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Determination of tallimustine in human plasma by high-performance liquid chromatography

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Abstract

A sensitive and reproducible HPLC method for the determination of tallimustine (I) in human plasma has been developed and validated. Compound I was extracted from plasma by solid-phase extraction using a C_{18} cartridge from which the test compound was eluted with a methanol–formic acid mixture. The methanol solution was evaporated to dryness and the residue dissolved in a 0.2 *M* formic acid in methanol–water (1:1, v/v) mixture, then injected onto the HPLC column. The chromatographic separation was performed isocratically by a reversed-phase column filled with ODS, using a 50 m*M* KH_2PO_4–acetonitrile mixture as the mobile phase. The flow-rate was 1 ml/min. The eluate was monitored at 314 nm. No peak interfering with that of I was observed when blank human plasma was assayed. Linearity was established in the concentration range 0.5–85.5 nanograms of I per millilitre of plasma. Four calibration curves in plasma, prepared and run on four different days, showed correlation coefficients higher than 0.99 and good reproducibility of the slope (C.V.=4.5%). The intra-day precision, evaluated at three concentrations (in the low, mid and high range of the standard curve) and expressed as C.V. ranged from 0.9 to 14.4%. The inter-day precision evaluated at the same concentrations was better than 10.2%. The inter-day accuracy evaluated in the same samples and expressed as the ratio of found/added amount of I, ranged from 86.2 to 108.5%. The limit of quantitation was 0.5 ng/ml plasma. The HPLC method described here was successfully employed for the determination of I in some plasma samples obtained during a phase I clinical trial with the test compound.

Keywords: Tallimustine

1. Introduction

Tallimustine (I), 3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[N,N-bis(2-chloroethyl)amino] benzene-4-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (code name FCE 24517, I, Fig. 1), is a distamycin A derivative bearing a benzoyl mustard moiety instead of the formyl group at the N-terminal [1]. In in vitro studies I has been found to display potent cytotoxic activity on human and murine tumour cell lines and to maintain activity on melphalan-resistant L1210 cells [2]. In vivo, I was found to possess evident antineoplastic activity on a series of murine transplanted solid tumours and human tumour xenografts [2].

The benzoyl mustard present in I seems to be essential in imparting an antiproliferative capacity to the molecule. Compound I binds to the minor groove of DNA, forming a strong but reversible complex

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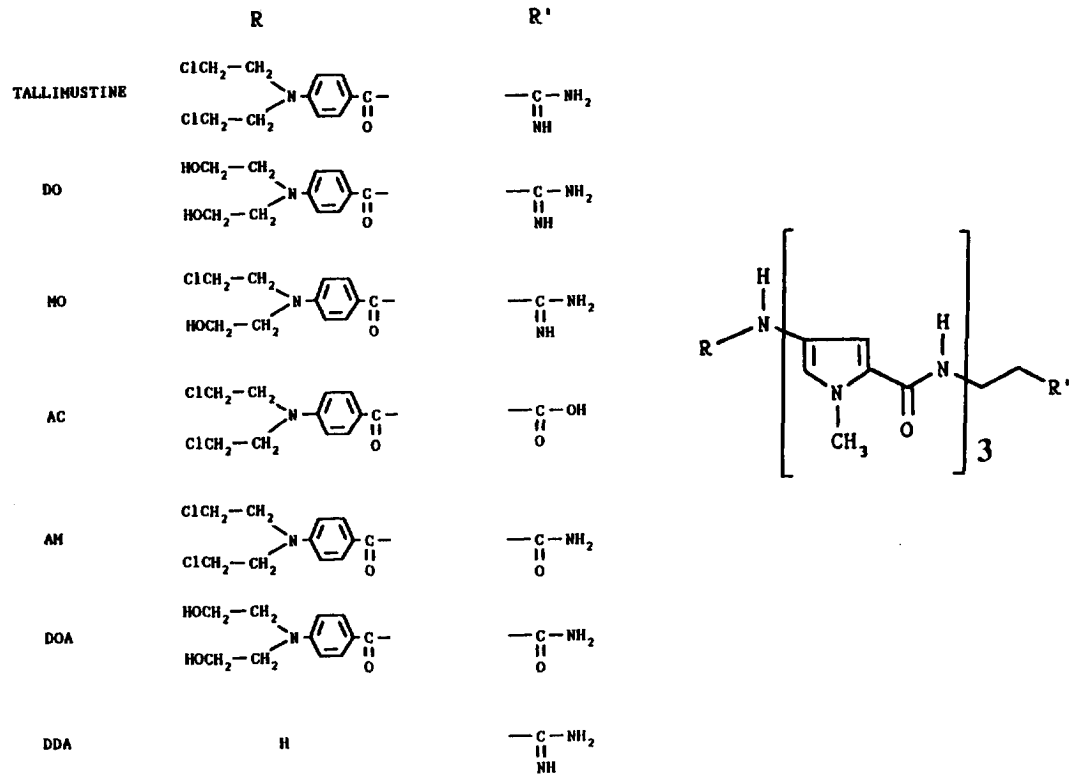

Fig. 1. Structural formulae of tallimustine and some of its derivatives.

with double helical B-DNA with high selectivity for adenine-thymine rich sequences. Alkylation of DNA by I would be weak, and in contrast to known nitrogen mustards, this compound does not induce alkylation at guanine N7 (the major site of alkylation for classical alkylating agents), but at selected adenines [3]. However the exact molecular mechanism(s) of I remains to be fully elucidated.

Because of its very promising preclinical antitumor activity I was selected for clinical development. Phase I studies are ongoing in patients with doses up to 800–1250 $\mu g/m^2$ [4]. Hence a sensitive analytical method was required to study the pharmacokinetics of this compound in humans. A radioimmunoassay for the determination of I in urine was previously reported [5]. This method, however, proved unsuccessful for plasma sample analysis because the specificity of the antiserum was not absolute and sample preparation involved several problems. Gas chromatography was inpracticable due to the poor volatility of I. High-performance liquid chromatography (HPLC) appeared to be an attractive alternative technique for analysis of I in plasma. Compound I has an UV spectrum characterized by two absorption maxima, the first at 314 nm and the second, whose intensity is about 60% that of the first, at 224 nm. Detection at 314 nm should therefore have provided higher sensitivity and better selectivity. Fluorimetric or electrochemical detection, often employed for selective and highly sensitive detection appeared inapplicable to this compound since I is practically non-fluorescent and only poorly electroactive. Several attempts to make the native molecule fluorescent or electroactive by reaction with different derivatizing reagents proved unsuccessful due to the poor reactivity of the compound. Our efforts were therefore directed toward the development of an analytical method based on HPLC with UV detection. The setting up and validation of this method are reported in the present paper.

2. Experimental

2.1. Chemicals

Compound I 95% pure (strength determined by HPLC), was used as the analytical standard. 3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[N,N-bis(2-chloroethyl)amino]benzene-4-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamide (AM), 3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[N,N-bis(2-hydroxyethyl) amino]benzene-4-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamide (DOA), 3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[N, N-bis(2-hydroxyethyl)amino]benzene-4-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine(DO),3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[(N-2-hydroxyethyl-N-2-chloroethyl)amino]benzene-4-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine (MO), 3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[N, N bis(2-chloroethyl)-amino]benzene-4-carboxamido]pyrrole-2-carbo-xamido]pyrrole-2-carboxamido]pyrrole-2-carbo-xamido] propionic acid (AC), and N-deformyl distamycin A dihydrochloride (DDA) were used as reference standards.

Tallimustine and its derivatives were supplied by Oncology-Immunology Preclinical Research, Department of Chemistry, B.A. Pharmaceuticals, Milan, Italy.

All reagents and solvents were of analytical or HPLC grade from Carlo Erba Reagents (Milan, Italy).

Extra Sep C_{18} cartridges (200 mg) were from Lida (Kenosha, WI, USA, code 3051-50).

2.2. Solutions

Phosphate buffer, 0.1 *M*, pH 7.4: Dissolve 1.36 g KH_2PO_4 in 90 ml H_2O, adjust to pH 7.4 with 0.1 *M* NaOH, then adjust the volume to 100 ml with water.

2.3. Chromatographic equipment

The HPLC system used in this study consisted of a pump (Model SP 8800, Thermo Separation Products, Fremont, CA, USA), an autosampler (Model 717 plus, Waters, Milford, MA, USA), a variable-wavelength UV detector (Model 975, Jasco, Tokyo, Japan) and a recorder-integrator (Model Chromjet, Thermo Separation Products) connected to a Labnet network (Thermo Separation Products). The detector was set at 314 nm and wired to send a 1 V signal to the integrator.

2.4. HPLC conditions

The chromatographic separation was performed by a 250 × 4 mm I.D. Hypersil BDS-C_{18} reversed-phase column (particle size 5 μm, Hewlett-Packard, Wilmington, DE, USA) with a LiChrospher 60 select B precolumn (4 × 4 mm I.D., particle size 5 μm).

The mobile phase consisted of a 50 m*M* KH_2PO_4–acetonitrile mixture (55:45, v/v). The mobile phase was prepared every day and degassed by He sparging. The flow-rate was 1 ml/min.

2.5. Preparation of standards

A stock solution of I was prepared by dissolving about 5 mg (exactly weighed) of the test compound in 20 ml dimethylsulfoxide (DMSO). From this stock solution five working solutions were prepared daily in DMSO at the final concentrations of about 0.5, 3, 10, 30 and 85 ng/10 μl. Aliquots of these working standards (10 μl) were spiked into blank human plasma (1 ml) and assayed to evaluate the linearity of the method. Another three working solutions in DMSO were prepared for the determination of precision and accuracy; aliquots (10 μl) of these solutions were added to 1 ml of blank human plasma to obtain tallimustine concentrations of 2.38, 10.45 and 54.62 ng/ml. Stock solution was stored at −20°C until use. Under these conditions, a stock solution of I is stable for at least one month.

2.6. Chromatographic performance

The suitability of the chromatographic system was checked by evaluating the column efficiency, the peak symmetry and the reproducibility of the response. In addition the resolution factor between the peak of I (retention time ca 5.3 min) and the merged peak of the two relative compounds (AM and AC),

having a retention time of about 5.9 min in the chromatogram was calculated.

This evaluation was carried out according to U.S.P. (XXII, 1990, 1566-1567) using the System Suitability Test software supplied by Thermo Separation Products.

2.7. Analytical procedure

Into a glass tube place 1 ml of the plasma sample and add 2 ml 0.1 *M* phosphate buffer, pH 7.4. Load the solution onto a 200 mg Extra Sep-C_{18} cartridge, pre-conditioned in sequence with 5 ml CH_3OH and 5 ml water, and aspirate the sample through the sorbent bed. Discard the exhausted plasma and wash the cartridge twice with 5 ml of water and twice with 0.5 ml of methanol, discard the washings and elute the test compound (slowly) with 0.5 ml of 0.2 *M* formic acid in methanol. Evaporate the methanol solution to dryness under N_2 at 37°C. Dissolve the residue in 220 μl of 0.2 *M* formic acid in a methanol–water (1:1, v/v) mixture. Centrifuge at 1200 *g* for 1 min and submit 200 μl of the final solution to the chromatographic analysis under the experimental conditions described above. Compound I elutes at about 5 min.

2.8. Calculations

The integrator determined the peak area of the analyte. Calibration curves were obtained by plotting the measured peak areas (counts) (y) vs. the concentration of the analyte spiked in plasma (ng/ml) (x). Weighted linear regression (weighting factor $1/y$) was used to calculate the concentration of I in quality control and unknown samples.

2.9. Evaluation of the extraction recovery

Blank human plasma (1 ml) was spiked with different amounts of the test compound (2.38, 10.45 and 54.62 ng) and assayed as described. Peak areas obtained were compared to the peak areas obtained by direct injection of unextracted standards.

3. Results and discussion

Representative chromatograms generated during the validation are presented in Fig. 2.

Under the chromatographic conditions adopted I gave a sharp peak with retention time of about 5.3 min. The suitability of the chromatographic system for the analysis of I was checked on different days by calculating the column efficiency, the peak symmetry and the system reproducibility. The column efficiency was expressed as the number of theoretical plates (N): this value must be higher than 6000. The tailing factor (used to evaluate the peak symmetry) must be less than 1.5. The reproducibility of the response, evaluated by repeated injections ($n=5$) of a standard solution containing ca. 10 ng of I, must give a C.V. of less than 5%.

Some possible metabolites were assayed under the chromatographic conditions adopted to check the selectivity of the separation. These drug-related compounds could be assigned roughly to two groups: the first group including the compounds poorly retained by the HPLC column (MO, DO, DDA, DOA, with a retention time of less than 3 min), and the second one including two compounds, AM and AC, having a retention time higher than that of FCE 24517 (5.9 vs. 5.3 min). As a whole, all the compounds assayed were well separated from the peak of I. A resolution factor higher than 1.5 was required between the peak of I and that of the acid and amide derivatives (giving a merged peak with a retention time of about 5.9 min, Fig. 2A) to ensure suitable determination of I.

Compound I, possessing an amidinic group, is a basic compound that should require high pH values (>9) for classical liquid–liquid extraction. Unfortunately, rapid degradation of I with formation of the corresponding amide has been shown to occur at pH 8 [6]. This instability discouraged the use of this extraction technique and prompted us to employ the solid-phase extraction technique for the selective extraction of the analyte from the complex plasma matrix before the chromatographic analysis.

The possible use of an internal standard has been explored during the development of the method. Efforts were directed towards the search for a possible candidate among the related substances

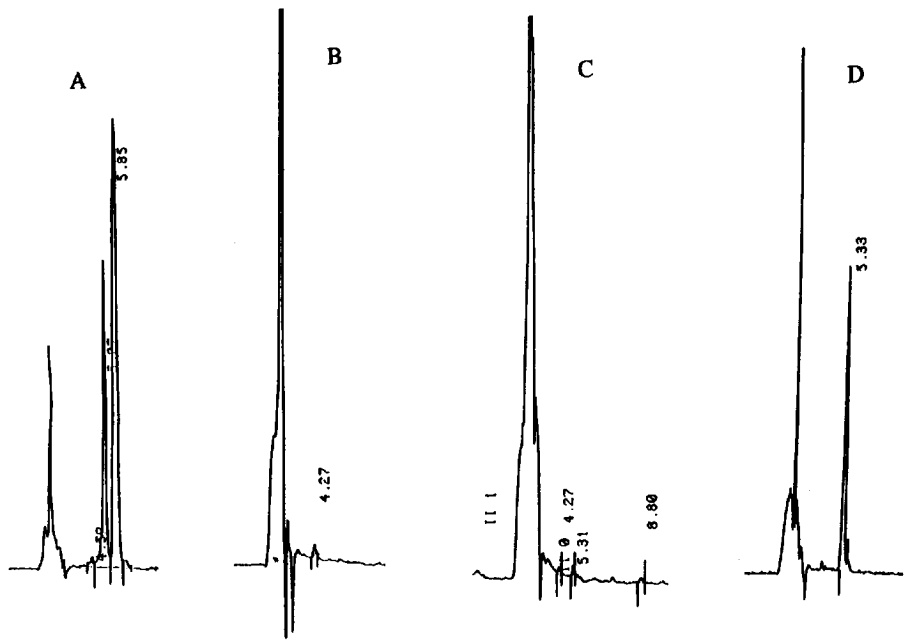

Fig. 2. Chromatograms obtained from (A) a standard solution of tallimustine (t_R = 5.27 min), AC (t_R = 5.85 min) and AM (t_R = 5.85 min); (B) from blank human plasma processed as described; (C) from 1 ml of blank human plasma spiked with 0.5 ng of tallimustine; (D) and from the plasma sample collected 15 min after dosing from a female patient with a solid tumour who had received a single i.v. dose of tallimustine (400 $\mu g/m^2$).

available. Unfortunately, the selective conditions needed for sample preparation before the chromatographic analysis made the search unsuccessful. The search for a compound to be used as internal standard was abandoned when it was established that the reproducibility of the method was satisfactory both in terms of chromatographic performance and extraction yield from the plasma matrix.

Strong adhesion of I to the glassware and to plastic containers (in the absence of plasma) was observed during the development of this method. This property of the test compound called for suitable handling procedures to ensure effective and reproducible determinations. The use of glassware pre-treated with dimethyldichlorosilane improved the recovery of I but was not sufficient to achieve complete recovery in all cases. In addition, pre-treatment of glassware with this passivating agent was time-consuming and dangerous since it is an extremely toxic compound. Dissolution of I in DMSO proved effective in preventing its absorption by the wall of the glass flasks used. A different approach was employed when the compound, after elution from the cartridge and evaporation to dryness of the eluting mixture, was to be redissolved for HPLC analysis. In this case a strongly acidic solution (used for the dissolution of the residue) proved effective in preventing absorption by the glass wall of the tube and by the wall of the microvials employed for sample injection. In addition, because of the better stability of I in acid solution [5] the final solution of the extracted analyte could be kept in the autosampler for longer (at least up to 16 h) at room temperature, permitting analysis of several samples within a working day. This, coupled with the short analysis time, improved the overall productivity of the assay.

Blank plasma samples collected from humans and from several different animal species and assayed as described showed no peak interfering with that of the test compound in the chromatogram.

Four calibration curves that were prepared and run

on different days with I in the concentration range 0.5–85.5 ng/ml plasma showed correlation coefficient (r) for the regression ranging from 0.9967 to 0.9995. The mean calibration curve obtained was described by the equation $y=7069x - 1414$ (slope C.V.=4.5%, $n=4$), where y is the peak area (counts) and x is the amount of I (ng) added to 1 ml plasma. The back-calculated standard values exhibited a C.V. of less than 13.2%.

The intra-day precision (expressed as C.V.) evaluated at three concentrations on four different days ranged from 0.9 to 14.4%. The inter-day precision evaluated at the same concentrations was better than 10.2%.

The accuracy of replicate determinations of I evaluated on the same samples and expressed as the ratio of found/added amount of I, ranged from 86.2 to 108.5%. The pooled accuracy (inter-day) ranged from 93.1 to 103.8% ($n=12$).

The limit of quantitation was 0.5 ng/ml plasma, corresponding to about 0.3 ng on the column. Spiked samples at this concentration showed a signal-to-noise ratio better than 5:1 and the C.V. of replicate determinations ($n=4$) was less than 7%.

The mean extraction recovery ($\pm$S.D., $n=9$) from plasma evaluated on three different days at 2.38, 10.45 and 54.62 ng/ml was $73.2 \pm 9.9\%$, $74.7 \pm 8.7\%$ and $71.4 \pm 4.9\%$, respectively (Table 1).

All the drug-related compounds submitted to chromatographic analysis under the conditions adopted for the assay of I could be distinguished from the peak of the unchanged drug (Fig. 2), showing the selectivity of the chromatographic separation. In addition the selective solid phase extraction performed before the chromatographic separation enabled us to obtain chromatograms free of interferences from plasma constituents. Furthermore the purification step carried out before the chromatographic analysis allowed us to concentrate the test compound, permitting significant sensitivity at the low nanogram level.

The analytical procedure described here was employed for the determination of the unchanged drug in the plasma samples obtained after a single intravenous administration of 0.76 mg (400 $\mu g/m^2$) of [^{14}C]FCE 24517 in a female patient with solid tumour from a study carried out at the Norwegian Radium Hospital (Montebello, Oslo, Norway) under the responsibility of Dr. N.P. Jørgensen. In these samples, despite the low dose administered, the unchanged drug could be measured up to 12 h after dosing. None of the possible metabolites described in Fig. 1, nor other metabolites, were found to interfere in the determination of FCE 24517 (Fig. 2D).

As a whole, this HPLC method proved to be suitable for the determination of FCE 24517 in human plasma and can be successfully employed in further clinical studies aimed at evaluating the pharmacokinetics of the test compound.

Table 1
Evaluation of the extraction recovery of I from plasma

Tallimustine concentration (ng/ml)	Day	n	Recovery (%)	S.D.	C.V.
2.38	1	3	61.9	6.96	
2.38	2	3	80.1	5.14	
2.38	3	3	77.7	4.32	
Mean value ±S.D.		9	73.2	9.86	13.46
10.45	1	3	65.3	4.76	
10.45	2	3	79.3	4.98	
10.45	3	3	79.6	7.34	
Mean value ±S.D.		9	74.7	8.70	11.65
54.62	1	3	70.4	1.98	
54.62	2	3	79.6	7.34	
54.62	3	3	70.3	8.10	
Mean value ± S.D.		9	71.4	4.90	6.87

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