Journal of Chromatography, 427 (1988) 166–171
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4105

Note

Reversed-phase high-performance liquid chromatography of dactinomycin

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(First received October 7th, 1987; revised manuscript received December 28th, 1987)

Dactinomycin (Actinomycin D, Cosmegen) is a naturally occurring antitumor antibiotic. It was desccribed in 1954 [1] and was first used clinically in 1956 [2]. The activity of the drug is attributed to dactinomycin's well known capacity to bind to DNA and inhibit synthesis of RNA by interfering with transcription [3,4]. However, precise characterization of the interaction of dactinomycin with DNA remains an area of active investigation. Dactinomycin is a stable drug and no significant metabolism has been demonstrated in vivo [5]. Dactinomycin is an effective chemotherapeutic agent for the treatment of several human malignancies and in particular for Wilm's tumor and gestational trophoblastic neoplasms [6]. It is also used, either alone or in combination with other cytotoxic agents, in isolated limb perfusion for the treatment of regionally advanced cutaneous melanoma and selected soft tissue sarcomas of the extremities [7–10].

We have begun to investigate the pharmacokinetics of various cytotoxic agents used in isolated limb perfusion to develop information which will be of value in the design of new drug regimens, or the modification of those presently in use [11,12]. There are no pharmacokinetic data on dactinomycin in the isolation perfusion system. Evaluation of the pharmacokinetics of dactinomycin in this system seems pertinent for several reasons. (1) The dosage recommended by investigators using this technique of drug administration has varied almost tenfold [7–10]. (2) Drug delivery may be affected by the fact that dactinomycin adsorbs to glass and plastics [13] and the oxygenators used in isolation perfusion are made of plastic. (3) Since it has been reported that hyperthermia (43°C)

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0378-4347/88/$03.50

decreases the uptake of dactinomycin in CHO cells [14], the mild hyperthermia (40–41°C) routinely employed in isolated limb perfusion may also affect drug uptake. (4) There is evidence to suggest that the binding of dactinomycin to DNA may be qualitatively different depending on the concentration of the drug used [15].

Most of the information on the pharmacokinetics of dactinomycin after systemic administration has been obtained by using tritiated dactinomycin [5,16–18]. UV absorbance detection has not been utilized in these studies presumably because the method is not sufficiently sensitive to measure the low plasma concentrations (0.1–100 ng/ml, 0.8–80.0 nmol/l) present after administration of the usual systemic dose of dactinomycin (0.015 mg/kg) [5,13]. With relatively high concentrations of drug, UV absorption detection should be satisfactory. A method for the separation of mixtures of actinomycins utilizing high-performance liquid chromatography (HPLC) and UV detection at 254 nm has been reported [19]. However, application of the method to quantitation of dactinomycin and the useful limit of detection were not described. Fluorescence detection of dactinomycin is not helpful because of low quantum yield [20]. A radioimmunoassay has also been recently described which permits quantitation to 5 ng/ml (0.1 pmol/l) [13].

Practical methods for the measurement of dactinomycin have therefore had to take into account the very low drug concentrations observed in the usual clinical setting. When administered in isolation perfusion, however, the amount of drug compared to volume of distribution is much larger. Thus dactinomycin levels in perfusate and perfused tissue would be expected to be substantially higher than the levels observed in plasma and tissue after systemic administration of the drug. This paper presents a simple reversed-phase HPLC technique with UV detection, which is sufficiently sensitive to permit evaluation of the pharmacokinetics of dactinomycin when administered in isolated limb perfusion.

EXPERIMENTAL

As supplied for clinical use dactinomycin (Cosmegen, Merck Sharp and Dohme, West Point, PA, U.S.A.) contains 20 mg mannitol per 0.5 mg dactinomycin. Dactinomycin of 98% purity was also obtained from Sigma (St. Louis, MO, U.S.A.) as a standard. No differences were observed between compounds from these two sources. After standard curves were run, factors that might possibly affect the reproducibility of the assay were evaluated. To estimate differences in adsorption of dactinomycin to surfaces, dactinomycin solutions of various concentrations were added to test tubes of similar size (approximately 14 mm×97 mm) but different composition [i.e., glass, commercially siliconized glass from Becton Dickinson Vacutainer Systems (Rutherford, NJ, U.S.A.), glass siliconized in the laboratory with AquaSil from Pierce (Rockford, IL, U.S.A.), polystyrene and polypropylene]. The tubes were constantly agitated for 15 min and the amount of dactinomycin remaining in solution was measured. Solutions of dactinomycin were also incubated in light and in darkness to determine the sensitivity of the drug to light. Percentage recovery of dactinomycin from protein-containing so-

lutions was determined by adding various amounts of dactinomycin to plasma and following the extraction procedure below.

Apparatus and technique

The HPLC equipment consisted of two Model 6000A solvent delivery pumps (Waters Assoc., Milford, MA, U.S.A.), a WISP 710B automatic injector (Waters), a 30 cm × 3.9 mm column, 10-μm μBondapak C_{18} silica-based packing (Waters), a plotter–integrator (Waters, Data Module 730) and a system controller (Waters 720). Absorbance detection (Waters, Model 441) was carried out at 436 nm.

Mobile phase

The mobile phase was acetonitrile–30 m*M* sodium acetate buffer pH 4.6 (65:35, v/v) at a flow-rate of 1.5 ml/min. The run time was 10 min. Buffer was prepared from sodium acetate (Fisher Scientific, Itasca, IL, U.S.A.) and HPLC-grade water. The acetonitrile had a UV cut-off of 188 nm (Burdick & Jackson Labs, Muskegon, MI, U.S.A.).

Sample preparation

Sample extraction with acidified ethyl acetate was carried out according to Weissbach et al. [16], with only slight modifications. In brief, after centrifugation to separate plasma from the cellular components of the perfusate, 1.0 ml of plasma was placed in a siliconized test tube, 0.1 ml of 2 *M* hydrochloric acid was added and the mixture was vortexed for 10 s. Then 3 ml of water-saturated ethyl aetate were added and the mixture was vortexed for 30 s and centrifuged at 300 *g* for 2 min. A 2-ml sample of the supernatant solution was transferred to a siliconized test tube and the aqueous phase was extracted with an additional 3.0 ml of water-saturated ethyl acetate. After centrifugation the supernatant solutions were pooled, dried under an air stream, and the residues reconstituted with 1.0 ml of methanol. Then 100 μl were injected into the HPLC apparatus. Quantitation was achieved by the use of external standards. Commercially siliconized tubes were utilized whenever possible. All other glassware was siliconized using AquaSil.

RESULTS AND DISCUSSION

Under the conditions described, dactinomycin elutes at 6.4 min. When dactinomycin was dissolved in distilled water the limit of detection of the system was 1.5 ng. Detector response was linear from 0.005 to 0.2 μg with a correlation coefficient of 0.999. Exposure of 10 and 25 ng/ml dactinomycin in balanced salt solution (pH 7.4) to light of 4800 lux or approximately three times ambient light in our laboratory resulted in no drug loss over a 2-h observation with aliquots of the solutions assayed every 10 min. These findings are in contrast to reports that have alluded to the decreased stability of dilute solutions of dactinomycin when exposed to light. The recovery (mean ± S.D.) of dactinomycin after extraction and chromatography of spiked plasma was 93.0 ± 3.0% for 1.0 μg/ml and 90.6 ± 4.3% for 0.1 μg/ml concentrations. The minimum plasma or perfusate concentration that can be reproducibly measured was 40 ng/ml. Extraction of a sample

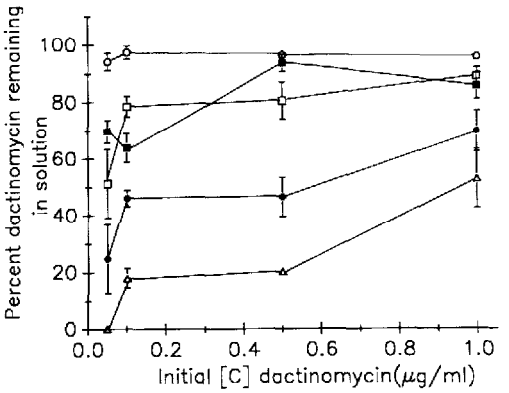

Fig. 1. Percentage of dactinomycin remaining in solution after 4.0 ml of various concentrations of dactinomycin in balanced salt solution (pH 7.2) were added to test tubes of different composition. (△) Polystyrene; (●) polypropylene; (□) glass; (■) glass siliconized with AquaSil; (○) commercially siliconized glass. The data are plotted with the 95% confidence limits; $n \geq 8$ for each data point.

of this concentration and injection of 100 μl places approximately 0.003 μg on the column. At this level the signal-to-noise ratio is 2:1.

Evaluation of adsorption of dactinomycin to test tubes of different materials revealed that adsorption was greatest with polystyrene and polypropylene, but there was marked loss of dactinomycin to the surfaces of all tubes (Fig. 1) except

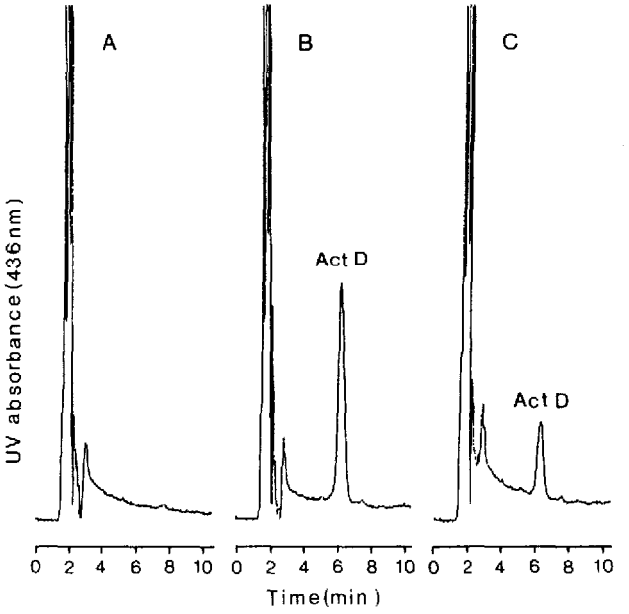

Fig. 2. Chromatograms of perfusate at (A) time 0 (before injection of dactinomycin); (B) 6 min after bolus injection of 2.0 mg (0.025 mg/kg body weight); perfusate concentration is 0.40 μg/ml; and (C) 60 min after injection; the concentration is 0.16 μg/ml. Detector sensitivity was 0.005 at 436 nm and the chart speed was 0.5 cm/min. Act D = dactinomycin.

those commercially siliconized. The percentage of dactinomycin lost was proportionally greater with more dilute solutions. Unfortunately, siliconization with AquaSil according to manufacturer's instructions did not uniformly decrease adsorption. This is being further evaluated because of the unavailability of commercially siliconized laboratory glassware. The use of small 2-ml Vacutainers to hold samples in the automatic injector instead of the usual glass inserts, for example, improved the reproducibility of the assay. In general, we have observed greater assay variation than might be expected, and we infer from the above data that the propensity of dactinomycin to adsorb to surfaces is probably a major factor.

Chromatograms of extracted perfusate samples that were obtained at 0, 6 and 60 min after injection of dactinomycin into oxygenator reservoir of the perfusion circuit during a clinical perfusion are shown in Fig. 2. There is satisfactory resolution of dactinomycin from endogenous plasma components. Initially, absorbance detection was done at 254 nm, but there were several small peaks in plasma and water blanks with retention times similar to that of dactinomycin. Resolution from dactinomycin was not readily achievable. Fortunately, these unknown compounds did not absorb at 436 nm and as spectrophotometric analysis showed that the signal height for dactinomycin at that wavelength was similar to that at 254 nm, the use of the longer wavelength obviated the problem. The absorbance maxima of dactinomycin in methanol are 244 and 441 nm.

CONCLUSION

Although dactinomycin is widely used for isolated limb perfusion there have been no pharmacokinetic studies of the drug in this system despite the potential importance of this information in developing new therapeutic regimens. This report describes a quantitative assay for dactinomycin that utilizes reversed-phase HPLC with UV detection. It is suitable for the determination of dactinomycin levels in plasma and particularly perfusate where drug concentration is relatively high. It should also prove satisfactory for the measurement of tissue levels. By avoiding the use of tritiated drug and the potential difficulties of radioimmunoassay, the study of pharmacokinetics of dactinomycin in clinical isolated perfusion is greatly facilitated.

ACKNOWLEDGEMENTS

Financial support was provided by National Institutes of Health, Grant PO1 CA31827, and P.P. Stathas Memorial Cancer Research Fund and Cancer Research Fund, University of Illinois Foundation.

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