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Determination of anti-cancer drug actinomycin D in human plasma by liquid chromatography–mass spectrometry

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on behalf of the UKCCSG Pharmacology Working Group

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Abstract

Actinomycin D is an anti-cancer drug commonly used in the treatment of paediatric malignancies such as Wilms' tumour, Ewing's sarcoma and rhabdomyosarcoma. Despite its long history of clinical use, little is known about the pharmacokinetics of actinomycin D in humans, largely due to problems in developing an analytical assay with the required sensitivity to measure relevant clinical concentrations. As actinomycin D treatment in children with cancer is associated with veno-occlusive disease (VOD), and as the dose intensity of actinomycin D treatment has been defined as a significant risk factor for the development of this potentially life-threatening hepatic toxicity, pharmacokinetic studies of actinomycin D may be beneficial in optimizing treatment with this drug. In order to investigate this issue, we developed a sensitive liquid chromatography–mass spectrometry (LC–MS) method for the determination of actinomycin D in human plasma samples. Extraction of analytical samples was carried out with acetonitrile and analysis performed on an API 2000 LC/MS/MS using an internal standard of 7-aminoactinomycin D. A limit of quantitation of 1.0 ng/ml was determined, allowing the reliable measurement of actinomycin D in plasma samples obtained from patients receiving this drug clinically. The method demonstrated good reproducibility, over the calibration curve range of 1.0–100 ng/ml, with intra- and inter-assay precision CVs of 2.7–11.3 and 2.3–7.8%, respectively. Accuracy data showed relative errors of 2.0–16.4 and 10.4–15.2% for intra-assay ($n = 10$) and inter-assay ($n = 7$) experiments, respectively. Initial results of actinomycin D pharmacokinetics in paediatric patients are shown.

Keyword: Actinomycin D

1. Introduction

Actinomycin D is an anti-tumour antibiotic used routinely to treat certain forms of cancer and is an important component of chemotherapies used during bone marrow transplantation. The mechanism of

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action of actinomycin D is thought to involve its intercalation with DNA, i.e. insertion into the DNA helix, and the subsequent inhibition of DNA transcription. This results in inhibition of RNA synthesis and, secondarily, protein synthesis [1,2]. Actinomycin D can also form single-strand DNA breaks through inhibition of topoisomerase II, a nuclear enzyme essential for cell replication due to its ability to create and reseal breaks in double-stranded DNA [3]. Actinomycin D is currently used to treat a number of paediatric malignancies, including Wilms' tumour, Ewing's sarcoma and rhabdomyosarcoma [4–6].

Despite its long history of clinical use, the pharmacokinetics of actinomycin D in humans have been the subject of a very limited number of studies in adults [7,8], and no reported studies in children. This compares strikingly with chemotherapeutic agents such as etoposide and carboplatin, which have been the subject of numerous pharmacokinetic studies in various patient populations [9–12]. The importance of these studies is best exemplified by carboplatin, whereby results from clinical pharmacokinetic studies have been used successfully to guide patient treatment.

The use of actinomycin D for the treatment of paediatric malignancies such as Wilms' tumour has led to a significant improvement in clinical response rates, with more than 80% of patients now being successfully cured of the disease [13]. However, treatment with actinomycin D is associated with hepatic veno-occlusive disease (VOD), a potentially life-threatening condition observed particularly in infants, with a reported incidence of 2–13.5% across different clinical studies [14,15]. Despite the introduction of amended actinomycin D dosing schedules in infants with Wilms' tumour, this background incidence of severe hepatic toxicity and associated treatment-related deaths remains a serious problem. As the prognosis following treatment is generally very good for Wilms' tumour, it is essential that the factors responsible for the occurrence of VOD in these patients are investigated. As the dose intensity of actinomycin D treatment has been clearly defined as a significant risk factor for the development of hepatotoxicity in Wilms' tumour patients [16], it would seem logical to carry out pharmacokinetic studies in these patients to investigate the extent of inter-patient variation in exposure to actinomycin D and potential correlations between drug exposure and toxicity.

There is a significant lack of data concerning analytical systems designed for the measurement of actinomycin D concentrations. Previously published assays include a thin-layer chromatography (TLC) assay to measure tritium-labelled actinomycin D [7] in addition to ELISA [17], flow cytometry [18] and radioimmunoassays [19]. None of these methods is suitable for specific determination of actinomycin D concentrations in clinical samples, either due to the use of radiolabeled drug or because of a lack of sensitivity or specificity for the parent drug as opposed to metabolites of actinomycin D.

We have developed a reliable and sensitive liquid chromatography–mass spectrometry (LC–MS) assay for the determination of actinomycin D in human plasma. We report on the validation of this assay and demonstrate its applicability to the quantitation of actinomycin D plasma concentrations in samples obtained from children receiving actinomycin D treatment for various paediatric malignancies.

2. Experimental

2.1. Reagents

Actinomycin D and 7-aminoactinomycin D were purchased from Sigma (Dorset, UK). Methanol, acetonitrile and acetic acid were obtained from Fisher (Loughborough, UK) and concentrated ammonia was purchased from BDH (Dorset, UK). Methanol and acetonitrile were of HPLC grade, all other chemicals were of analytical grade. Blank plasma obtained from citrate phosphate dextrose (CPD)-anticoagulated whole blood, was generously provided by the Blood Transfusion Service, Newcastle upon Tyne.

2.2. Preparation of stock solutions and working standards

Actinomycin D was weighed and dissolved in methanol to produce a stock solution of 1 mg/ml. This was diluted in blank plasma to obtain a 1 μg/ml intermediate stock solution which was used to prepare working standards of 100, 50, 20, 10, 5, 2 and 1 ng/ml in plasma. Fresh intermediate stock

solutions and working standards were prepared at monthly intervals.

7-Aminoactinomycin D was weighed and dissolved in methanol to produce a stock solution of 1 mg/ml. This was diluted in methanol to give a working internal standard solution of 5 μg/ml. All stock solutions, intermediate stock solutions and working standards were stored at −20 °C.

2.3. Instrumentation and chromatographic conditions

An API 2000 LC/MS/MS from Applied Biosystems (California, USA) was used for analysis, with a Series 200 Micro pump, autosampler and Peltier column oven, all from Perkin Elmer (Beckonsfield, UK). The analytical column, a Luna C_8 (3 μm, 50 mm × 2 mm) column from Phenomenex (Cheshire, UK), was fitted with a Genesis C_8 (4 μm, 20 mm × 2 mm) guard column from Jones Chromatography (Glamorgan, UK). The analysis was performed with a gradient as shown in Table 1, at a flow rate of 0.2 ml/min and an injection volume of 50 μl. Electrospray ionization was performed in the positive ion mode with nitrogen as the auxillary, nebulizer and curtain gas, with optimum values set at 50, 50 and 30 psi, respectively. The temperature of the heated nebulizer was set at 450 °C. The pause time was 5.0 ms and the dwell time 150 ms. A Q1 method (positive ion mode) was used to monitor protonated precursor ions for actinomycin D (*m*/*z* 1256) and internal standard 7-aminoactinomycin D (*m*/*z* 1271). No suitable product ions were discovered, during method development, with improved sensitivity over that for actinomycin D itself. LC–MS data were analysed using Analyst Software 1.1 from Applied Biosystems. The structures of actinomycin D and 7-aminoactinomycin D are shown in Fig. 1.

Table 1
Composition of mobile phase and gradient conditions

Time (min)	A (%)	B (%)
0	35	65
9	0	100
10	0	100
12	35	65
20	35	65

A: acetate buffer (1% acetic acid, adjusted to pH 4.0 with strong ammonia); B: methanol.

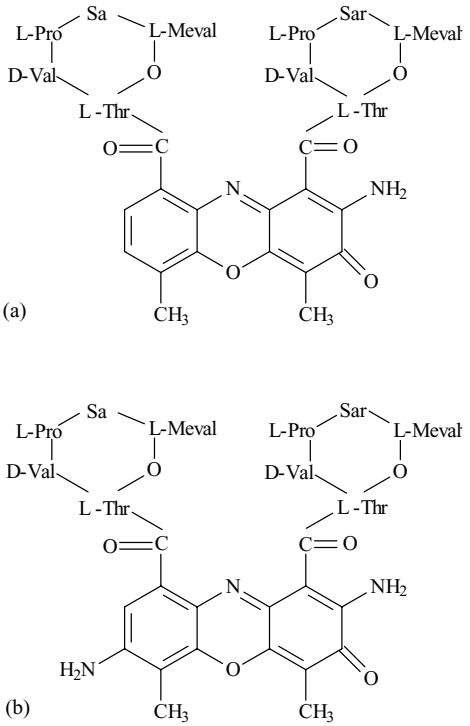

Fig. 1. Chemical structures of (a) actinomycin D (MW 1255) and (b) internal standard 7-aminoactinomycin D (MW 1270).

2.4. Sample collection and extraction

Blood samples for pharmacokinetic analysis were obtained prior to administration of a single intravenous dose of actinomycin D (0.75 or 1.50 mg/m^2) and at 15 min, 30 min and 1, 2, 4, 6 and 24 h post-administration. Blood samples (2 ml) were collected in heparinised tubes and centrifuged at 1200 g for 10 min at 4 °C. Plasma was separated and frozen at −20 °C prior to analysis. Extraction of actinomycin D from human plasma (500 μl) involved addition of 100 μl of the internal standard working solution (5.0 μg/ml), and 3 ml of acetonitrile, added in three 1 ml aliquots while vortex mixing the tube for approximately 30 s. Following extraction, samples were centrifuged at 1200 × *g* for 5 min at room temperature and 3 ml of supernatant transferred to clean glass tubes and evaporated to dryness under nitrogen at 37 °C. Samples were resuspended in 200 μl of mobile phase (acetate buffer pH 4.0:methanol; 35:65), transferred to Eppendorf tubes and microfuged at

6500 g for 3 min. Aliquots of supernatant (150 μl) were transferred to small volume insert tubes and 50 μl was injected for analysis.

2.5. Calibration and system validation

Calibration graphs were obtained by plotting peak area ratios of actinomycin D and internal standard against actinomycin D concentration and analysis of linearity was performed by non-weighted linear least-squares regression analysis. The standard curve for actinomycin D incorporated concentrations of 1, 2, 5, 10, 20, 50 and 100 ng/ml with analysis of linearity determined from five separate assays. The limit of quantification for the assay was defined as the lowest concentration which could be measured with precision and accuracy coefficients of variation of less than 20%, as determined from spiked plasma samples in five separate assays. The limit of detection for the assay was defined as the lowest concentration of actinomycin D that gave a signal-to-noise ratio of 3. Reproducibility of the assay was tested using spiked human plasma samples with actinomycin D concentrations of 5, 20 and 100 ng/ml. A minimum of 10 replicates per concentration were analyzed for intra-assay precision, with inter-assay precision determined from seven separate experiments carried out on different days. Actinomycin D recovery was determined at concentrations of 5, 20 and 100 ng/ml, with five replicates at each concentration, by comparing the peak areas of actinomycin D extracted from spiked plasma samples with those obtained from direct injection of actinomycin D standard solutions in mobile phase (acetate buffer pH 4.0:methanol; 35:65). The stability of extracted samples was determined with samples extracted from spiked plasma at 5, 20 and 100 ng/ml being stored for 24 h at 4 °C or room temperature prior to LC–MS analysis. Long-term storage stability, of spiked plasma samples at actinomycin D concentrations of 5, 20 and 100 ng/ml at −20 °C, was determined by analysis of samples at time intervals up to 7 weeks from preparation by using a freshly spiked calibration standard curve. Freeze/thaw stability was also tested with aliquots of spiked plasma at concentrations of 5, 20 and 100 ng/ml being subjected to one, two or three freeze/thaw cycles prior to analysis.

3. Results and discussion

An LC–MS assay to allow the quantitation of actinomycin D levels in clinical samples has been developed and validated. Fig. 2 shows a representative blank plasma chromatogram and chromatograms of plasma spiked with actinomycin D (50 ng/ml) and the internal standard 7-aminoactinomycin D (1.0 μg/ml), with retention times of 7.5 and 6.0 min, respectively. Examination of linearity over the concentration range 1.0–100 ng/ml yielded a linear correlation of ≥0.9988 from five separate assays. The relative standard deviation (R.S.D.) of the slope calculated with calibration curve data was 14.5%, indicating good repeatability (Table 2). The limit of quantification for the assay was 1.0 ng/ml (CV 12.1%). The limit of detection was also determined as an actinomycin D concentration of 1.0 ng/ml. Intra-assay precision studies showed coefficients of variation ranging from 11.3% at a concentration of 5.0 ng/ml to 2.7% at 100 ng/ml, with relative errors of 16.4, 5.15 and 2.7% at 5, 20 and 100 ng/ml,

Table 2
Inter-day precision in the slope and intercept of standard curves (1.0–100 ng/ml)

Day	Slope	Intercept	Correlation
1	0.0055	0.0017	0.9996
2	0.0054	0.0005	0.9998
3	0.0061	0.0029	0.9988
4	0.0062	0.0006	0.9997
5	0.0076	0.0019	1.0000
Mean ± S.D.	0.0062 ± 0.0009	0.0015 ± 0.0010	0.9996 ± 0.0005
R.S.D. (%)	14.5	65.5	0.05

Data obtained from five replicates at each concentration. S.D.: standard deviation; R.S.D.: relative standard deviation.

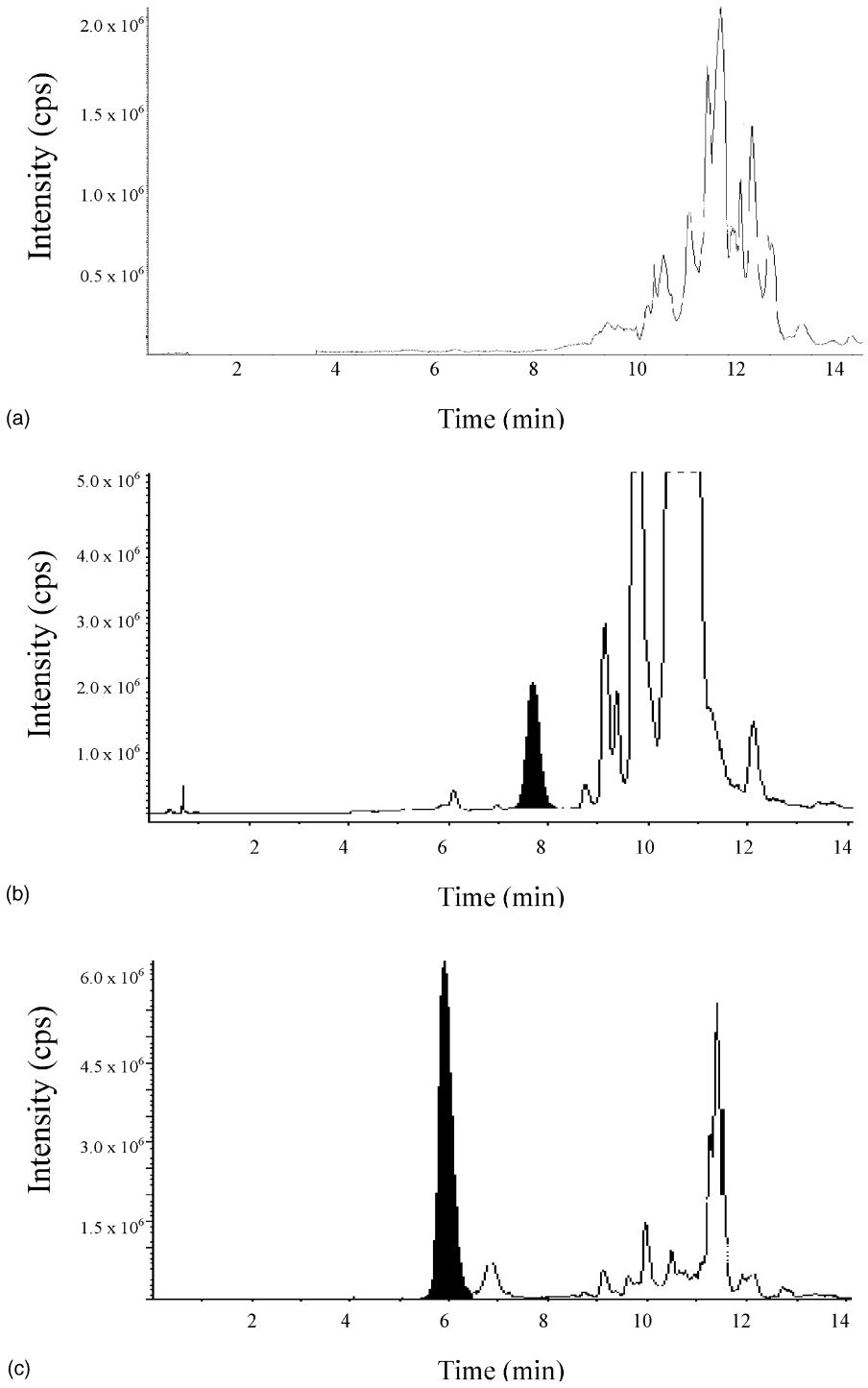

Fig. 2. Chromatograms of (a) blank plasma, (b) plasma spiked with 50 ng/ml actinomycin D and (c) plasma spiked with 1.0 μg/ml of internal standard 7-aminoactinomycin D.

Table 3
Intra- and inter-assay precision and accuracy data

Added (ng/ml)	Intra-assay precision ($n = 10$)			Inter-assay precision ($n = 7$)		
	Found	CV (%)	R.E. (%)	Found	CV (%)	R.E. (%)
5.0	5.80 ± 0.66	11.3	16.4	5.76 ± 0.13	2.33	15.2
20.0	21.0 ± 1.05	5.0	5.15	22.1 ± 1.72	7.81	10.7
100.0	101 ± 2.70	2.7	2.01	110 ± 8.37	7.58	10.4

Data expressed as mean ± S.D. for 'found' values, mean data shown for R.E. (%).

Table 4
Absolute recovery rates following extraction

Added (ng/ml)	Recovery (ng/ml)	CV (%)	Recovery (%)
5.0	4.45 ± 0.225	5.1	89.0 ± 4.5
20.0	17.2 ± 1.88	10.9	86.2 ± 9.4
100.0	83.5 ± 7.82	9.4	83.5 ± 7.8

Data obtained from five replicates at each concentration. Recovery data expressed as mean ± S.D.

respectively ($n = 10$). Coefficients of variation for the inter-assay precision studies were 2.33% at 5 ng/ml, 7.81% at 20 ng/ml and 7.58% at 100 ng/ml, with relative errors of 15.2, 10.7 and 10.4%, respectively ($n = 7$) (Table 3).

Actinomycin D recovery rates, determined by comparing the peak areas of spiked plasma samples, following extraction, with those obtained from direct injection of actinomycin D standard solutions in mobile phase, varied from 83.5 ± 7.8% (mean ± S.D.) at 100 ng/ml to 89 ± 4.5% at 5 ng/ml, with CV values ranging from a 5.1% at 5 ng/ml to 10.9% at 20 ng/ml (Table 4). Recovery of internal standard 7-aminoactinomycin D (1.0 μg/ml) was 81.9±4.3% (mean±S.D.; $n = 10$). The stability data of extracted samples at concentrations of 5, 20 and 100 ng/ml, showed no significant degradation, with <10% reductions in actinomycin D peak areas and peak area ratios when stored at either 4 °C or room temperature for a 24 h period. Long-term storage stability of spiked plasma samples at −20 °C indicated mean percentage decreases of 1.7% at an actinomycin D concentration of 5 ng/ml, 1.3% at 20 ng/ml and 6.8% at 100 ng/ml over a period of 7 weeks. Exposure of spiked plasma samples to three freeze/thaw cycles prior to extraction had no significant effect on analysis of actinomycin D levels.

Analysis of plasma samples obtained from two patients receiving actinomycin D treatment, indicated that the assay could successfully be used to quantify actinomycin D in clinical samples. Fig. 3 shows concentration–time curves for the two patients studied following administration of actinomycin D by bolus intravenous infusion of 0.75 mg/m^2 (patient 1) and 1.50 mg/m^2 (patient 2). Pharmacokinetic analysis using WinNonlin indicated AUC values of 7.70 and 15.3 μg/ml min and estimated C_{max} values of 12.3 and 72.9 ng/ml for patients 1 and 2, respectively.

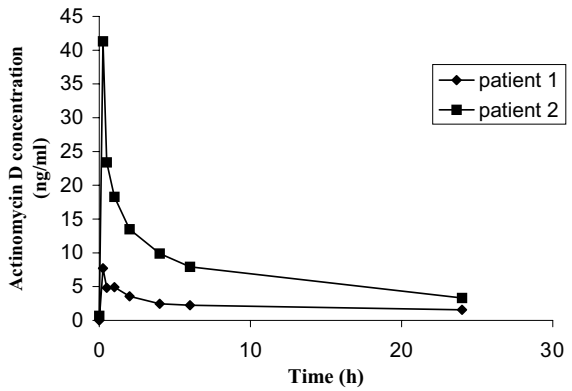

Fig. 3. Plasma pharmacokinetics of actinomycin D following doses of 0.75 mg/m^2 (patient 1) and 1.5 mg/m^2 (patient 2).

4. Conclusion

We have developed a reliable and sensitive LC–MS assay for the determination of the anti-cancer agent actinomycin D in human plasma. The observed limit of quantification of 1 ng/ml allows the determination of actinomycin D concentrations in plasma samples taken from patients receiving this drug for the treatment of various different malignancies.

Data concerning the pharmacokinetics of actinomycin D will provide useful information for investigating the importance of systemic exposure to this agent in determining clinical response and toxicity. This may be particularly valuable when we consider the dose-related incidence of VOD observed in patients receiving actinomycin D treatment. Pharmacokinetic studies in children of different age groups may give some indication why infants are more susceptible to this type of hepatotoxicity than older children. These studies are currently being performed by the UKCCSG in various clinical centres across the United Kingdom.

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