of blank plasma, plasma sample spiked with ecabet at 10 ng/mL, and plasma sample from a healthy volunteer 1 h after an oral administration of an ecabet disodium tablet containing 1 g ecabet. There was no endogenous peak interference observed with the analyte and I.S. at their retention times, demonstrating the selectivity and specificity of the assay.

Although, matrix effect is generally a significant problem in the LC–MS/MS analysis of biological samples, in this assay, the ratios of the peak responses were $92.5 \pm 2.3\%$, $96.7 \pm 2.5\%$, and $96.6 \pm 1.6\%$ at 20, 600, and 4800 ng/mL concentrations for ecabet and $92.7 \pm 5.5\%$ for I.S. respectively, indicating no co-eluting endogenous substances interfering with the ionization of ecabet or I.S. under the conditions of the assay.

3.4.2. Linearity and sensitivity

The peak area ratios of analyte to I.S. in human plasma varied linearly with concentrations over the range tested (10–6000 ng/mL). The correlation coefficient (r) is in the range of 0.9990–0.9994. Good linearity was shown in the stated concentration ranges. A typical calibration curve on a validation day is shown in Fig. 3.

The LLOQ and LOD of ecabet were determined to be 10 and 2 ng/mL, respectively. The LLOQ was sufficient for clinical pharmacokinetic studies following an oral administration as shown in Section 3.5.

3.4.3. Accuracy and precision

A summary of inter- and intra-day precision and accuracy at three QC concentrations (20, 600, and 4800 ng/mL) is shown in

Table 1
Precision (R.S.D., %) and accuracy (R.E., %) at three concentrations of QC samples (six replicates each day, on three different days)

Nominal concentration (ng/mL)	Measured concentration (mean, ng/mL)	Inter-day R.S.D. (%)	Intra-day R.S.D. (%)	R.E. (%)
20.00	20.03	2.55	2.09	0.15
600.0	605.4	2.43	1.87	0.90
4800	4821	1.87	1.77	0.44

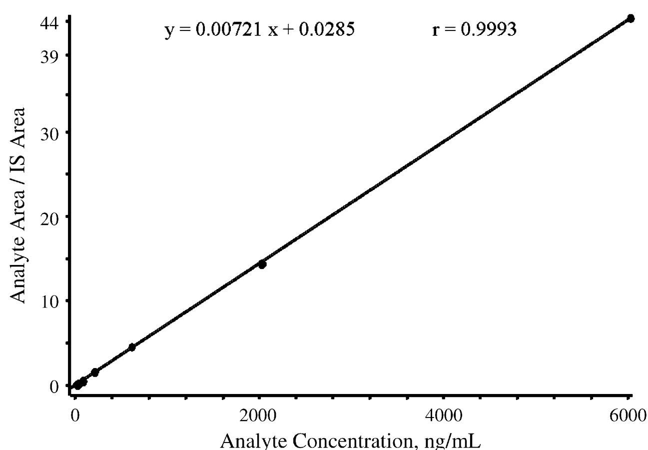

Fig. 3. A typical calibration curve on a validation day.

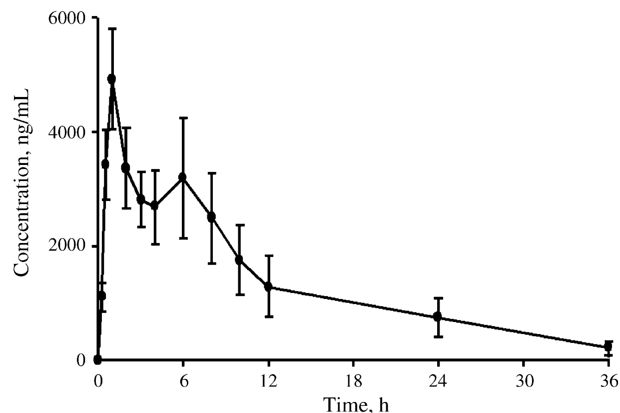

Fig. 4. Mean plasma concentration-time profile of ecabet after a single oral administration of an ecabet disodium tablet (containing 1 g ecabet) to each of the 10 healthy volunteers. All data are expressed as mean ± S.D. ($n = 10$).

Table 1. The precisions (R.S.D., %) were all less than 3% and the accuracy (R.E., %) was within ±1.0%. The results indicated that the assay had very desirable reproducibility with reasonable accuracy and precision.

3.4.4. *Extraction recovery*

The extraction recoveries of ecabet from human plasma were 94.1 ± 5.6%, 94.8 ± 5.0%, and 97.8 ± 5.8% for the QC samples at concentration of 20, 600, and 4800 ng/mL, respectively, whereas 96.4 ± 8.4% for I.S.

3.4.5. *Analyte stability*

In the stability evaluation experiments, the concentration variations (summarized in Table 2) were within ±1.5%, ±3.6%, and ±1.5% of nominal concentrations, respectively, indicating no significant substance loss during these procedures.

3.5. *Clinical pharmacokinetic study in healthy volunteers*

This validated assay was applied to a clinical pharmacokinetic study in 10 healthy volunteers following a single oral administration of an ecabet disodium tablet containing 1 g ecabet. The mean plasma concentration-time profile is shown in Fig. 4. The plasma concentration maximum (C_{max}) of ecabet was 4926 ± 880 ng/mL at 1 h, the plasma elimination half-life was 6.63 ± 2.24 h, and the area under the plasma concentration-time curve (AUC) was 36943 ± 5764 ng h/mL. The assay was sensitive enough for the pharmacokinetic studies.

It was possible that the difference between pharmaceutical formulations and compounds of ecabet disodium (used in this

Table 2
Results of stability experiments

Storage conditions	Nominal concentration (ng/mL)	Pre-procedure ($n = 3$)			Post-procedure ($n = 3$)		
		Measured concentration (mean, ng/mL)	R.S.D. (%)	R.E. (%)	Measured concentration (mean, ng/mL)	R.S.D. (%)	R.E. (%)
In human plasma at −20 °C for 6 weeks	20.00	19.85	2.08	−0.75	19.74	4.92	−1.30
	600.0	606.9	3.17	1.15	595.3	3.99	−0.78
	4800	4768	1.35	−0.67	4748	2.88	−1.08
In human plasma after freezing-thawing for 3 cycles	20.00	19.95	1.56	−0.25	19.82	2.36	−0.90
	600.0	598.0	3.28	−0.33	615.5	2.42	2.58
	4800	4777	4.59	−0.48	4629	0.89	−3.56
In reconstitution solutions in the autosampler at room temperature for 24 h	20.00	19.93	0.67	−0.35	20.14	1.50	0.70
	600.0	603.8	1.17	0.63	596.1	4.00	−0.65
	4800	4810	0.92	0.21	4737	1.74	−1.31

report) and ecabet monosodium (TA-2711) and the difference between volunteer populations resulted in the disparity between the pharmacokinetic parameters shown above and those reported in the accompanying documents on TA-2711 (T_{max} 2–5 h, C_{max} 1.0 μg/mL, $t_{1/2}$ 8 h) [14].

4. Conclusions

In conclusion, a simple, robust and cost-effective LC–MS/MS method is reported for the determination of ecabet in human plasma. The assay has been successfully applied to evaluate plasma concentration-time profiles in a single oral dose pharmacokinetic study of ecabet. The assay allows high sample throughput because of its simple sample preparation and short run time.

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