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Quantification of (+)-calanolide A, a novel and naturally occurring anti-HIV agent, by high-performance liquid chromatography in plasma from rat, dog and human

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Abstract

A HPLC method was validated for quantification of (+)-calanolide A (**1**), a novel anti-HIV agent, in rat, dog and human plasma. The synthetic intermediate (±)-12-oxocalanolide A (**2**) was found to be a suitable internal standard. Compounds were extracted from plasma using a solid-phase C_{18} cartridge and quantified over the assay range of 12.5 to 800 ng/ml. The method was utilized to determine (+)-calanolide A pharmacokinetics in rats, dogs and humans. This is the first report of a validated HPLC assay for determination of (+)-calanolide A concentrations in rat and dog plasma as well as human plasma obtained from clinical trials. There was no evidence of in vivo epimerization of (+)-calanolide A to its inactive epimer (+)-calanolide B (**3**). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Calanolide A

1. Introduction

(+)-Calanolide A (**1**), a natural product isolated from several tropical plants of the genus *Calophyllum*, has been demonstrated to be active against HIV-1 [1]. Further evaluation has shown that (+)-calanolide A provides cytoprotection to human cells against all laboratory and clinical isolates of HIV-1, with a unique drug-resistance profile [2–10]. Significant in vivo anti-HIV activity of (+)-calanolide A, either alone or in combination with AZT, was demonstrated in the hollow fiber mouse study. Following oral or parenteral administration on a once- or twice-daily treatment schedule, the compound was capable of suppressing virus replication in two distinct and separate physiologic compartments (peritoneal cavity and subcutaneous site) [11]. Due to its lipophilic nature, (+)-calanolide A has been demonstrated to readily distribute into viral reservoir sites such as brain and lymph after oral and intravenous administration to rats [12].

Currently, (+)-calanolide A is in clinical trials to evaluate its safety and pharmacokinetics in both normal healthy and HIV-infected volunteers. After

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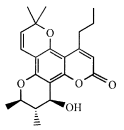
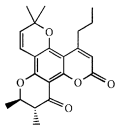
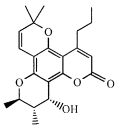
oral administration, the drug was generally well tolerated and no patterns indicative of a safety concern were observed in healthy HIV-negative people [13]. Plasma drug concentrations in humans were higher than anticipated from animal data and area under the curve (AUC) and C_{max} increased proportionally with increasing dose. It appeared that therapeutic levels can be achieved in humans, based on the in vitro EC_{90} values of (+)-calanolide A, concentrations required to inhibit 90% of virus-induced cell killing in cultured cells. Herein, we wish to communicate a validated high-performance liquid chromatographic (HPLC) method for quantification of (+)-calanolide A in rat, dog and human plasma. Also presented is the application of this method for the determination of pharmacokinetic parameters in these three species for the first time (during the preparation of this manuscript, we became aware that the (+)-calanolide A plasma pharmacokinetics profile was determined in mice after the drug levels were quantified using an HPLC method [14]). Furthermore, (±)-12-oxocalanolide A (**2**), the internal standard used in the assay, is also a promising antiviral agent [15] and the reported method should be suitable for quantification of (±)-12-oxocalanolide A (**2**) as well.



(+)-Calanolide A (**1**)



12-Oxocalanolide A (**2**)



(+)-Calanolide B (**3**)

2. Experimental

2.1. Reagents and chemicals

(+)-Calanolide A (**1**) and internal standard (±)-12-oxocalanolide A (**2**) were synthesized according to the published methods [2]. Rat and dog plasmas were purchased from Pelfreeze (Rogers, AR, USA) and heparinized human plasma from the Interstate Blood Bank (Memphis, TN, USA). Acetonitrile (UV grade) and water (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI, USA).

Stock solutions of **1** and **2** (1 mg/ml) were prepared in 100% acetonitrile and stored at −20°C. Under these conditions, the solutions were stable for at least 1 month.

2.2. Extraction procedure

A 10-μl aliquot (rat and dog) or 50-μl aliquot (human) of the internal standard solution (4 μg/ml) was mixed with the plasma sample to be analyzed. A volume of 100 μl of rat plasma, 200 μl dog plasma and 1.0 ml of human plasma samples were employed. The sample mixture was loaded onto a Varian Bond Elut column (Harbor City, CA, USA) (6 ml containing 500 mg of C_{18} packing material for the rat and dog assay and 1 g for the human samples). The C_{18} column was pre-conditioned with 5 ml of acetonitrile followed by 5 ml of water flowing through the column by gravity. The column was then eluted with 5 ml of acetonitrile under vacuum and the eluents collected and dried under N_2 at 50–60°C. The residue was reconstituted with 300 μl of acetonitrile, vortex-mixed, sonicated and filtered (0.2 μm, 13 mm PTFE Gelman filter attached to a 1 ml disposable syringe) into an autoinjector vial for HPLC analysis.

2.3. Chromatography

The liquid chromatograph (Hewlett-Packard 1050 HPLC) was operated at a flow-rate of 1.3 ml/min and the autoinjector (Spectra-Physics 8775/3506) was set to deliver 100 μl. The analytical column was a 250×4.6 mm Zorbax ODS C_{18} with 5 μm particle size at ambient temperature, preceded by a C_{18} guard column (Brownlee Newguard, 15×3.2 mm, 7 μm particle size). The system was also equipped with an in-line filter (Fisher Scientific, 0.5 μm) and a fluorescence detector (Applied Biosystems 980), which was set for excitation at 285 nm with an emission cut-off filter at 418 nm. Mobile phase A was acetonitrile–water (70:30) and mobile phase B was acetonitrile. The gradient profile of the mobile phase was as follows:

0–2 min	Hold at 100% A
2–5 min	Linearly increased to 95% B in A
5–10 min	Hold at 95% B in A
10–12 min	Linearly decreased to 100% A

2.4. Calibration curve and linearity

Calibration curves were prepared by spiking normal rat, dog or human plasma with increasing amounts of (+)-calanolide A. The same value of standard working solution was used to prepare the the plasma standards. The linearity in each of these matrices was assessed with concentrations of (+)-calanolide A ranging from 12.5 to 800 ng/ml (12.5, 25, 50, 100, 200, 400 and 800 ng/ml). The ratios of the peak areas for (+)-calanolide A and internal reference 12-oxocalanolide A were plotted against the (+)-calanolide A concentration to check for linearity, and the correlation coefficient was calculated. Curves with a correlation coefficient of >0.98 from the unweighted regression analysis were accepted.

2.5. Reproducibility and accuracy

Both intra- and inter-day reproducibilities were determined with the same set of samples used for linearity. For intra-day reproducibility, quadruplicates of each sample were tested on the same day and the resulting relative standard deviation (RSD) (reproducibility) and percent relative error (accuracy) determined. To measure inter-day reproducibility, three quality control (QC) samples at (+)-calanolide A concentrations of 30, 160 and 500 ng/ml for low-, middle- and high-level, respectively, were run in quintuplicate on each of 3 separate days. RSD and percent error were determined.

2.6. Freeze–thaw stability

The freeze–thaw stability study was done on three QC samples at (+)-calanolide A concentrations of 30, 160 and 500 ng/ml for low-, middle- and high-level, respectively. The samples were subjected to three freeze–thaw cycles prior to HPLC analysis and analyzed in duplicate for rat and dog samples and in quintuplicate for human plasma samples.

2.7. (+)-Calanolide A plasma pharmacokinetics in rats, dogs and humans

Plasma pharmacokinetics of (+)-calanolide A were determined in rats, dogs, and humans. A total of 32 Sprague–Dawley male and female rats (eight per sex group) were administered (+)-calanolide A at 15 mg/kg intravenously (i.v.) or 50 mg/kg orally and randomized for plasma sampling groups (see the following table for details). Plasma samples from four male and four female rats at each timepoint were obtained at 0.5, 1, 2, 4, 6, 8, 12 and 24 h after oral dosing or at 10 min and 0.5, 1, 2, 4, 6, 12 and 24 h after intravenous dosing.

Group	Route/dose (mg/kg)	Males/females	Timepoints of plasma sampling (h)
1A	Oral/50	4/4	0.5, 2, 6, 12
1B	Oral/50	4/4	1, 4, 8, 24
2A	IV/15	4/4	10 min, 1, 4, 12
2B	IV/15	4/4	0.5, 2, 6, 24

Four male and four female beagle dogs were dosed (+)-calanolide A at a mean dosage of 31.6 mg/kg by oral gavage and plasma samples were taken prior to treatment and at approximately 0.5, 1, 1.5, 2, 6, 8, 12, 24, 48 and 72 h after dosing. After a 7-day wash-out period, the same four male and four female dogs received (+)-calanolide A at 5 mg/kg intravenously and plasma samples were obtained at pre-dose and approximately 10 and 30 min and 1, 1.5, 2, 6, 12, 24, 48 and 72 h after dosing.

A cohort of 12 healthy HIV-negative human volunteers (six males and six female) were enrolled in the study (protocol CA-96-025) after each signed a written informed consent approved by the Institutional Review Board and administered orally a single dose of 800 mg (+)-calanolide A. Plasma samples were obtained at pre-dose and approximately 30 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 32, 36 and 48 h after dosing.

All plasma samples were frozen at $-20^{\circ}C$ until analysis. In all species, when concentrations were above the upper limit of quantification, the samples were measured with dilution. The formulation used for the intravenous dosing in rats and dogs was propylene glycol–ethanol (80:20), while the oral dosing for all three species was in the oil-based clinical formulation.

3. Results and discussion

Shown in Fig. 1 are typical chromatograms obtained from analyses of (+)-calanolide A in rat, dog

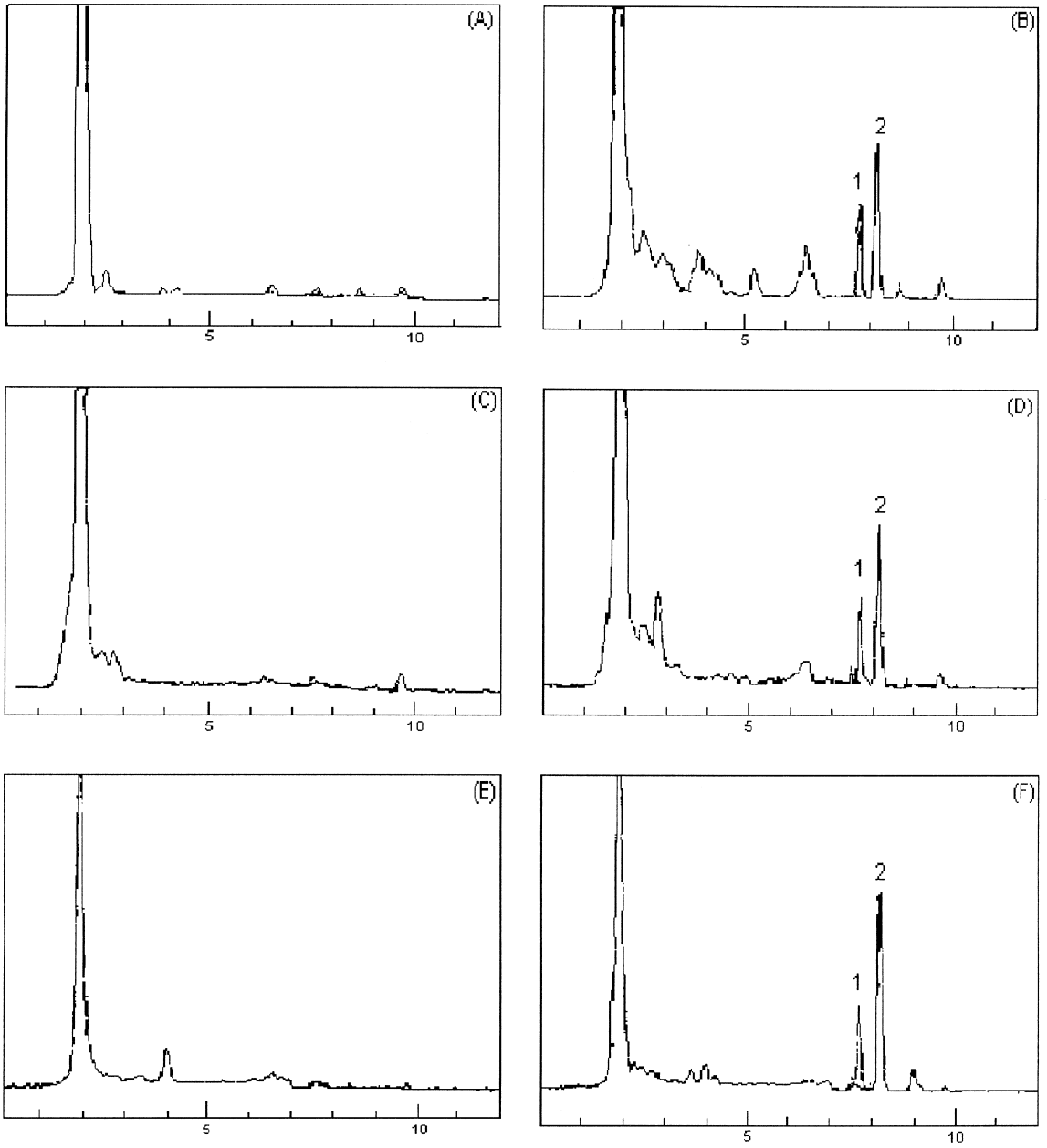


Fig. 1. HPLC chromatograms of (A) human blank plasma, (B) human plasma containing the internal and reference standards, (C) dog blank plasma, (D) dog plasma containing the internal and reference standards, (E) rat blank plasma, (F) rat plasma containing the internal and reference standards. Peak 1 was the internal standard (±)-12-oxocalanolide A and peak 2 was reference standard (+)-calanolide A.

and human plasma. Chromatograms of extracted blank plasma samples and of plasmas containing (+)-calanolide A and (±)-12-oxocalanolide A are presented. It was observed that both (+)-calanolide A (1) and (±)-12-oxocalanolide A (2) were resolved from endogenous peaks. Retention times were 8.1

Table 1
Intra-day reproducibility and accuracy

	800 (ng/ml)	400 (ng/ml)	200 (ng/ml)	100 (ng/ml)	50 (ng/ml)	25 (ng/ml)	12.5 (ng/ml)
Rat plasma							
Mean±SD	802±21	399±31	194±6	99±12	55±7	29±3	11±8
RE (%)[a]	0.2	−0.3	−2.8	−1.2	10.7	14.9	−9.2
RSD (%)[b]	2.6	7.8	3.2	11.9	13.5	8.8	67.3
Human plasma							
Mean±SD	800±66	401±31	198±8	100±2	48±6	27±4	13±2
RE (%)[a]	−0.1	0.3	−0.8	0.1	−3.4	7.2	0.5
RSD (%)[b]	8.3	7.7	4.3	1.8	12.8	14.8	19.3

[a] RE: Relative error = [(mean − nominal)/nominal] × 100.
[b] RSD: Relative standard deviation = (standard deviation/mean) × 100.

min for (+)-calanolide A and 7.7 min for (±)-12-oxocalanolide A in rat, dog and human plasma.

3.1. Assay reproducibility and accuracy

Intra-day reproducibilities for rat and human and inter-day reproducibilities for rat, dog and human plasma (+)-calanolide A concentrations are shown in Tables 1 and 2. The acceptable relative error (RE) and RSD values were set to be ≤15% for middle- and high-level standards and ≤20% for low-level standards. Therefore, the lowest (+)-calanolide A standard with acceptable RSD and RE values, as shown in the intra-day assay, was 25 ng/ml for rat plasma and 12.5 ng/ml for human plasma, respectively (Table 1). The intra-day reproducibilities for dog plasma were not determined. For all three types of plasma, inter-day reproducibilities indicated that all RSD and RE values were acceptable for three QC samples at (+)-calanolide A concentration of 30, 160 and 500 ng/ml for low-, middle- and high-level, respectively (Table 2). In a representative trial, high

Table 2
Inter-day reproducibility and accuracy

	Day 1			Day 2			Day 3		
	30 (ng/ml)	160 (ng/ml)	500 (ng/ml)	30 (ng/ml)	160 (ng/ml)	500 (ng/ml)	30 (ng/ml)	160 (ng/ml)	500 (ng/ml)
Rat plasma									
Mean±SD	34±6	140±9	540±36	34±4	159±8	510±20	32±6	155±7	524±13
RE (%)[a]	13.9	−12.36	7.91	13.2	−0.65	2.04	7.16	−2.82	4.79
RSD (%)[b]	16.3	6.69	6.64	11.4	4.87	4.00	18.8	4.34	2.56
Dog plasma									
Mean±SD	32±4	165±8	539±17	35±5	153±4	503±20	30±7	156±7	514±24
RE (%)[a]	5.32	3.39	7.76	16.8	−4.16	0.65	−0.82	−2.56	2.87
RSD (%)[b]	12.6	4.71	3.07	13.0	2.63	4.07	22.8	4.28	4.58
Human plasma									
Mean±SD	34±3	163±5	529±31	32±3	166±5	553±29	31±3	168±4	528±9
RE (%)[a]	12.66	1.94	5.75	5.94	3.76	10.52	4.63	−5.28	5.66
RSD (%)[b]	9.87	3.34	5.81	8.03	2.81	5.34	10.7	2.49	1.77

[a] RE: Relative error = [(mean − nominal)/nominal] × 100.
[b] RSD = Relative standard deviation = (standard deviation/mean) × 100.

absolute recoveries of (+)-calanolide A (91.2%) and internal standard (93.6%) were obtained.

Freeze–thaw stability studies were done on three QC samples at (+)-calanolide A concentrations of 30, 160 and 500 ng/ml for low-, middle- and high-level, respectively. The results presented in Table 3 indicated that re-freezing the rat, dog and human plasma over three cycles did not influence the outcome, since all the RE and RSD values were within the acceptable ranges, with an exception of the RSD value for the low-level standards in rat plasma.

3.2. Linearity

The linearity study was carried out over 3 days with concentrations of (+)-calanolide A ranging from 12.5 to 800 ng/ml in rat, dog and human plasma. The correlation coefficients between the peak area ratio of (+)-calanolide A and internal standard (±)-12-oxocalanolide A with the (+)-calanolide A concentration in these matrices were 0.995, 0.999 and 0.999, respectively. The linearities resulting from the analysis of the unweighted regression plots can be expressed by these equations: $y=0.00314x+0.02575$ (in rat plasma), $y=0.00297x+0.03283$ (in dog plasma), and $y=0.00140x+0.01086$ (in human plasma).

3.3. Pharmacokinetics in rats, dogs and humans

Drug concentration versus time curves are presented in Fig. 2 and the mean plasma pharmacokinetic parameters listed in Table 4. As can be seen [a more detailed analysis of (+)-calanolide A plasma pharmacokinetics will be presented elsewhere] (+)-calanolide A in human exhibited a relatively long elimination half-life, with $t_{1/2}$ of 19.8 h; humans also exhibited the highest total drug exposure in terms of both $AUC_{0-\infty}$ and C_{max} after oral dosing. The order of dose-normalized $AUC_{0-\infty}$ and dose-normalized C_{max} among the species tested after oral administration of a single dose of (+)-calanolide A were as follows: human (19-fold)>dog (three-fold)>rat for $AUC_{0-\infty}$ and human (15-fold)>dog (three-fold)>rat for C_{max}.

3.4. No evidence of epimerization of (+)-calanolide A after oral administration in humans

It was found that, under acidic conditions, (+)-calanolide A may be epimerized at 12-OH, leading to the formation of (+)-calanolide B (**3**) which is devoid of anti-HIV activity. Since the pH in human stomach is <2.0, it is possible for such epimerization to occur and it would be crucial to determine if the HPLC method developed could distinguish (+)-calanolide A (**1**) and (+)-calanolide B (**3**). Thus,

Table 3
QC plasma sample freeze–thaw stability

	30 (ng/ml)	160 (ng/ml)	500 (ng/ml)
Rat plasma			
Mean±SD	31±8	150±1	518±9
RE (%)[a]	2.81	0.18	3.58
RSD (%)[b]	26.1	0.35	1.72
Dog plasma			
Mean±SD	35±4	159±3	514±27
RE (%)[a]	18.0	−0.61	2.74
RSD (%)[b]	10.7	2.04	5.18
Human plasma			
Mean±SD	30±4	164±4	507±26
RE (%)[a]	0.09	2.71	1.38
RSD (%)[b]	13.7	2.47	5.15

[a] RE: Relative error=[(mean−nominal)/nominal]×100.
[b] RSD=Relative standard deviation=(standard deviation/mean)×100.

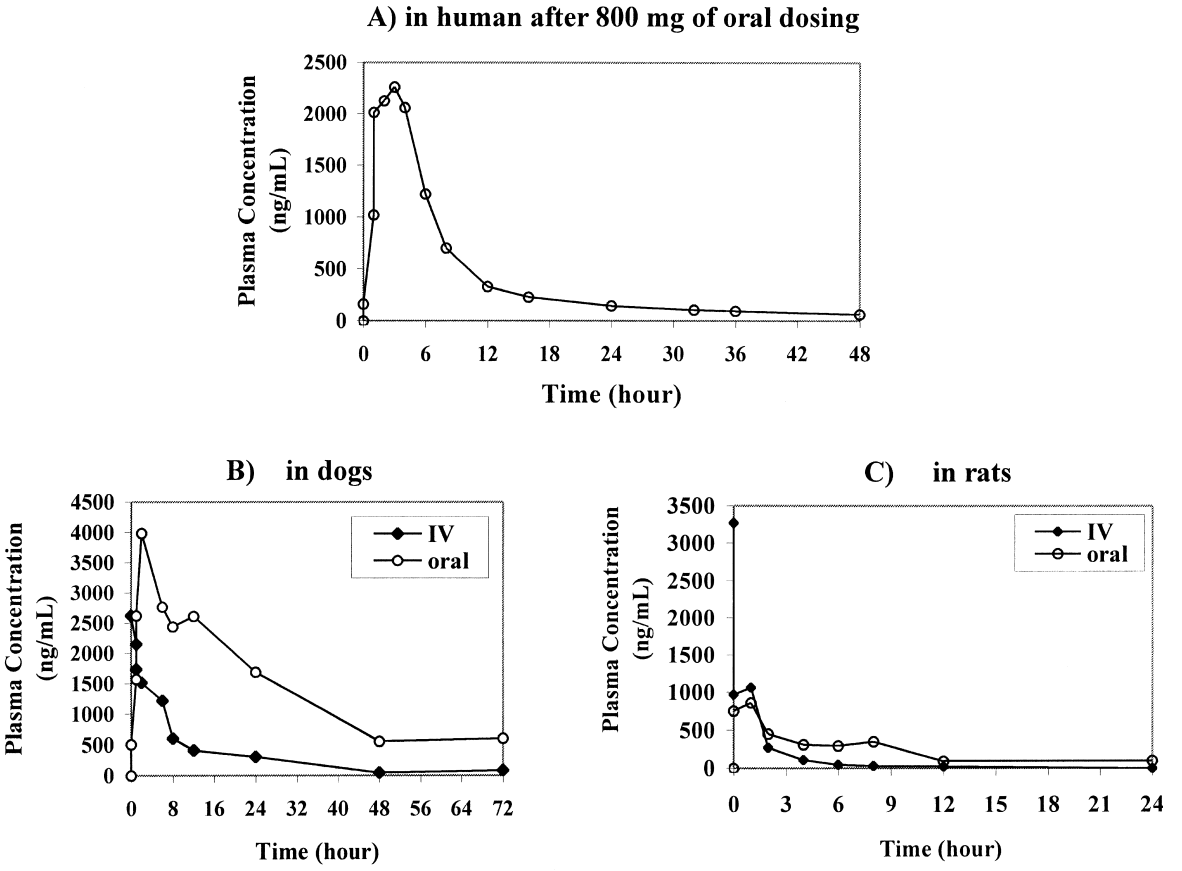


Fig. 2. Drug concentration versus time plot of (+)-calanolide A in plasma after administration to human, dog and rat, respectively.

Table 4
Mean plasma pharmacokinetic parameters of (+)-calanolide A

Species (route)	Dose (mg/kg)	$AUC_{0-\infty}$ (μg h/ml)	C_{max} (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	Cl (l/h/kg)	V_d (l/kg)	F (%)
Rat (i.v.)	15	3.42	NA	NA	3.8	4.24	23.36	NA
Rat (oral)	50	5.14	0.86	1.0	NE	7.72	NE	45
Dog (i.v.)	5	4.0	NA	NA	7.3	1.40	15.17	NA
Dog (oral)	31.6	8.42	1.89	2.1	2.5	4.96	13.84	34
Human (oral)	10.8[a]	20.59	2.81	2.4	19.8	NE	NE	NE

[a] Mean dose converted from a cohort of 12 subjects who received 800 mg (+)-calanolide A individually.
NA: Not applicable.
NE: Not estimated.

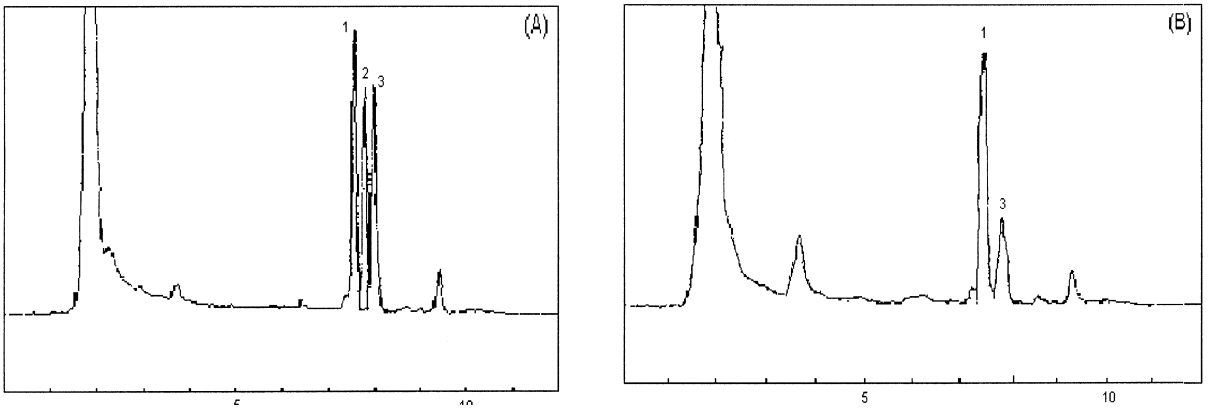


Fig. 3. HPLC chromatograms of (A) human plasma containing the internal and reference standards as well as (+)-calanolide B, (B) plasma sample obtained from clinical trials spiked with internal reference standard (subject 305, 4 h post-dose). Peak 1 was the internal standard (±)-12-oxocalanolide A, peak 2 was (+)-calanolide B, and peak 3 was reference standard (+)-calanolide A.

(+)-calanolide A (**1**) and (+)-calanolide B (**3**) were spiked with internal standard (±)-12-oxocalanolide A (**2**) in human plasma. As shown in Fig. 3A, the three compounds were distinguishable by the HPLC analysis, with retention times of 7.9, 7.7, and 7.5 min, respectively. There were no measurable amounts of (+)-calanolide B in plasma samples obtained from clinical trials (Fig. 3B), indicating no epimerization of (+)-calanolide A in the in vivo environment to its inactive epimer had occurred. Furthermore, LC–MS analysis confirmed that the peak which eluted at 7.9 min in plasma samples obtained from clinical trials possessed an ion mass of 371, identical with M+H of (+)-calanolide A.

In summary, this is the first report of a validated HPLC assay for determination of (+)-calanolide A concentrations in rat, dog and human plasma. The methods were utilized to determine (+)-calanolide A pharmacokinetics in rats and dogs, as well as in humans enrolled in clinical trials.

Acknowledgements

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