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Short communication

High performance liquid chromatographic determination of thalidomide in patients affected by hepatocellular carcinoma

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Abstract

The present study developed a validate and precise reversed-phase high performance liquid chromatography (HPLC) method for the determination of thalidomide (T) in plasma, to quantify T in patients affected by hepatocellular carcinoma. Twelve male subjects aging from 62 to 82 years and weighting 66–88 kg, were orally administered with single dose of T (200 mg/BW). Two ml of stabilizer-solution (CH_3OH/CH_3CN, 1/1 (v/v) + CH_3COOH 2%) were added to 1 ml of human plasma and stoked to −80 °C until analyses. This moisture (1.38 μl) was added with 20 μl of CF_3COOH and 100 μl of phthalimide (IS) 1.75 μg/ml, vortexed and centrifuged. Surnatant (800 μl) was dried under vacuum at room temperature, added with 50 μl of appropriate solution and injected onto HPLC. T and IS were detected at UV wavelength of 220 nm with a run time of 10 min. Mobile phase was 10 mM pH 5.5 $NH_4^+CH_3COO^-/CH_3CN$, 75/25 (v/v) buffer at flow rate of 1.5 ml/min. Inter-day and intra-day variation coefficient was <10% with an error of accuracy <10%. The present detection method was able to quantify T to every withdrawal time period (LOD 0.05 μg/ml). The IS used in the present study had the same wavelength maximum absorption of T, differently from early UV detection methods reported in literature where phenacetin was used. Pharmacokinetic parameters belonging from the present study are not significantly different from those calculated in previously studies performed in human health subjects and patients affected by other pathology.

Keywords: Thalidomide; Phthalimide; HPLC; Pharmacokinetic; Hepatocellular carcinoma

1. Introduction

Thalidomide (T) was originally used as an antiemetic in pregnant women. In the 1960s it was withdrawn from the market because of its teratogenic effect [1]. In 1991 it was found out that T inhibits the Tumour Necrosis Factor-alpha (TNF-alfa) [2], activity important in the treatment of leprosy knotty rash, condition associated with extremely high haematic and dermatologic TNF-alfa levels. Such discovery took to the use of T in several illnesses associated with increased TNF-alfa production, among which various rheumatologic chronic diseases and the AIDS related Kaposi sarcoma. In 1994 Folkmann found out that T had also an anti-angiogenic activity, in particular inhibiting basic Fibroblast Growth Factor (bFGF) and VEGF [3]. This last discovery opened the doors to clinical trials using T in cancer treatment.

In 1999 it was reported that T is effective in the management of multiple myeloma [4]. Ever since it was evaluated in clinical trials in several types of solid tumours with different degrees of success.

Hepatocarcinoma is one of the most common cancers in the world and still represents today one of the main causes of death for cancer. In 80% of cases it hits a cirrhotic liver. Surgery represents the only effective therapy, but only 10–25% of the patients are eligible for resection [5], but the risk of recidivism remains extremely high (about 80% at 5 years) [6]. Among the inoperable patients some are eligible for local or locoregional treatments, as chemoembolization, radio frequency, alcolization treatment and hepatic intra-artery chemotherapy. However, most patients have advanced disease, which usually requires

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a systemic therapy. The hormonotherapy does not represent a "standard" treatment, since literature data are differing. The systemic chemotherapy produced objective answers lower than 20%, with any used agent, and mono-chemotherapy direction did not show superiority of the poly-chemotherapy. Above all, a statistically significant advantage in terms of survival never emerged from the mentioned therapies, so, a series of experimental therapies is at present being studied in the clinical trials context. In the last years T was sperimentally used in clinical practice, against hepatocarcinoma, at different dose regimens [7,8] but further studies will be necessary to evaluate the effectiveness of the drug. Therefore a rapid, sensible and simple method for the T detection in human plasma is necessary to avoid severe side effect. Previous methods report LC–MS [9–11], polarographic [12], phosphorimetry [13], spectrofluorimetric [14] and (high performance liquid chromatography) HPLC UV [15–20] plasma detection. The aim of the present study was to evaluate the pharmacokinetics of T following a single oral dose (200 mg/BW) in patients affected by advanced hepatocellular carcinoma using an improved HPLC UV method.

2. Experimental

2.1. Material and methods

Thalidomide (T) and phthalimide (P), Fig. 1, used as internal standard, were provided by Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Carlo Erba (MI, Italy). Trifluoroacetic acid (98%), chloridric acid (37%) and glacial acetic acid were acquired from (J.T. Baker, Phillipsburg, NJ, USA). Methanol and acetonitrile were of super-hplc grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore Corporation, MA, USA). All the other reagents and materials were of analytical grade and supplied from commercial source.

2.2. Apparatus and chromatographic conditions

A Thermo Finnigan HPLC system was used to quantify T in human plasma. The HPLC system consisted of a Thermo Finnigan SpectraSystem SN4000 system controller, coupled with P2000 pump, a SCM1000 degasser and a UV2000 UV detector at an operation wavelength of 220 nm. Data were monitored and analyzed using ChromQuest software (Thermo Finnigan, Waltham, MA, USA). Separation of compounds was carried out at ambient temperature on a BDS HYPERSIL column (250 mm × 4.6 mm i.d., 5 μm) from Thermo Finnigan, preceded by a C18 guard column. The mobile phase, delivered at a flow rate of 1.5 ml/min, consisting of acetonitrile–10 mM ammonium acetate buffer (pH 5.5) (25/75, v/v) was filtered through a 0.45 μm membrane and degassed before use.

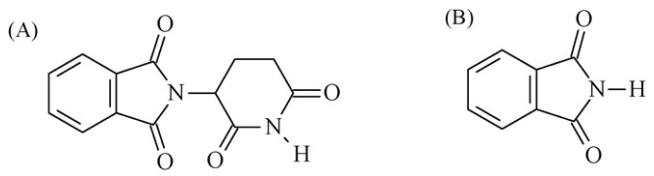

Fig. 1. Molecular structures of Thalidomide (A) and Phthalimide (B).

Stock solutions of T and P (1 mg/ml) were freshly prepared in acetonitrile–methanol (50:50, v/v). Working solutions (0.05–50 μg/ml) of T and P were prepared by diluting stock solutions with acetonitrile–methanol (50:50, v/v). All working solutions were freshly prepared. Analysis was performed by mixing 2 ml of stabilizer-solution (CH_3OH/CH_3CN, 1/1 (v/v) + CH_3COOH 2%) with 1 ml of human plasma and samples frozen to −80 °C. Two aliquots (1.38 ml) of each sample were added by 20 μl trifluoroacetic acid (deproteinizing agent) into a tube containing 100 μl IS, mixed by vortexing for 10 s and centrifuged at 3000 rpm for 5 min. Surnatant (800 μl) was dried under vacuum trap (SpeedVac, Savant, Ramsey, MN, USA), at room temperature. The residue was reconstituted with 50 μl of H_2O–HCl–CH_3CN–CH_3OH–CF_3COOH (60:10:5:5:20, v/v/v/v/v) and 20 μl injected onto HPLC. When plasma standards were prepared the same procedure was followed, previous adding 100 μl of T appropriate working solution. Typical standard samples contained T ranging from 0.05–50 μg/ml.

2.3. Calibration curves

The calibration curves over concentration range of 0.05–50 μg/ml were constructed by plotting the peak area ratio of the analyte over IS *vs.* the concentrations spiked. Five concentration points (0.05, 0.5, 5, 10 and 50 μg/ml) were used to obtain the linearity and independent calibration curve was constructed during each run of experiment. The equations were calculated using linear regression. The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Concentrations in unknown samples were obtained from the resulting peak area ratios and the regression equation of the calibration curve using back calculation. The limit of quantification was defined as the lowest drug concentration that could be determined with a coefficient of variation (C.V.) ≤20% and a recovery of 100 ± 20% on a day-to-day basis.

All validation runs were performed on 3 consecutive days and all samples used for validation were prepared as standard samples. Five different plasma concentrations (0.05, 0.5, 5, 10 and 50 μg/ml) of T were investigated for recovery, whereas P was measured at the concentration used in sample preparation. The recovery was determined by comparing the peak areas of plasma samples with those replaced by an equal volume acetonitrile–methanol (50:50, v/v) after the same sample handling. Intra-day and inter-day precision and the mean accuracy were determined by repeated analysis ($n = 5$) of T at five different concentrations on a single day and on 3 consecutive days, respectively.

2.4. Stability of T in plasma human samples and P in stock solutions

Torano et al. [16] reported that T stock solutions stored at room temperature and 3–8 °C was degraded of 5–14% within a one-week period, whereas plasma samples stoked at −20 °C after 6 days had a stability of 95%. In the present study samples were stocked at −80 °C and analyzed within 6 days.

P stock solutions were tested for stability for at least 30 days both at room temperature as well as at 3–8 °C.

2.5. Patients treatment

Twelve male subjects effecting to hepatocarcinoma aging from 62 to 82 years and weighting 66–88 kg, were orally administered with single dose of T (200 mg/BW). The haematic samples (5 ml) were withdrawn at the following times: 0 (basal), 0.5, 1, 2, 3, 4, 6, 8, and 24 h after dosing, blood was centrifuged to separate plasma, immediately added to the exact volume of stabilizer-solution and frozen to −80 °C until analysis.

2.6. Pharmacokinetic analysis

The blood concentration–time curve for each subject was evaluated. The area under curve (AUC), the maximum blood concentration (C_{max}), elimination half-life ($T_{1/2}$), time to achieve maximum blood concentration (T_{max}), absorption and elimination coefficients (K_a, K_{el}), renal clearance (Cl/F) and volume of distribution ($V_{d/F}$), were calculated. Semi-logarithmic plots of plasma T concentration *vs.* time were constructed for each patient. Pharmacokinetic analysis was performed for each data set, using a computer program (Easy Fit, Mario Negri Institute, Milano Italy). Akaike's information criterion [21] was used to select the best-fitting model (monoexponential *vs.* biexponential).

One-way analysis of variance (ANOVA) was applied to the principal pharmacokinetic parameters obtained from patients after oral administration. $P < 0.05$ was taken as being significant. The times of peak plasma concentration (T_{max}) from individual patients were analyzed using the Wilcoxon's non-parametric test.

3. Results and discussion

In hepatocellular carcinoma the evaluation of the dose-limiting toxicities, maximum tolerate dose and pharmacokinetic of T are important parameters for a effective therapy; they should be supported by a rapid, sensible and simple method for the T plasma detection. Representative chromatograms for T are shown in Fig. 2. Under the chromatographic conditions used for the analysis the retention times for IS and T were 4.0 and 5.0 min, respectively. This method employed simple liquid–liquid extraction partially modified [18]. Extraction efficiency (recovery) of the drug was >90% within 0.05–50 μg/ml and no variation concentration dependent was observed. The recovery of the IS, determined at the concentration used, was 92.3 ± 6.3% ($n = 3$). The validation data in terms of intra-day and inter-day precision

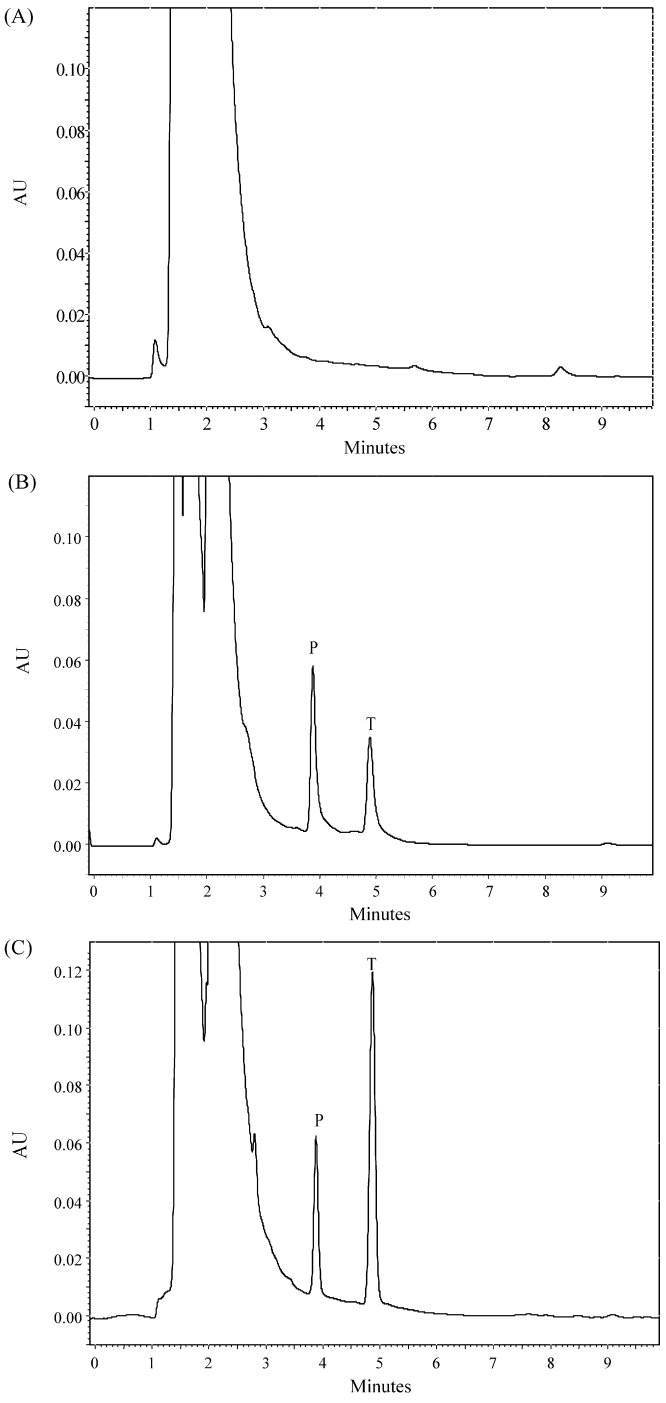

Fig. 2. Chromatographic courses of: (A) drug free plasma; (B) T spiked plasma (0,5 μg/ml); (C) patient plasma sample.

and accuracy are shown in Table 1. The differences between the theoretical and the actual concentration and the coefficient of variation were less than 10% at any quality control sample concentrations.

P stock solutions were found to have maximal stability for at least 27 days both at room temperature as well as at 3–8 °C. Appropriate handling of biological samples containing T is crucial to avoid degradation. The degradation rate of T in plasma blood samples at pH 7.4 is dependent on pH and temperature

Table 1
Intra-day ($n = 5$) and inter-day ($n = 3 \times 5$) precision and accuracy of the HPLC determination of T in human plasma

Concentration (μg/ml)	No. of samples (n)	C.V. (%)	Accuracy (%)
Intra-day			
0.05	5	8.9	89.7
0.5	5	5.7	92.3
5	5	4.3	96.5
10	5	6.8	91.4
50	5	3.5	97.1
Inter-day			
0.05	15	9.2	90.9
0.5	15	6.4	96.8
5	15	5.8	93.5
10	15	9.2	94.3
50	15	2.5	96.6

[18,20,22], therefore a stabilizing solution was added to plasma samples.

In the pharmacokinetic studies of T present in literature, chromatographic methods used phenacetin as IS [22–26]. This substance has a maximum absorption wavelength of 244 nm while T at 220 nm; this difference could cause a lack of sensibility. In the present study phenacetin was replaced by P having the same maximum absorption wavelength of T. Limit of quantization and detection resulted of 0.1 and 0.01 μg/ml, respectively and more performing respect to the other methods using UV single wavelength [15,16,27,28]. Although retention time of P was of 4.0 min it did not interfere with impurity of matrices (Fig. 2). The present method shown good limits of detection and quantization and was able to detect T in every withdrawal time period (0.5–24 h) though differences among patients were high (Fig. 3). These concentrations are in accordance with a previous pharmacokinetic study performed in patients with hepatocarcinoma [15]. Table 2 reports the main pharmacokinetic parameters calculated after drug administration. C_{max} reported in the present study is in accordance with pharmacokinetic studies of T administered to healthy patients [28] or affected to graft-versus-host disease [29], colon rectal cancer [30], HIV [31] and leprosy [32], but significant different from those conducted in patients with prostate cancer [24] and multiple myeloma [9]. On the other, probably due to the high standard deviation, no significant differences were shown among T_{max} reported in the present study

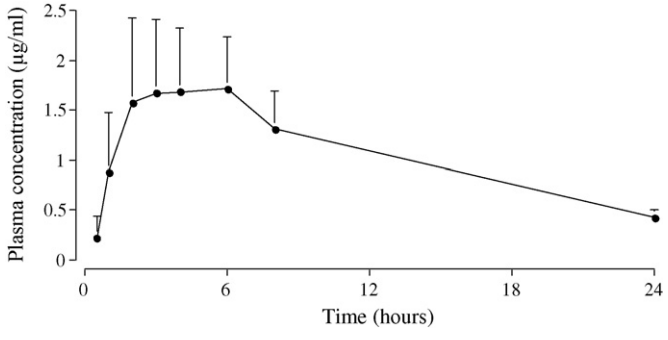

Fig. 3. Plasma concentration *vs.* time of *T* following singular oral dose administration (200 mg/BW).

Table 2
Pharmacokinetic parameters (means ± standard deviations) calculated after dosing (200 mg/BW) in patients affected by advanced hepatocellular carcinoma ($n = 12$)

Parameters	
r^2	0.936 ± 0.077
AUC (μg h/ml)	29.18 ± 6.60
C_{max} (μg/ml)	1.93 ± 0.63
T_{max} (1/h)	4.18 ± 2.5
K_a (1/h)	0.903 ± 0.722
K_{el} (1/h)	0.115 ± 0.070
$V_{d/F}$ (1/kg)	77315 ± 37953
Cl/F (1/(h kg))	7220 ± 2048
$T_{1/2}$ (h)	7.99 ± 4.58

and those reported in both healthy and disease affected patients [9,15,28–32,24]. Half-life estimated in the present study seems to be longer than those reported in previous studies [27]: it could be due to both the few withdrawal points in the elimination phase and the patients' variability. Other considerations about pharmacokinetic parameters could be too speculative due to their high variable values. Although T is not well tolerated and confers limited disease control in advanced hepatocellular carcinoma [7] its combination with other anti-neoplastic drug could increase efficacy in this pathology. Therefore the results reported in the present study, could be useful to assess the pharmacokinetic information for the design of future studies of T in cancer patients. This pharmacokinetic information is important to determine the contribution of the drug, the pharmacokinetic parameters to allow the establishment of dosing regimes to achieve targeted plasma concentrations which are clinically effective.

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