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High-performance liquid chromatographic assay for the determination of Aloe Emodin in mouse plasma

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Abstract

An isocratic high-performance liquid chromatography (HPLC) method was developed and validated to determine Aloe Emodin (AE) in mouse plasma. The analysis required 0.3 ml of plasma and involves extraction with dichloromethane. The HPLC separation was carried out on Symmetry Shield RP18, a mobile phase of methanol–water–acetic acid (65:35:0.2) and fluorescence detection at $\lambda_{ex} = 410$ nm and $\lambda_{em} = 510$ nm. The retention time of AE was 11.7 min. The assay was linear from 10 to 1000 ng/ml ($r^2 \geq 0.999$), showed intra- and inter-day precision within 7.8 and 4.7%, and accuracy of 87.3–105.7%. Detection limit (LOD) and quantification limit (LOQ) were 4.5 and 5 ng/ml, respectively. The method was applied to determine for the first time the pharmacokinetic of AE in mice.

Keyword: Aloe Emodin

1. Introduction

Aloe Emodin (AE) is an hydroxy-anthraquinone (Fig. 1) naturally present in the leaves of some species of *Aloe* that has shown antitumor and antiproliferative activity in neuroectodermic tumors in vitro and in vivo [1–3]. The growth of this tumor transplanted in mice with severe combined immunodeficiency SCID was highly inhibited by AE administered as a suspension in DMSO and saline solution (1:25). The findings that AE possesses a selective activity against neuroectodermic tumors and particularly against neuroblastoma, that is a tumor exhibiting low susceptibility to the available drugs is of potential clinical interest. For this reason it is important to perform preclinical pharmacokinetic studies of AE that can optimize the dosage-schedule to be investigated. In addition it would be of interest to evaluate whether the selectivity of action is related to an enhanced distribution of AE in neuroectodermic tumors.

The need of studies on the distribution of AE prompted us to developing an analytical assay for the

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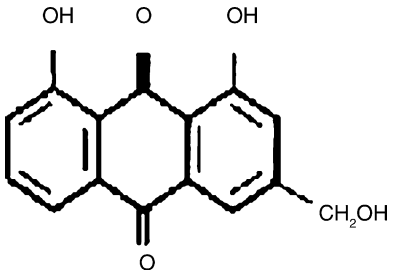

Fig. 1. Chemical structure of Aloe Emodin.

determination of this agent in biological fluids and tissues.

As far as we know, only method on the isolation, identification and purification of AE from natural extract were published in literature [4,5].

This paper reports a novel method to extract and analyze AE by high-performance liquid chromatography (HPLC) that has been applied to investigate the pharmacokinetic proprieties of this compound in mice.

2. Experimental

2.1. Chemicals

- Aloe Emodin (95% purity) was obtained from Sigma–Aldrich, St. Louis, MO, USA (lot #119H1333).
- Control mice plasma was obtained from IFFA, Credo, France.
- Methanol of HPLC grade was obtained from J.T. Baker B.V., Deventer, Holland.
- Acetic acid glacial for analysis was obtained from Carlo Erba, Milan, Italy.
- Dichloromethane for analysis was obtained from Merck, Darmstadt, Germany.
- Water of HPLC grade was obtained from Milli Ro 60 Water System, Millipore, Milford, MA, USA.
- PEG300 was obtained from Fluka Chemie GmbH, Buchs, CH.

2.2. Instrumentation and materials

The HPLC equipment comprised a model 717 WISP autosampler, a model 510 pump, a fluorescence detector model 474 and an acquisition system Millenium 32 Software Chromatography Manager were obtained from Waters Associates (Milford, MA, USA).

Symmetry Shield RP18 (3.5 μm) 4.6 mm × 150 mm HPLC column, guard column of the same material (5 μm × 4.6 mm × 20 mm), vials and limited volume insert for WISP 717 autosampler were obtained from Waters.

The HPLC filter, 0.4 μm Nucleopore PC membrane filter, was obtained from Nucleopore Italia (Milan, Italy).

Disposable borosilicate glass tubes, 16 mm × 100 mm, were from Corning (New York, USA).

2.3. Animals

The experiment was performed with male Swiss mice (body weight 25 ± 2 g), 3 months old, that were born, housed and handled in the animal facility according to the institutional guidelines.

2.4. Preparation of standard solutions

AE stock solution was prepared weighting 5 mg of AE on a microbalance and transfer it to a 50 ml volumetric flask and dissolve in an appropriate amount of methanol to yield a 100 μg/ml solution. The stock solution was further diluted with methanol to obtain working standard solutions (wAE) at concentrations of 0.1, 0.5, 1, 5 and 10 μg/ml.

2.5. Preparation of plasma standards

Every run analysis, seven plasma standards at different AE concentrations in the range 10–1000 ng/ml were prepared combining 270 or 285 μl of control mouse plasma with different amount of AE working solutions according to the following scheme:

Standard (ng/ml)	Preparation
10	30 μl wAE 0.1 μg/ml + 270 μl plasma
25	15 μl wAE 0.5 μg/ml + 285 μl plasma + 15 μl methanol
50	30 μl wAE 0.5 μg/ml + 270 μl plasma
100	30 μl wAE 1.0 μg/ml + 270 μl plasma
250	15 μl wAE 5.0 μg/ml + 285 μl plasma + 15 μl methanol
500	30 μl wAE 5.0 μg/ml + 270 μl plasma
1000	30 μl wAE 10.0 μg/ml + 270 μl plasma

Each point was prepared in duplicate.

2.6. Preparation of quality control samples

Plasma was divided into three fractions of 6 ml (A, B and C) to prepare quality controls (QC) samples for AE. A and B were added with 180 μl of wAE of 1 and 10 μg/ml and C with 75 μl of the working solution at 100 ng/ml in methanol to obtain a final plasma concentration of 30, 300 and 750 ng/ml, respectively.

Several aliquots of 300 μl of the three fractions were stored at −20 °C, as a control for future assay and to check the stability under storage conditions.

2.7. Extraction procedure

Plasma samples (0.3 ml) were diluted 1:1 (v/v) with water and extracted with 5 ml of dichloromethane. After 30 min of shaking samples were centrifuged at 4000 rev./min for 10 min at 4 °C. 4.5 ml of the organic phase were accurately measured, separated into borosilicate tubes and dried under nitrogen. The residue was dissolved in 200 μl of methanol, vortexed for 1 min and centrifuged at 14,000 rev./min for 10 min. A volume of 50 μl of the supernatant was injected onto the HPLC column for quantitative analysis.

2.8. Chromatographic conditions

HPLC analyses were carried out using a Symmetry Shield RP18 (3.5 μm × 4.6 mm × 150 mm) column equipped with a guard column of the same material and a mobile phase of methanol–water–acetic acid glacial (65:35:0.2) previously filtered through 0.45 μm filters and degassed. The flow rate was 1.2 ml/min and peak was detected with fluorimetric detector at $\lambda_{ex} = 410$ nm and $\lambda_{em} = 510$ nm.

At the end of the daily analyses the HPLC column was washed with acetonitrile–water (1:1) for 30 min at the flow rate of 0.5 ml/min.

2.9. Validation study

Precision and accuracy were evaluated by determining AE in three replicate of three QC samples at the nominal concentration of 30, 300 and 750 ng/ml (prepared as shown in Section 2.6) on three different days. To quantify the QCs, three different standard calibration curves (see Section 2.5) of seven plasma concentrations (10, 25, 50, 100, 250, 500, 1000 ng/ml) of AE were prepared in duplicate and processed as described in Sections 2.7 and 2.8.

Chromatograms were evaluated for the peaks area of AE.

To check the linearity of the standard curves, the peak area (expressed as detector response in millivolts) for AE was plotted versus nominal concentrations. The linearity of the standard curves was determined by the correlation coefficient (r^2) and by comparison of the nominal and back-calculated concentrations of the calibration standards.

The precision of the method at each concentration was expressed as a coefficient of variation (R.S.D.%) by expressing the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the measure was determined by expressing the mean calculated concentration as percentage of the nominal concentration.

The percentage extraction recovery of AE was determined at three different plasma concentrations (10, 50 and 100 ng/ml) in triplicate. Peak area of the analyte of chromatograms obtained from extracted plasma samples was compared to that of the external standards prepared in methanol.

The detection limit (LOD) was defined as the concentration at which the signal-to-noise ratio was 3. The quantification limit (LOQ) was defined as the lowest amount of the analyte which can be determined in a sample with a precision expressed as intra-day R.S.D. $< 20\%$ and an accuracy of $100 \pm 20\%$. To prepare samples at LOQ, an aliquot of 1.900 ml of control plasma were combined with 100 μl of 100 ng/ml working solution of AE to produce a nominal plasma concentration of 5 ng/ml of the analyte. Five replicates of the obtained LOQ samples were processed and analyzed by HPLC according to the previously described procedure together with a freshly prepared standard curve with a blank plasma control in duplicate.

The stability of plasma samples under storage conditions was also checked for analyzing five replicates of QCs, prepared on day 1 of the validation study, after 1 week or 3 months of storage at −20 °C and after three repeated thaw/freeze cycles.

2.10. Application of the method

Mice were treated i.p. with the dose of 20 mg/kg of AE dissolved in the vehicle PEG300. Animals

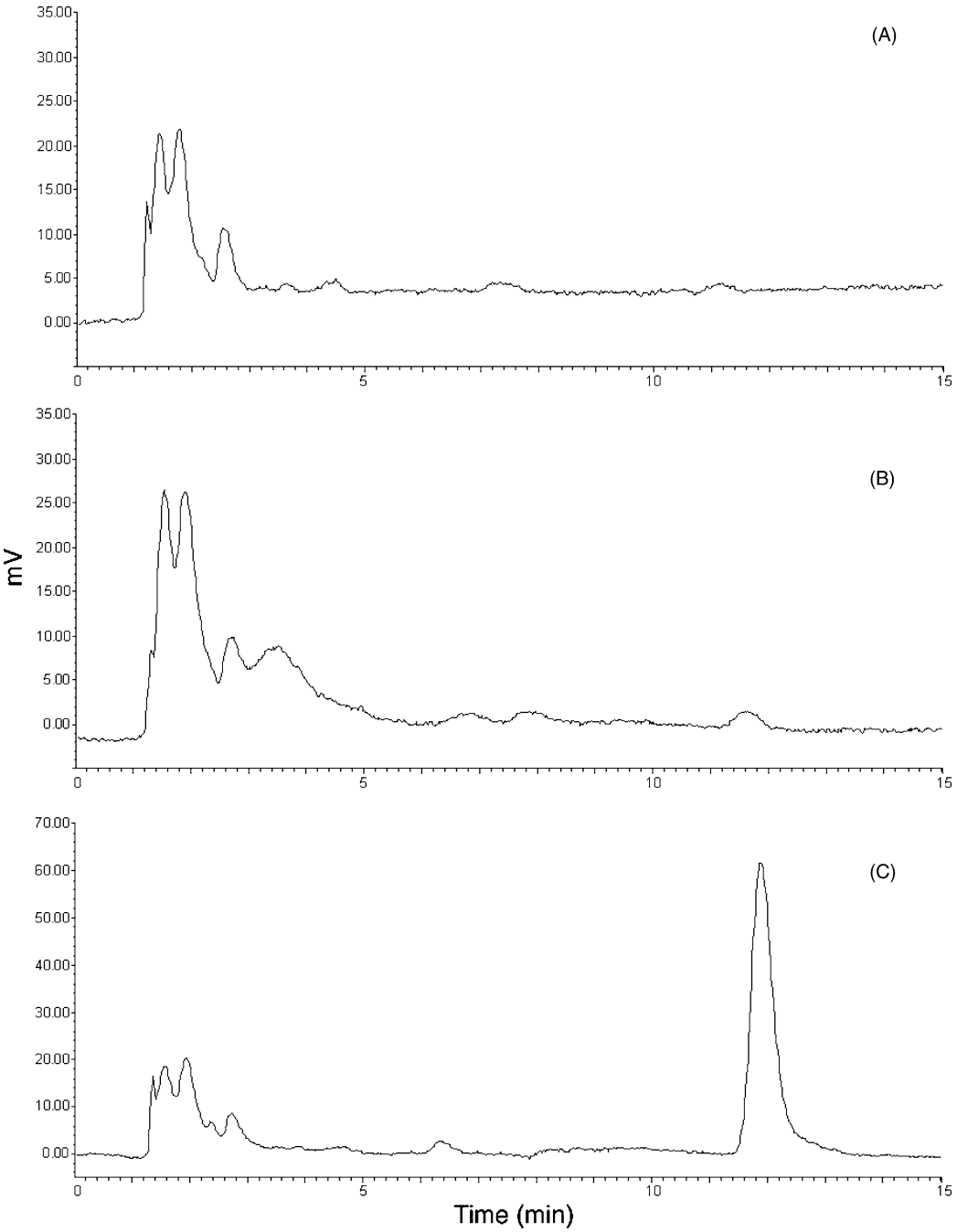

Fig. 2. Chromatograms of mouse blank plasma sample (A); a sample with 5 ng/ml of AE (LOQ) (B) and a sample taken 1 h after the i.p. administration of AE (calculated concentration, ng/ml) (C).

were anesthetized (xilazine 20 mg/kg and ketamine 25 mg/kg) and blood samples were collected in heparinized tubes at selected times of 5, 15 and 30 min, 1, 2 and 4 h after the AE administration. Four animals were used for time points at 5, 15 min and 2 and 3 h at times 30 min, 1 and 4 h. Animals were sacrificed by cervical dislocation.

The plasma fraction was immediately separated by centrifugation (10 min, 2000 × *g*, 4 °C) and stored at −20 °C until analysis of AE.

The experimental area under the curve of the plasma concentration versus time points (AUC) of AE was calculated by the linear trapezoidal rule. The constant of elimination (K_e) of the drug was obtained by the fitting of the concentration time points and the half-life with the following formula: $t_{1/2} = 0.693/K_e$.

3. Results and discussion

3.1. Chromatography

Fig. 2A shows a typical chromatogram of extracted control mouse plasma only treated with the vehicle PEG300. No endogenous interfering substances of plasma were present at the retention time of AE.

Fig. 2B shows a chromatogram of a plasma sample containing AE at the concentration of 5 ng/ml (i.e. LOQ). Significant differences in the peak area of AE were obtained between the responses for the blank and the LOQ sample in the five replicates injected.

Fig. 2C shows a chromatogram of plasma sample taken 1 h after the i.p. treatment with 20 mg/kg of AE and corresponds to an amount of 102.3 ng/ml of AE. The retention time of AE was 11.7 min and the chromatographic separation and resolution from plasma matrix appears very good.

3.2. Validation study

The plasma standard curves for AE in the concentration range of 10–1000 ng/ml determined on three different days were linear, with a r^2 always more than 0.999 and a mean slope of 1.01E+4 ± 1.85E+2 (Table 1).

Table 1 also reports the results of the calibration curves accuracy in the 3 days of the validation study. Mean accuracy values were always around 100%, and the percentage of the relative standard deviation (R.S.D.) was in the range of 0.4–3.1%.

The reproducibility of the method was evaluated analyzing three replicates of three QC samples containing AE at the nominal concentrations of 30, 300 and 750 ng/ml on three different days. The intra- and inter-day precision and accuracy are reported in Table 2. The method was found to be highly precise, with a R.S.D. = 7.8% and accuracy in the range of 87.3–105.7% for each of the concentrations tested.

The mean extraction recovery of AE from plasma performed at three representative concentrations of 10, 50 and 100 ng/ml were 99.6% (R.S.D. = 1.6%), 96.3% (5%) and 93.6% (5.9%), respectively.

The LOD was defined as the concentration at which the signal-to-noise ratio was 3. The mean of the noise recorded in the intervals between 10 and 13 min (comprising the retention times of the analyte) was

Table 1
Correlation coefficients (r^2) and comparison of nominal and back-calculated concentration standards for Aloe Emodin in mouse plasma

Day	10[a]	25	50	100	250	500	1000	r^2	Slope
Calibration curve accuracy (%)[b]									
1	104.1	107.5	103.6	109.7	107.5	97.0	100.2	0.999	1.02E+4
2	107.2	106.9	101.3	109.2	108.1	97.0	100.1	0.999	1.02E+4
3	104.1	102.9	96.6	104.8	107.5	100.7	99.3	0.999	9.88E+3
Mean	105.1	105.8	100.5	107.9	107.7	98.2	99.9		1.01E+4
S.D.	1.8	2.5	3.6	2.7	0.4	2.1	0.5		1.85E+2
R.S.D. (%)	1.7	2.3	3.5	2.5	0.3	2.2	0.5		1.8

[a] Nominal concentration of standards (ng/ml).
[b] Accuracy (%): (calculated concentration/nominal concentration) × 100%.

Table 2
Summary of intra- and inter-assay precision and accuracy data for Aloe Emodin in quality control samples

Day	*N*	Mean observed	R.S.D. (%)	Accuracy (%)[a]
1	3	28.4	7.78	94.8
	3	286.09	3.87	95.4
	3	654.68	4.15	87.3
2	3	29.06	4.11	96.9
	3	286.78	2.68	95.6
	3	655.23	3.88	87.4
3	3	31.72	2.12	105.7
	3	311.61	1.96	103.9
	3	703.38	5.65	93.8
Overall	3	29.74	4.67	99.14
	3	294.83	2.83	98.28
	3	671.10	4.56	89.48

[a] Accuracy (%): at nominal QC concentration: (calculated concentration/nominal concentration) × 100.

equivalent to an amount of 1.5 ng/ml of analyte, the resulting LOD is 4.5 ng/ml.

The LOQ was fixed at 5.0 ng/ml for AE; at this nominal concentration (very close to the LOD) the mean observed concentration was 4.7 ng/ml. The within-day R.S.D. and accuracy were 5.5 and 93.9%, respectively.

The drug appears stable in frozen mouse plasma; >85% of the original concentration of AE was found in the QC samples after 3 months at −20 °C. AE was found stable also after three repeated thaw/freeze cycles of plasma samples; the amount was >87.6% (R.S.D. = 9.3%) of the original concentration.

The feasibility of the present method was verified also in tissues of mice. The mean extraction recovery of AE assessed at the concentrations of 10 and 100 ng/ml was >80.1% (R.S.D. = 9.5%) in liver, and >78.1% (R.S.D. = 6.1%) in brain. Standard curves made in the range 25–1000 ng/ml were linear with $r^2 > 0.998$.

3.3. Application of the method

The HPLC method described was used to assess, for the first time, the pharmacokinetic of AE in mice. Fig. 3 shows the pharmacokinetic profile of AE formulated in PEG300 in plasma of Swiss mice after i.p. administration of 20 mg/kg of the drug.

AE achieved a Cmax of 654.6 ng/ml, was rapidly distributed and was eliminated from plasma with a terminal half-life of 78 min. The experimental $AUC_{0–4\,h}$ was 1474.9 ng/ml h and the drug was still detectable at 4 h at concentration of 22 ng/ml.

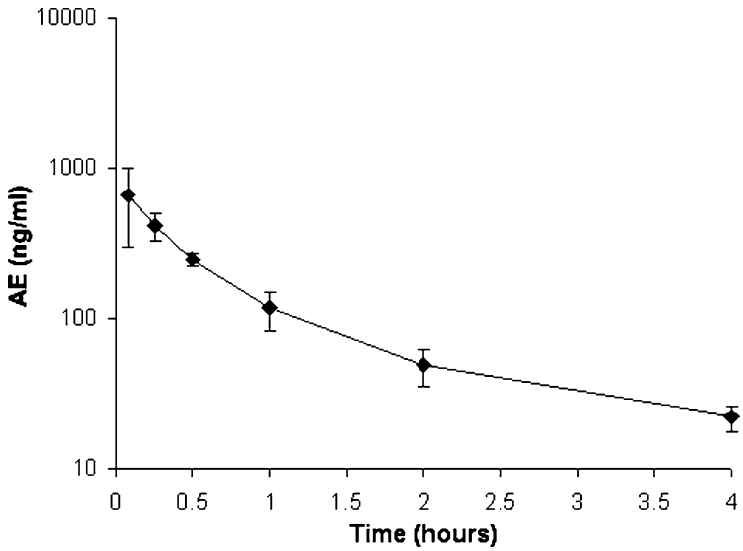

Fig. 3. Aloe Emodin plasma concentrations vs. time profile after an i.p. administration of 20 mg/kg of drug.

4. Conclusion

This report describes the procedure to measure AE in mouse plasma. The method shows great linearity and has a high degree of selectivity, sensitivity and good precision and accuracy.

It should be noted that the method is very rapid requiring simple dichloromethane extraction procedure and HPLC analyses of only 14 min run time.

The method is suitable for use in determining plasma and tissue levels in preclinical investigations and will prove useful for evaluating the pharmacokinetic properties of this antitumor agent.

References

[1] T. Pecere, M.V. Gazzola, C. Mucignat, C. Parolin, F. Dalla Vecchia, A. Cavaggioni, G. Basso, A. Diaspro, B. Salvato, M. Carli, G. Palù, Cancer Res. 60 (2000) 2800.
[2] H.Z. Lee, S.L. Hsu, M.C. Liu, C.H. Wu, Eur. J. Pharmacol. 431 (2001) 287.
[3] L. Wasserman, S. Avigad, E. Beery, J. Nordenberg, E. Fenig, Am. J. Dermatopathol. 24 (2002) 17.
[4] M. Rychener, W. Steiger, Pharmaceutica Acta Helvetiae 64 (1989) 8.
[5] Y. Li, H. Liu, X. Ji, J. Li, Electrophoresis 21 (2000) 3109.