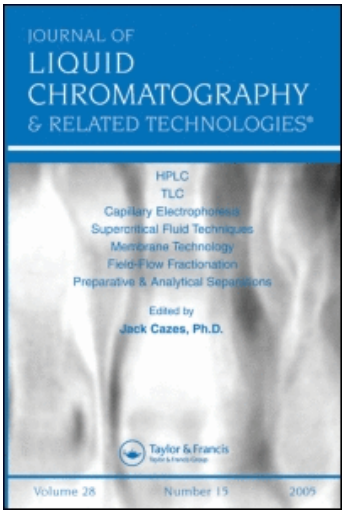

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Echinomycin (NSC-526417) in Human Plasma: Comparison of Conventional HPLC to a Capillary HPLC, Electrospray Ionization and Mass Spectrometry System

R. A. Newman[a]; A. Fuentes[a]; T. Y. Minor[a]; K. T. McManus[b]; D. A. Garteiz[b]
[a] Department of Clinical Investigation, The University of Texas M. D. Anderson Cancer Center, Houston, Texas [b] TEXms Inc., Houston, Texas

To cite this Article Newman, R. A. , Fuentes, A. , Minor, T. Y. , McManus, K. T. and Garteiz, D. A.(1994) 'Determination of Echinomycin (NSC-526417) in Human Plasma: Comparison of Conventional HPLC to a Capillary HPLC, Electrospray Ionization and Mass Spectrometry System', Journal of Liquid Chromatography & Related Technologies, 17: 2, 403 — 417
To link to this Article: DOI: 10.1080/10826079408013360
URL: http://dx.doi.org/10.1080/10826079408013360

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(2), 403-417 (1994)

DETERMINATION OF ECHINOMYCIN (NSC-526417) IN HUMAN PLASMA: COMPARISON OF CONVENTIONAL HPLC TO A CAPILLARY HPLC, ELECTROSPRAY IONIZATION AND MASS SPECTROMETRY SYSTEM

R. A. NEWMAN*[1], A. FUENTES[1], T. Y. MINOR[1],
K. T. McMANUS[2], AND D. A GARTEIZ[2]
[1]*The University of Texas M. D. Anderson Cancer Center*
Department of Clinical Investigation
1515 Holcombe Boulevard
Box 52
Houston, Texas 77030
[2]*TEXms Inc.*
Houston, Texas

ABSTRACT

We have compared the use of conventional HPLC to that of capillary HPLC used together with electrospray ionization and mass spectrometry (capHPLC/ESPI/MS) as a means of achieving picogram sensitivity for the analyses of a potent model anticancer compound, echinomycin. Using conventional HPLC, a lower limit of quantitation (LLQ) of 10 ng/ml plasma was obtained but capHPLC/ESPI/MS permitted a LLQ of 100 pg/ml. The latter method was found to be accurate and reproducible and provided a broad range of drug detection capability (0.1 ng/ml to 1 μg/ml). Comparison of analytical assay parameters using the capHPLC/ESPI/MS methodology to that of conventional HPLC are provided and discussed.

*Author to whom requests for reprints should be sent.

INTRODUCTION

The development of potent anticancer compounds in conjunction with their administration in the clinic setting by prolonged infusion schedules has made analyses of these drugs in biological fluids by conventional HPLC or GC extremely difficult. While liquid chromatography (especially HPLC) combined with thermospray/mass spectrometry has been an essential analytical tool for the pharmaceutical industry for drug metabolism studies, it has rarely been applied to the routine determination of experimental drug levels. The recent development of extremely potent anticancer compounds, however, has brought forth a need for analytical equipment with detection capabilities down to at least the picogram/ml level. The introduction of capillary electrophoresis and capillary HPLC columns and related equipment (e.g. flow splitters and microinjectors) has provided an opportunity to combine these types of chromatography with efficient, nondestructive methods of sample introduction (i.e. electrospray ionization) into the mass spectrometer to achieve extremely sensitive and specific drug detection and assay capabilities.

In this study we have compared an analytical assay procedure for echinomycin (Figure 1) using conventional HPLC with UV detection to that using capHPLC together with electrospray ionization and mass spectrometry (Figure 2). Echinomycin (NSC 526417, quinomycin A) is one of a family of quinoxaline antibiotics originally isolated from Streptomyces echinatus (1,2). It is thought to act as a bifunctional intercalator in DNA thereby resulting in inhibition of DNA-directed RNA synthesis. The drug's specific interaction with DNA results

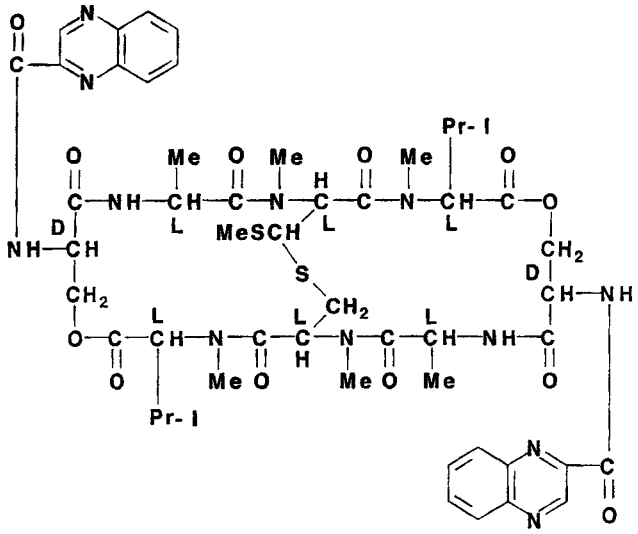

Figure 1. Structure of Echinomycin (Quinomycin A; NSC-526417).

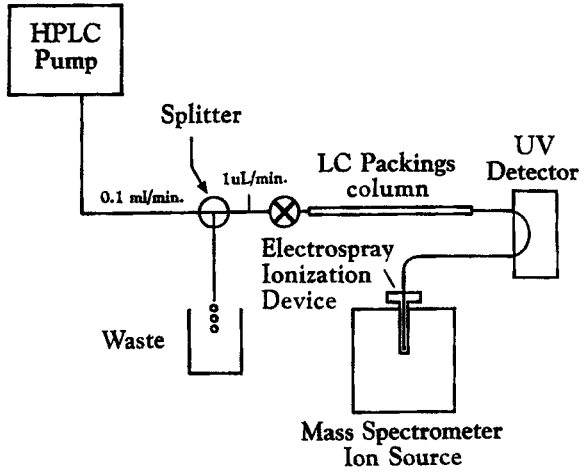

Figure 2. Schematic diagram of the capillary HPLC/electrospray ionization/mass spectrometry system.

in both inter- and intramolecular crosslinking (3,4). Although a number of Phase II studies have been completed with this drug, attempts to develop a sensitive and specific assay have been unsuccessful and, hence, there are no published reports on the clinical pharmacology of this potent anticancer compound.

In the present study we report that capHPLC with ESPI/MS can be used in an assay method which extends the limit of quantitation down to pg/ml levels with excellent reproducibility and accuracy. It is suggested that this combination of techniques may be appropriate for a wide variety of pharmaceutical compounds and their metabolites where detection limits are important and cannot be met by conventional HPLC instrumentation or methodology and where, as opposed to methods such as RIA or ELISA, qualitative knowledge of a compound's identity (via mass weight) is considered important.

MATERIALS AND METHODS

Conventional HPLC

The instrumentation consisted of a Waters Assoc. (Milford, MA) Model 6000A pump, Model U6K injector and a Model 990 photodiode array detector. The separations were performed on a Waters μBondapak C18 column (3.9 x 300 mm; Waters Assoc., Milford MA). A μBondapak C18 guard column was routinely used to protect the analytical column.

CapHPLC/ESPI/MS

The capillary HPLC equipment consisted of a fused 320 um x 150 mm C18 capillary column and an Acurate flow splitter (LC Packings, Zurich,

Switzerland), a Valco microinjector valve (Valco, Houston, TX), an electrospray interface (Analytica Inc., Branfort, CT) and a Nermag R-30-10 triple quadrapole mass spectrometer (Paris, France).

Reagents and Materials

Methanol and acetonitrile were of HPLC grade (Curtis Matheson, Scientific Inc., Houston, TX). Phosphoric acid was obtained from Fisher Scientific (Fairlawn, NJ). Echinomycin was obtained from the National Cancer Institute (Bethesda, MD). Taxol was purchased from Sigma Chemical Co. (St. Louis, MO).

Drug Extraction

BondElut C2 minicolumns (Varian Associates, Palo Alto, CA) were activated by rinsing with methanol and water as per the manufacturer's instructions. Using a low vacuum (approximately 7 psi), 1 ml of plasma containing echinomycin and the internal standard, taxol, was added to the BondElut columns. They were then rinsed with 2 ml of water followed by 2 ml of 10% acetonitrile in water. These eluants were discarded and the drug was eluted using 1 ml of 100% acetonitrile which was then dried under nitrogen. Samples were reconstituted in either 250 μl acetonitrile: water (60:40, v/v) for conventional HPLC analyses or 20 μl methanol for analyses by capHPLC/ESPI/MS.

Analytical Assay (capHPLC/ESPI/MS)

Reconstituted drug (1 μl) was injected into the capillary HPLC system. As seen in Figure 2, mobile phase (methanol) from the pumps goes through the splitter and then onto the capillary column's injector. This splitting brings the

flow rate into the 2-3 μl/min range which is the optimum flow rate for 0.32 mm (ID) capillary columns. In addition, these flow rates are also needed for optimum electrospray ionization sensitivity. Higher flow rates reduce electrospray ionization efficiency and, therefore, sensitivity. Rention times for echinomycin and taxol on the capillary HPLC column under the conditions described were 11.8 min and 12.3 min, respectively (Fig. 3).

Analytical Assay (Conventional HPLC)

Reconstituted drug (50 μl) was injected onto the HPLC column using a mobile phase of 48% acetonitrile, 6% methanol and 46% 0.1 M H_3PO_4 (pH 7.1). Flow rate was 1.5 ml/min and the detector was set at 243 nm and 0.001 AUFS. Under these conditions, echinomycin eluted at approximately 6.5 min and taxol eluted at 7.8 min (Fig. 4).

RESULTS

Extraction efficiency from human plasma was examined over a wide range of drug concentrations. Concentrations below 25 ng/ml produced recoveries which were lower (64% to 70%) than those at concentrations exceeding this value; concentrations above 25 ng/ml, however, yielded recoveries which were consistently greater than 87%. Increasing either the volume or strength of the 10% acetonitrile minicolumn wash lowered the recovery of echinomycin substantially; however, reducing the volume of the 10% acetonitrile wash resulted in retention of UV absorbing material on the C2 minicolumn which was subsequently eluted with echinomycin. While the extent of recovery from plasma was felt to be adequate, the concentration-dependent variability in recovery made

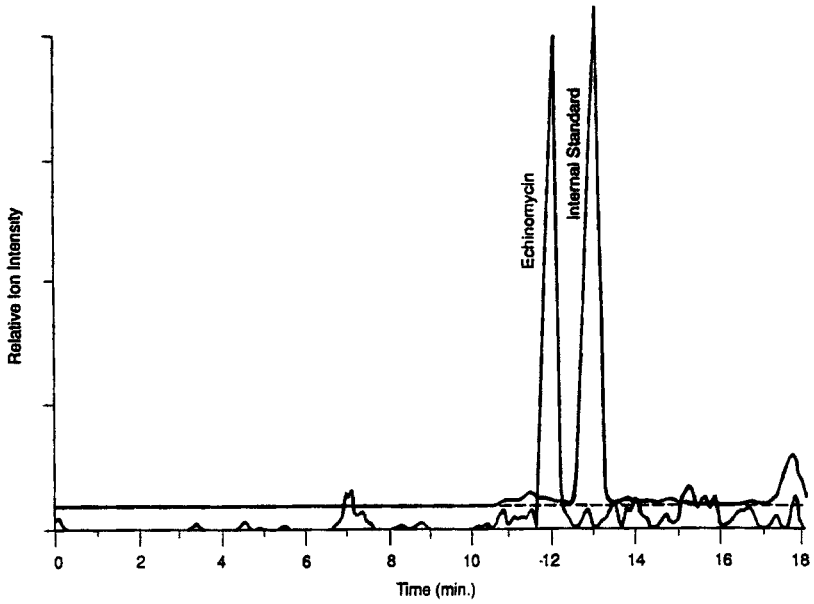

Figure 3. Mass spectrometry analyses of echinomycin (*m/z* 110) and taxol, the internal standard (*m/z* 854). The figure depicts relative ion intensities for 2 ng (on column) echinomycin and 50ng (on column) taxol.

the use of an internal standard necessary. Taxol, a structurally unrelated compound, was determined to be a useful internal standard based on its similar retention characteristics on C2 minicolumns and elution time on a conventional reverse phase C18 analytical column.

Excellent separations of internal standard and echinomycin were achieved on both the C18 analytical column (conventional HPLC) and on the capillary HPLC column. As shown in Figure 4, taxol is well resolved from echinomycin with no interfering peaks. Using taxol as an internal standard, standard curves were prepared over a wide concentration range. The lower limit of detection

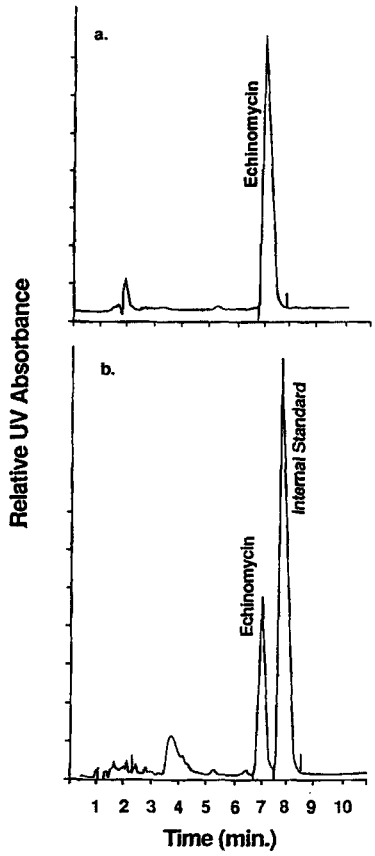

Figure 4. Representative conventional HPLC chromatogram of a) echinomycin (200 ng on column) extracted from human plasma, and b) echinomycin (200 ng) and the internal standard, taxol (500 ng), extracted from human plasma.

using the conventional HPLC approach (UV detection) was approximately 10 ng/ml. However, 50 ng/ml was determined to be the actual lower limit of quantitation. Excellent linearity of standards was obtained up to a concentration of 500 ng/ml (Figure 5).

The standard curves for echinomycin obtained using capHPLC/ESPI/MS are shown in Figures 6 and 7. While this analytical method could determine drug

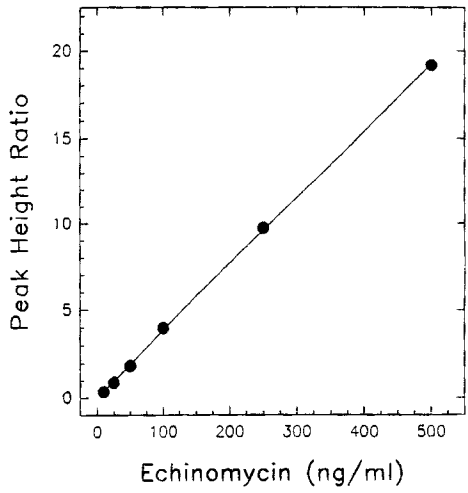

Figure 5. Standard curve for echinomycin obtained using conventional HPLC and the peak height ration method. Data are representative of curves run in triplicate on each of three different days; $r^2 = 0.98$.

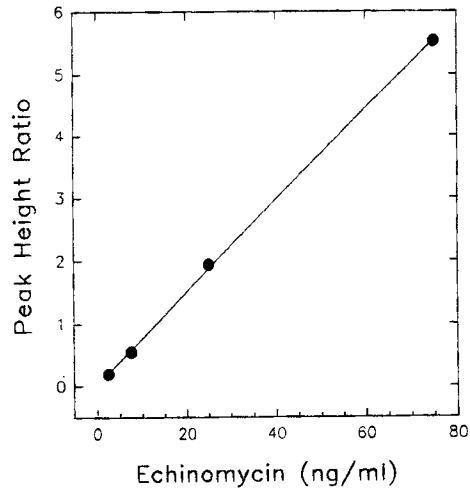

Figure 6. Standard curve (total assay range) for echinomycin obtained using capHPLC/electrospray ionization/MS. Data are presented as the average of duplicate determinations per point; $r^2 = 0.99$.

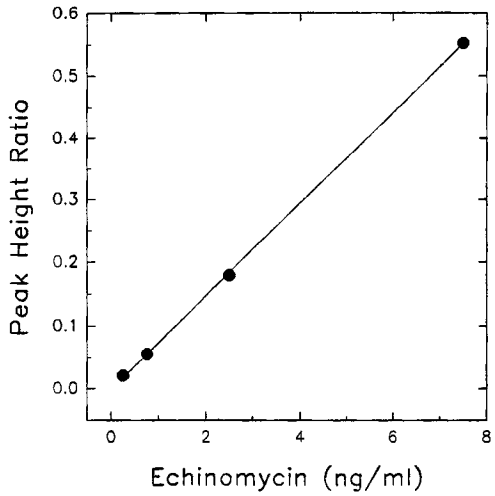

Figure 7. Standard curve (working range) for echinomycin obtained using capHPLC/electrospray ionization/MS. Data are presented as the average of duplicate determinations per point; $r^2 = 0.99$.

in plasma extracts over a wide range (2.5 to 75 ng/ml; Figure 6), the lower range of 0.25 to 7.5 ng/ml was chosen as the more useful working analytical range (Figure 7). This is also the most likely range of values which can be expected from administration of low doses of echinomycin to patients.

Assay precision and accuracy were examined for both analytical methods. Within day precision for the conventional HPLC assay was within 8% of the mean value for both concentration levels examined while precision for the capHPLC/ESPI/MS was also excellent with all relative standard deviations less than 10.5% (Table 1). Both analytical assays also showed excellent accuracy as all concentrations assayed by either method were within 4% of their respective nominal values. Between day accuracy and precision for both methods was also

TABLE 1

Within-day Accuracy and Precision

Method	N	Concentration (ng/ml) Nominal	Observed	%RSD	% Deviation from Nominal
HPLC	6	25.0	25.6	7.1	2.4
	6	250	254	4.4	1.6
capHPLC/ESPI/MS	6	0.25	0.26	10.2	3.2
	6	7.5	7.4	5.9	1.7
	6	75.0	74.0	7.0	1.3

considered as acceptable; data are presented in Table 2. The %RSD and % deviation of observed values from nominal values were all less than 12% for both analytical methods.

DISCUSSION

Echinomycin (Quinomycin A; NSC-526417) is a fermentation product derived from Streptomyces echinatus. It is a large (mol. wgt. = 1101) and extremely potent natural product which has been administered to humans with cancer at low $\mu g/m^2$ doses making it nearly undetectable by conventional chromatography methods. Echinomycin is, in fact, representative of a number of natural products from several sources including plants, marine organisms and bacteria, which have been identified, isolated and purified for potential use as anticancer drugs. Their

TABLE 2

Between-day Accuracy and Precision

Method	Day	Concentration (ng/ml) Nominal	Observed	%RSD	% Deviation from Nominal
HPLC	1	25.0	25.5	9.4	2.0
		250	257	3.1	2.8
	2	25.0	22.6	10.6	9.6
		250	255	2.9	2.0
	3	25.0	23.7	8.2	5.2
		250	251	3.0	0.4
capHPLC/ESPI/MS	1	0.25	0.26	11.2	3.5
		25.0	26.8	2.2	7.2
	2	0.25	0.24	2.9	2.0
		25.0	25.0	3.8	0.2
	3	0.25	0.26	11.5	3.2
		25.0	26.1	4.4	4.5

N = 3/day

potency presents an analytical problem especially when these agents are combined with prolonged infusion schedules. Anticipated blood levels are often far below the usual detection limit for conventional HPLC with UV or even fluorescence detection capabilities. To overcome this problem we have evaluated an analytical system which combines capillary HPLC with electrospray and mass spectrometry. As shown in the present series of studies, the capHPLC/ESPI/MS system has been found to provide detection capabilities down to at least 100 pg/ml with excellent accuracy and precision. We believe that even lower levels of compound could be easily determined if extracts derived from larger initial plasma volumes had been used.

The combination of capillary HPLC and ESP ionization has rapidly become the techniques of choice for the quantitation of "difficult to do" pharmaceutical compounds. Compounds presenting analytical challenges include those which exhibit one or more of the following characteristics: high potency with respect to pharmacodynamic action(s), poor volatility, high polarity, thermal lability, and poor UV absorbance or fluorescence capabilities.

ESP ionization has the advantage of ionizing molecules and driving them into a gas phase thorough an electrical repulsion mechanism rather than through the use of heat. This results in large currents of ions of only one mass (i.e. the molecular ion adduct). With respect to echinomycin, the molecular species m/z 1102^{+1} carries all the current. In addition to great enhancement of sensitivity, specificity of detection is significantly improved.

The low solvent flow (1-10 μl/min) typically used with capillary HPLC is ideally suited to ESP ionization procedures which works optimally at these low flow rates. Capillary columns also provide a very high theoretical plates (typically 15,000 to 30,000) and permit short retention times which are typically under 10 min. The resulting narrow peaks provide greater sensitivities. On theoretical grounds, the gain in sensitivity using a 0.32 mm column, instead of a 4.6 mm column is about 200 fold. From our experience, it also appears that currently available capillary columns are durable and stand up well to repetitive injections of biological extracts. The principal disadvantage to the use of capillary HPLC columns, however, is the limited injection volume which must be used. In our system a maximum of 1 μl can be injected without loss of retention characteristics and peak homogeneity. A second disadvantage is that for most applications using capillary HPLC, gradient elution programs are required to adequately separate the eluant of choice from other contaminating species. Coeluting compounds can interfere with the ESP ionization process resulting in lower sensitivities than expected. Better separation and/or cleaner sample preparations are therefore often required for capillary HPLC analyses than for conventional HPLC. Given these disadvantages, however, the present work demonstrates that capHPLC/ESP/MS is a useful analytical approach for the quantitation of pharmaceutical compounds in biological matrices which must be determined at extremely low levels.

ACKNOWLEDGEMENTS

We are grateful to Beatrice Leech for help in the preparation of this manuscript.

REFERENCES

1. Martin, D.G., Misak, S.A., Biles, J.C., Baczynskyi, L., Meulman, P.A. Structure of quinomycin antibiotics. J. Antibiot. 28: 332-336, 1975.

2. Foster, B.J., Clagett-Carr, K., Shoemaker, D.D, Suffness, M., Plowman, J., Trissel, L.A., Grieshaber, C.K., Leyland- Jones, B. Echinomycin: the first bifunctional agent in clinical trials. Invest. New Drugs 3: 403-410, 1985.

3. Wakelin, L.P.G, Waring, M.J. The binding of echinomycin to deoxyribonucleic acid. Biochem. J. 157: 721-740, 1976.

4. Warin, M.J., Wakelin, L.P.G. Echinomycin: a bifunctional intercalating antibiotic. Nature 252: 653-657, 1974.

Received: January 20, 1993
Accepted: June 16, 1993