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Short communication

High-performance liquid chromatographic method for the quantification of unbound evernimicin in human plasma ultrafiltrate

Ruyun Zhong[a,*], Abraham Hernandez[b], Kevin B. Alton[a], Narendra S. Kishnani[a], James E. Patrick[a]

[a]*Department of Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA*

[b]*Perkin-Elmer Instruments, 761 Main Street, Norwalk, CT 06859, USA*

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Abstract

A rapid HPLC method was developed for quantification of unbound evernimicin in human plasma. Protein-free samples prepared by ultrafiltration were injected directly onto a polymeric reversed-phase column and the eluent monitored at 302 nm. Evernimicin that eluted within 3.5 min was well resolved from endogenous components. Linearity was established between peak height and evernimicin concentration from 25 to 2500 ng/ml. Assay precision (C.V.) was within 5% while bias was no greater than 3%. This method has been used for the ex vivo assessment of evernimicin protein binding in human plasma from safety and tolerance as well as liver dysfunction and renal insufficiency studies.

Keywords: Evernimicin

1. Introduction

Evernimicin is a member of the everninomicin class of oligosaccharide antibiotics isolated from *Micromonospora carbonacea* [1–3]. It has in vitro bacteriostatic activity against a wide spectrum of gram-positive organisms, including highly resistant organisms such as methicillin-resistant *Staphylococcus aureus* and vancomycin-intermediate-sensitivity *S. aureus*, both penicillin-susceptible and non-susceptible *Streptococcus pneumoniae*, and vancomycin-resistant enterococci [4]. Evernimicin specifically inhibits protein synthesis in *S. aureus* and *E. coli* [5,6]. The prolonged half-life and high exposure (area under the concentration-time curve) to evernimicin in tissues have contributed to its in vivo activity [7].

Evernimicin is highly bound (>95%) to human plasma proteins [8]. Based on US and international regulatory agency guidelines, determination of protein binding for drugs that are ≥80% bound is imperative when administered to subjects with impaired renal and/or hepatic dysfunction. This is the rationale of this paper, which describes a simple and rapid method for determining unbound concentrations of evernimicin in human plasma ultrafiltrate.

*Corresponding author. Tel.: +1-908-740-3038; fax: +1-908-740-3966.

E-mail address: ruyun.zhong@spcorp.com (R. Zhong).

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The method involves direct injection of plasma ultrafiltrate and differs from an earlier method described for the determination of total evernimicin concentration in plasma [9]. In addition to differences in the nature of the sample and its preparation, ammonium acetate was used instead of ammonium phosphate as a mobile phase buffer. Also, the mobile phase consisted of a lower proportion of acetonitrile and had a higher pH (8.75 vs. 7.8) relative to the earlier method [9]. This method was useful for determination of unbound concentration of evernimicin in clinical samples with total plasma levels greater than 2 μg/ml, typically obtained following intravenous infusion doses of 0.3 mg/kg or greater. Unbound and total concentrations of evernimicin were used to estimate plasma protein binding from such clinical samples.

2. Experimental

2.1. Chemicals

Evernimicin (Fig. 1) was supplied as white powder (purity 96.7%) by Schering-Plough Research Institute (Kenilworth, NJ, USA). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Springfield, NJ, USA). Ammonium acetate and triethylamine (reagent grade) were obtained from Sigma Chemical Co (St. Louis, MO, USA). High-purity water was prepared using a Millipore Milli-Q® Water Purification System (Bedford, MA, USA).

2.2. Preparation of calibration standards and quality control samples

Ultrafiltrate was prepared by centrifuging human plasma (1350 *g* for 1.5 h) using a Centriprep-30 Centrifugal Filter Device (Millipore Corp., Bedford, MA, USA; nominal molecular mass cutoff=30 000 daltons, nominal capacity ~15 ml). A series of purity corrected standard working solutions (25, 50, 250, 500, 1000, 2000 and 2500 ng/ml) were prepared by adding an aliquot (40 μl) of evernimicin stock solutions, prepared in acetonitrile, to 100 μl of analyte-free ultrafiltrate. Each standard (100 μl) was injected onto the HPLC system. Evernimicin quality control samples were prepared similarly to obtain 64, 1280 and 1880 ng/ml.

2.3. Sample preparation

On the day of analysis, an aliquot (1 ml) of each plasma sample was subjected to ultrafiltration by centrifugation (1500 *g* for ~5 min at 37 °C), using a Centrifree® Micropartition Device (Millipore, nominal molecular mass cutoff=30 000 daltons, nominal capacity ~1 ml). To minimize non-specific binding losses to polypropylene-based components used in the handling of ultrafiltrate, the receptacle cups and pipette tips were treated with 0.1% Tween-20 prior to use. About 150 μl of ultrafiltrate was obtained for each sample. An aliquot (100 μl) of the ultrafiltrate was mixed with 40 μl of acetonitrile, transferred into an autosampler vial then injected (100 μl) onto the HPLC system.

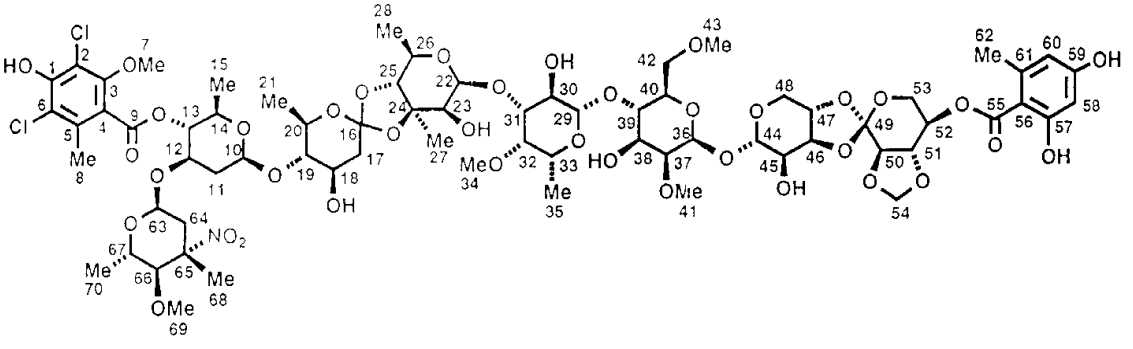

Fig. 1. Chemical structure of evernimicin (SCH 27899).

2.4. Chromatography

A Waters (Waters Corp., Milford, MA) HPLC system consisting of a Model 590 pump, a Model 6K pump, a 717 plus auto-sampler and a Model 486 tunable UV detector was used in this study. The detector was connected to Waters 860 Expert Ease Version 3.2 chromatographic data acquisition system. The analytical column was a 5-μm polymeric reversed-phase PRP-1™ (150×4.1 mm) column from Hamilton (Reno, NV, USA). The column was maintained at 40 °C using a CH-30 column heater and TC-50 temperature controller (Eppendorf, Madison, WI, USA). The mobile phase, consisting of 57% 0.2 *M* ammonium acetate (pH 8.75, adjusted with triethylamine) and 43% acetonitrile, was delivered at 1.0 ml/min. The eluent was monitored at 302 nm to simultaneously maximize evernimicin absorption signal and minimize matrix-related interference.

2.5. Method evaluation

Precision and accuracy, measured by C.V. and bias, respectively, were calculated for calibration curve standards (n=3/level) and QC samples (n=6/level) prepared in plasma ultrafiltrate and assayed on each of 3 days. The limit of quantitation was established as the lowest concentration in the standard curve (25 ng/ml).

Recovery of evernimicin from plasma ultrafiltrate was evaluated by comparison to equivalent amounts of evernimicin dissolved in mobile phase.

The stability of processed evernimicin plasma ultrafiltrate samples containing acetonitrile following storage at ambient temperature for 24 h was evaluated by comparison to the corresponding values from previously analyzed samples. This assessed stability of samples during extended HPLC runs.

3. Results and discussion

Preliminary pilot studies had indicated a propensity for non-specific binding losses of evernimicin (~12–50%) to polypropylene-based surfaces when evaluated at concentrations between 0.5 and 20 μg/ml. Treatment of polypropylene-based components with 0.1% Tween-20 consistently reduced such losses to within limits of experimental variability of approximately less than 20%. Typical chromatograms of analyte-free human plasma ultrafiltrate and ultrafiltrates spiked with evernimicin standards (50 and 1500 ng/ml) are shown in Fig. 2. Evernimicin eluted with a relatively short retention time (~3.5 min) and was well resolved from peaks resulting from endogenous constituents of plasma ultrafiltrate. A representative chromatogram of a plasma ultrafiltrate sample from a subject following intravenous

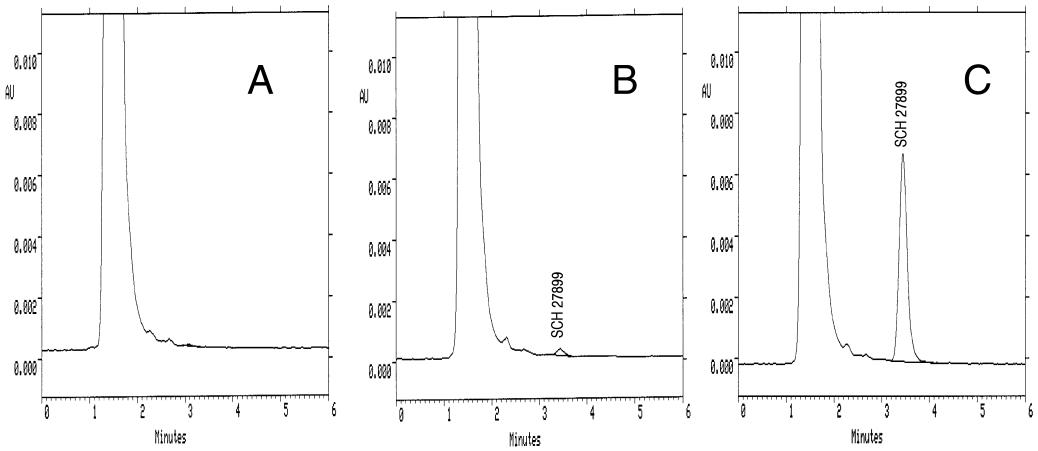

Fig. 2. Typical chromatograms of: (A) blank human plasma ultrafiltrate; (B) blank human plasma ultrafiltrate spiked with evernimicin (SCH 27899, 50 ng/ml); and (C) blank human plasma ultrafiltrate spiked with evernimicin (1500 ng/ml).

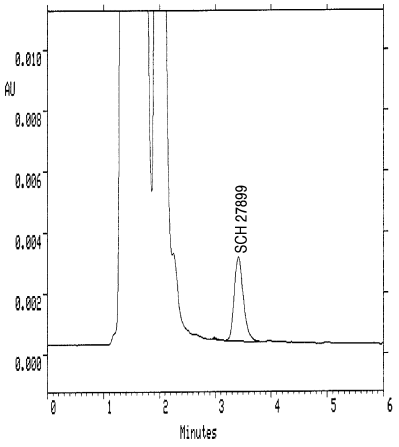

Fig. 3. A chromatogram of a human plasma ultrafiltrate sample obtained from a subject 1 h after intravenous infusion (6 mg/kg, plasma concentration 34.1 μg/ml) of evernimicin (SCH 27899).

administration (6 mg/kg) of evernimicin is shown in Fig. 3.

An external calibration method was used in this assay. Criteria for precision (should not exceed 15% of the C.V. except at the lower limit of quantitation, where it should not exceed 20% of the C.V.) and accuracy (mean value should be within 15% of the actual value except at the lower limit of quantitation where it should not deviate more than 20%) determined at each concentration were selected based on guidelines acceptable to regulatory agencies [10]. Standard curves were generated by plotting peak height vs. the concentration of evernimicin and data subjected to weighted ($1/x$) linear least-squares analysis. The results showed that standard curves were linear from 25 to 2500 ng/ml with a mean correlation coefficient (r) of 0.999. The sensitivity of detector response (indicated by slope) varied by less than 7.5% over 3 days (Table 1). Mean inter-assay accuracy (%bias) and precision (% C.V.) for all calibration standards ranged between ±7% and 0.9–6.1%, respectively.

The intra- and inter-day performance of the method was also assessed using quality control samples prepared at evernimicin concentrations of 64, 1280 and 1880 ng/ml (Table 2). Intra-assay C.V. ranged from 1.73 to 4.69% while bias varied from −2.98 to −0.16%. Inter-assay C.V. ranged from 2.03 to 3.09% and bias varied from −1.54 to 1.09%. Recovery of evernimicin from human plasma ultrafiltrate ranged between 99.4 and 103% (Table 3). The stability of evernimicin in processed samples stored for 24 h at ambient temperature was acceptable and did not change by more than 10%. The short run-time of 6 min allowed a high sample throughput (~100 samples, including calibration standards and quality control samples, in an overnight run) without any significant loss of resolution and deterioration of column.

In summary we have developed an HPLC method with UV detection for the rapid analysis of unbound evernimicin in human plasma ultrafiltrate. This highly reproducible and accurate assay has been successful-

Table 1
Daily calibration curve parameters for evernimicin in human plasma ultrafiltrate

Batch	Slope[a]	Intercept	r
1	4.01	−8.33	0.999
2	3.83	−10.4	0.999
3	4.54	−0.340	0.999
Mean	4.12	−6.35	0.999
% C.V.	7.35	NC[b]	NC[b]

[a] Weight: $1/x$; Regression: $y = Ax + B$.
[b] NC, Not calculated.

Table 2
Precision and accuracy of evernimicin quality control samples in human prepared plasma ultrafiltrate

	Concentration added (ng/ml)	Concentration found (mean±SD)[a]	C.V. (%)	Bias (%)
Intra-day	64	63.9±3.0	4.69	−0.16
	1280	1262±21.8	1.73	−1.41
	1880	1824±36.8	2.02	−2.98
Inter-day	64	64.7±2.0	3.09	1.09
	1280	1267±25.7	2.03	−1.02
	1880	1851±38.1	2.06	−1.54

[a] $n = 6$ for intra-day analysis and $n = 18$ for inter-day analysis.

Table 3
Recovery of evernimicin from human plasma ultrafiltrate

Concentration added (ng/ml)	% Recovery (mean±SD, $n = 4$)	C.V. (%)
64	102±4.0	3.90
1280	103±1.1	1.06
1880	99.4±1.0	1.04

ly used to support single- and multiple-rising dose safety and tolerance studies in the clinic as well as to assess the effect of liver dysfunction or renal insufficiency on the binding of evernimicin to human plasma proteins.

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