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Journal of Chromatography B, 862 (2008) 237–241
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous high-performance liquid chromatographic determination of *Cedrus deodara* active constituents and their pharmacokinetic profile in mice

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Received 28 August 2007; accepted 19 December 2007
Available online 28 December 2007

Abstract

A specific and sensitive high-performance liquid chromatographic (HPLC) method with photodiode-array (PDA) ultraviolet detection was developed for the simultaneous determination of three bioactive constituents of *Cedrus deodara* namely wikstromol, matairesinol and dibenzylbutyrolactol in mouse plasma. In solid-phase extraction (SPE) these constituents were successfully separated using a C_{18} column by isocratic elution using acetonitrile:water containing hexanesulphonic acid, 32:68 (v/v). The flow rate was set at 1 ml/min and detector wavelength at 225 nm. Good linearity ($r^2 > 0.999$) was observed over the studied range of 0.015–5.0 μg/ml for wikstromol and 0.030–5.0 μg/ml for matairesinol and dibenzylbutyrolactol. The CV values of intra-day precision for wikstromol, matairesinol and dibenzylbutyrolactol were in between 1.8–6.9, 1.7–4.9 and 1.6–4.2% and values of inter-day precision were in between 10.4–12.2, 9.7–11 and 10–11.2%, respectively. The extraction recoveries at low to high concentration were greater than 98, 83 and 87% for each analyte, respectively. The LOQ for wikstromol was 0.015 μg/ml and for both matairesinol and dibenzylbutyrolactol it was 0.030 μg/ml. The developed method was used to determine the pharmacokinetics of the three analytes in mice after intraperitoneal administration of CD-3.

Keywords: *Cedrus deodara*; Dibenzylbutyrolactol; Matairesinol; Pharmacokinetics; Wikstromol

1. Introduction

Cedrus deodara (Himalayan cedar, 'devadaru'; family: Pinaceae) is a large evergreen tree. Bark of this plant has been included in several traditional Ayurvedic polyherbal preparations for indications like inflammation, arthritic pain and dermal diseases [1]. Several botanical products from the *C. deodara* have shown diverse pharmacological activities: anti-spasmodic and anti-cancer against human epidermoid carcinoma of nasopharynx [2], spasmolytic [3], anti-fungal [4], anti-arthritic [5], anti-allergic [6,7], anti-inflammatory and analgesic [8,9], anti-oxidant [10], anti-filarial [11], and molluscicidal [12].

Chemical investigation of *C. deodara* has revealed the presence of sesquiterpenes and glycosides [13,14]. Recently, wood of *C. deodara* plant has revealed the presence of three lignans, namely wikstromol, matairesinol and dibenzylbutyrolactol (Fig. 1). A standardized herbal mixture derived from *C. deodara* (CD-3) comprising of 78% wikstromol, 11% matairesinol and 11% dibenzylbutyrolactol (w/w) was found to possess cytotoxic activity against several cancer cell lines and showed ability to induce tumor regression *in vivo* [15,16]. It was shown that CD-3 exhibited anti-cancer effect via modifying nitric oxide-mediated cellular signaling events in leukemia cells [17]. Earlier (−)-wikstromol has been reported from *Wikstroemia viridiflora* [18,19]. Matairesinol has also been shown to occur in various food sources [20]. Dibenzylbutyrolactol has been isolated from the wood of *Abies pinsapo* [21]. The anti-tumor and anti-oxidant activity of wikstromol and matairesinol is also reported [22,23]. In our ongoing programme for the development of herbal anti-

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doi:10.1016/j.jchromb.2007.12.015

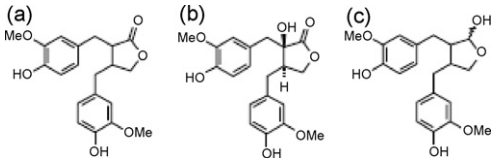

Fig. 1. Chemical structures of matairesinol (a), wikstromol (b) and dibenzylbutyrolactol (c).

cancer agents, CD-3 is being actively investigated for a possible *in vivo* activity in several animal models.

The present report deals with HPLC assay for the simultaneous determination of wikstromol, matairesinol and dibenzylbutyrolactol and their pharmacokinetic profile after intraperitoneal administration of CD-3 in mice.

2. Experimental

2.1. Chemicals

Samples (97% pure) of wikstromol, matairesinol and dibenzylbutyrolactol were received as a gift from Dr. J.M. Rao, Indian Institute of Chemical Technology, Hyderabad, India. HPLC grade water and acetonitrile were purchased from Rankem, Mumbai, India.

2.2. Animals

Swiss mice (20–25 g) were obtained from the Animal House of this Institute, and kept in regulated environmental conditions (temperature: $25 \pm 2\,^{\circ}C$, humidity: $60 \pm 5\%$, 12 h dark/light cycle). Animals were fed on standard pelleted diet (Ashirwad Industries, Chandigarh, India) and water was provided *ad libitum*. Animals were fasted overnight before the experiment. Animal experiments were approved by Institutional Ethics Committee.

2.3. Instrumentation

Chromatographic analysis was performed on Shimadzu HPLC system (LC10 ATVP) equipped with a diode array detector, solvent delivery module, online degasser and an autosampler using a reversed-phase HPLC column (RP-18, 25 cm × 4.6 mm, 5 μm particle size, Waters, USA). Data analysis was carried out using Class VP V6.12 SP2 software (Shimadzu, Japan).

2.4. Chromatographic conditions

Mobile phase consisted of acetonitrile:water containing 20 mM hexanesulphonic acid (32:68, v/v). It was filtered under vacuum through a 0.45 μm membrane filter before use. The flow rate was adjusted at 1 ml/min; detection wavelength at 225 nm and column oven temperature at 30 °C.

2.5. Preparation of standards

Stock solution (1 mg/ml) of each analyte was prepared individually in acetonitrile. Stock solutions were diluted with acetonitrile:water containing 20 mM hexanesulphonic acid, 32:68 (v/v) and stored at −80 °C (stable for >2 months; data not shown). For standard curves, blank mouse plasma samples (200 μl) were spiked to yield the following concentrations: 0.015, 0.03, 0.6, 1.25, 2.5, 5.0 and 10 μg/ml. The studies showed that all the three analytes were stable at room temperature (in fresh mouse plasma) for the entire duration of the experiment (Table 1). Quality control samples for determination of intra-day and inter-day variations, accuracy, precision, extraction recovery

Table 1
Stability data for wikstromol, matairesinol and dibenzylbutyrolactol

	Nominal concentration (μg/ml)	R.S.D. (%)		
		Wikstromol	Matairesinol	Dibenzylbutyrolactol
Storage stability (−80 °C)				
0 month	0.030	9.5	10.6	9.7
	0.5	5.1	6.7	5.9
	2	4.8	3.6	2.9
2 months	0.030	8.4	9.1	9.1
	0.5	7.2	5.4	5.9
	2	3.1	2.9	4.2
Freeze–thaw stability				
0 cycles	0.5	6.3	7.2	5.8
3 cycles	0.5	9.6	9.2	9.3
Autosampler stability				
0 h	0.5	5.1	4.7	7.1
24 h	0.5	6.9	4.9	4.8
Short-term stability in plasma at room temperature				
0 h	0.5	5.1	4.3	6.9
24 h	0.5	7.3	9.6	10.1

and stability were prepared in the same way as the calibration samples. Concentrations of each analyte (μg/ml) were calculated by relating to their respective calibration curves (peak area versus concentration) which were generated by weighted linear regression ($1/y^2$).

2.6. Collection of samples

CD-3 was administered as a suspension in 1% sodium carboxymethyl cellulose to the adult male Swiss mice intraperitoneally at a dose of 100 mg/kg [15]. Blood (750 μl from retro-orbital plexus using sterile glass capillary) was collected in heparinized tubes at the time interval of 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 24 h post-dosing (six mice/time point). The blood samples were centrifuged (1500 × *g*; 10 min; 15 °C) and plasma was stored in a set of pre-labeled glass tubes at −80 °C until analysis.

2.7. Extraction procedure

A semi-automated vacuum chamber and vacuum pump system (Supelco, USA) were used for the extraction of the analytes using following steps: (a) conditioning of the solid-phase extraction (SPE) column (C-18 cartridge, 3 ml capacity, 100 mg bed, Samprep-Ranbaxy, Mumbai, India) with 1 ml methanol followed by 1 ml HPLC grade water; (b) dispensing 2 ml of diluted plasma (1:10 dilution with HPLC grade water) into SPE column and drying under positive pressure; and (c) eluting the samples with 1 ml of mobile phase. The eluents were carefully collected in clean glass tubes and 50 μl of each sample was injected into HPLC system for analysis.

2.8. Pharmacokinetic analysis

Concentration–time curve (0–12 h) was established for each analyte and the pharmacokinetic parameters were determined by a non-compartmental analysis using TOPFIT software Version 1.1 (Gustav Fischer, Stuttgart).

3. Results and discussion

3.1. Method development and specificity

The HPLC method described in this paper evolved from the optimization of parameters for the sample preparation, chromatographic conditions and detection of wikstromol, matairesinol and dibenzylbutyrolactol. The UV spectrum of the three analytes showed distinct maxima at 225 nm.

To establish the chromatographic separation, various mobile phases and analytical columns were tested under reversed-phase conditions and validated with regard to peak characteristics, achievable LOQ and interferences from endogenous substances. Optimum results were obtained with the Waters RP-18 column in combination with a mobile phase consisting of acetonitrile:water containing 20 mM hexanesulphonic acid (32:68, v/v).

Different techniques like liquid–liquid extraction, plasma protein precipitation and solid-phase extraction were used to recover the analytes from the plasma. The liquid–liquid extraction and plasma protein precipitation techniques were found to be irreproducible and resulted in unquantifiable chromatograms. The solid-phase extraction method was found to be highly reproducible with high percent recovery of the analytes.

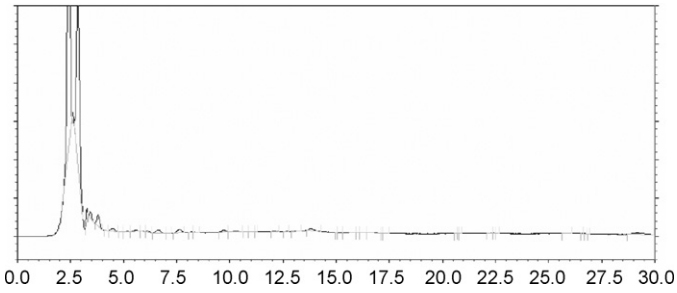

Fig. 2. Chromatogram of extracted unspiked mouse plasma.

The specificity of the method was demonstrated by comparing the chromatogram of analyte-free plasma (Fig. 2) and plasma spiked with the three analytes (Fig. 3). A comparison of the chromatograms did not show any interference at the retention time of analytes as determined from six plasma samples collected from as many mice. The retention time of wikstromol, matairesinol and dibenzylbutyrolactol was 10.325, 15.500 and 6.133 min, respectively.

3.2. Recovery

The average peak area obtained after spiking the plasma samples with 0.15 and 2 μg/ml of wikstromol, 0.5 and 2 μg/ml each of matairesinol and dibenzylbutyrolactol were compared with standards containing equivalent concentrations of each analyte. The extent of recovery for wikstromol, matairesinol and dibenzylbutyrolactol was 98–102, 83–89.2 and 87–93%, respectively (analyzed from eight samples collected from as many mice).

3.3. Linearity

Calibration curves were linear over the low and high concentration range of 0.015–5.0 μg/ml for wikstromol ($r^2 > 0.9998$, slope 0.0109, *y*-intercept −5.6624); 0.030–5.0 μg/ml each for matairesinol ($r^2 > 0.9999$, slope 0.0012, *y*-intercept 0.7701) and dibenzylbutyrolactol ($r^2 > 0.9998$, slope 0.0059, *y*-intercept 1.6890).

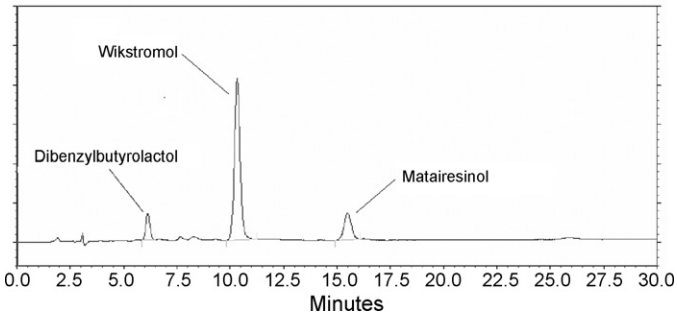

Fig. 3. Chromatogram of plasma sample spiked with 0.7 μg/ml of wikstromol, 0.15 μg/ml each of matairesinol and dibenzylbutyrolactol.

Table 2
Intra-day and inter-day precision and accuracy for the determination of three analytes in mouse plasma

Compound	Nominal concentration (μg/ml)	Precision CV (%)		Accuracy (diff.%)	
		Intra-day ($n=8$)	Inter-day ($n=8$)	Intra-day ($n=8$)	Inter-day ($n=8$)
Wikstromol	0.015	6.9	–	9.8	3.2
	0.5	2.9	10.4	−4.3	−2.9
	2	1.8	12.2	−2.6	–
Matairesinol	0.030	4.9	–	−9.2	−10.1
	0.5	3.1	9.7	−2.1	−6.4
	2	1.7	11	1.2	–
Dibenzylbutyrolactol	0.030	4.2	–	−8.1	2.1
	0.5	2.7	10	−3.9	2.6
	2	1.6	11.2	0.13	–

3.4. Precision

Intra-day variation was assessed by using eight replicates of spiked plasma: each at three concentrations: 0.015, 0.5 and 2 μg/ml for wikstromol and 0.030, 0.5 and 2 μg/ml each for matairesinol and dibenzylbutyrolactol. Coefficients of variation (CVs) were 1.8–6.9, 1.7–4.9 and 1.6–4.2% for wikstromol, matairesinol and dibenzylbutyrolactol, respectively. Inter-day precision was assessed by spiking the plasma samples each with two concentrations: 0.5 and 2 μg/ml, for all analytes over 30 days (Day 1, 2, 3, 15 and 30) under same experimental condition. CVs were 10.4–12.2, 9.7–11 and 10–11.2% for wikstromol, matairesinol and dibenzylbutyrolactol, respectively (Table 2).

3.5. Accuracy

The accuracy of the intra-day analysis was evaluated by using eight replicates of spiked plasma each at three concentrations: 0.015, 0.5, 2 μg/ml for wikstromol and 0.030, 0.50, 2.0 μg/ml for matairesinol and dibenzylbutyrolactol. The differences (diff.%) between mean measured and nominal concentrations were calculated as follows: [(mean concentration × nominal concentration)/nominal concentration] × 100. The intra-day diff.% for wikstromol was between −2.6 and 9.8%, for matairesinol −9.2 and 1.2% and for dibenzylbutyrolactol −8.1 and 0.13%. Inter-day accuracy was assessed by assaying plasma samples spiked at two different concentrations (0.5 and 2 μg/ml) for wikstromol, matairesinol and dibenzylbutyrolactol over 1 month (Day 1, 2, 3, 15 and 30) under the same experimental conditions. The inter-day diff.% for wikstromol was between −2.9 and 3.2%, for matairesinol −10.1 and −6.4%, and for dibenzylbutyrolactol 2.1 and 2.6% (Table 2).

3.6. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ (defined as the lowest concentration of a calibration curve which could be analyzed with acceptable accuracy and precision) was 0.015 μg/ml for wikstromol (CV: 6.9%); 0.030 μg/ml each for matairesinol (CV: 4.2%) and dibenzylbutyrolactol (CV: 4.9%). The LOD of the method was 0.005 μg/ml for the wikstromol and 0.010 μg/ml each for matairesinol and dibenzylbutyrolactol.

3.7. Stability

The stability of wikstromol, matairesinol and dibenzylbutyrolactol in plasma was demonstrated after 24 h storage at room temperature, after three freeze–thaw cycles and after 1 month frozen storage at −80 °C. The stability of the processed samples in the autosampler at room temperature was confirmed after 24 h storage. These data are summarized in Table 1.

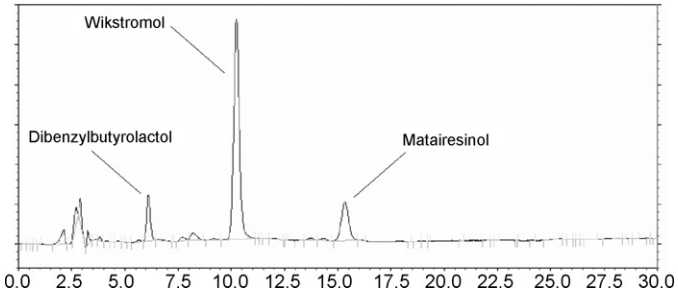

Fig. 4. Chromatogram of the three analytes extracted from plasma 2 h post-dosing of CD-3 (100 mg/kg, i.p.).

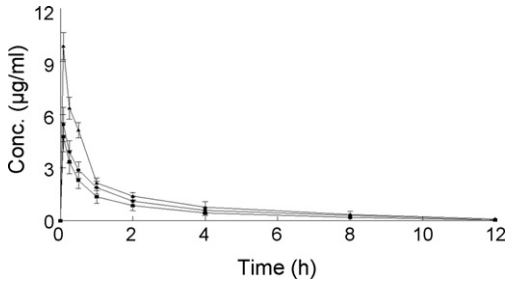

Fig. 5. Plasma concentration vs. time curve (mice) following i.p. administration of CD-3 (100 mg/kg); wikstromol (▲), matairesinol (●), and dibenzylbutyrolactol (■). Each data point is mean ± S.E. ($n=6$).

Table 3
Pharmacokinetic parameters

Parameter	Wikstromol	Matairesinol	Dibenzylbutyrolactol
C_{max} (μg/ml)	9.87	5.43	4.73
$t_{1/2}$ (h)	2.02	1.98	1.89
$AUC_{0–12}$ (μg/ml h)	12.37	8.76	6.67
Cl (ml/min)	135	190	250
V_d (l)	23.5	32.6	40.8

Values are derived from Fig. 5.

3.8. Pharmacokinetic studies

Fig. 4 represents the typical chromatographic profile of CD-3 in plasma of mice after intraperitoneal (i.p.) administration (2 h post-dosing). The rate and extent to which the active constituents reach the systemic circulation (bioavailability) was determined by pharmacokinetic analysis. Fig. 5 shows the concentration–time curves for the three analytes which were detectable only up to 12 h of sampling time. The data were fitted to a non-compartmental model using TOPFIT software to determine various pharmacokinetic constants such as peak plasma concentration (C_{max}), extent of absorption (AUC), half-life ($t_{1/2}$), clearance (Cl), and volume of distribution (V_d) (Table 3). The results showed that the bioavailability indices (C_{max} and AUC) were highest for wikstromol followed by matairesinol and dibenzylbutyrolactol: C_{max} and $AUC_{0–12}$ for wikstromol were 9.87 μg/ml and 12.37 μg h/ml, respectively; for matairesinol 5.43 μg/ml and 8.76 μg h/ml and for dibenzylbutyrolactol 4.73 μg/ml and 6.67 μg h/ml, respectively. The Cl of dibenzylbutyrolactol was rapid compared to the other two analytes. However V_d was low for wikstromol compared to the other two analytes.

4. Conclusion

The present results demonstrated a specific, accurate and precise HPLC assay for simultaneous determination of wikstromol, matairesinol and dibenzylbutyrolactol in a composition (CD-3) from *C. deodara* which would further help in ongoing pharmacological studies for the development of CD-3 as new anti-cancer herbal lead.

Acknowledgement

The authors are grateful to Dr. G.N. Qazi, Director of this institute for providing constant guidance and critical advice.

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