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Short Communication

Rapid and sensitive high-performance liquid chromatographic assay for novobiocin in human serum

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Abstract

In this paper we present a new HPLC method for the determination of novobiocin in human serum. The assay uses mitomycin C as an internal standard, protein precipitation with acetonitrile, an ODS reversed-phase column with an isocratic mobile phase of acetonitrile–0.01 *M* phosphoric acid (80:20, v/v), and UV detection at 340 nm. The assay has a lower limit of quantitation of 1 μg/ml and is linear over the range of 1–1000 μg/ml. The assay is ideally suited for use in clinical trials as it requires minimal amounts of serum, is highly sensitive and reproducible, is performed with minimal sample preparation, and involves a short run time. It should prove important in evaluating the potential of novobiocin as a means to modulate resistance to antineoplastic chemotherapy and in therapeutic drug monitoring of the growing number of patients receiving novobiocin to control methicillin-resistant *Staphylococcus aureus* infections.

1. Introduction

Novobiocin is an antibiotic obtained from cultures of *Streptomyces niveus* or *Streptomyces spheroids*. Initial work with novobiocin identified a number of susceptible microorganisms, including many Gram-positive cocci, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, and Gram-positive bacilli, such as *Bacillus anthracis*, *Clostridium perfringens* and *Corynebacterium diphtheriae*. Antibacterial activity was also documented against Gram-negative organisms, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* [1]. Widespread use of novobiocin has largely been supplanted by the availability of more efficacious antibiotics. Renewed interest in novobiocin, however, has been brought about by the increasing problem posed by methicillin-resistant *Staphylococcus aureus* [2–4] and has led to the use of novobiocin, at a dosage of 500 mg by mouth twice a day, to eradicate the methicillin-resistant *Staphylococcus aureus* carrier state [5]. Additionally, the observation that novobiocin can overcome cellular resistance to certain antineoplastic agents *in vitro* has led to a number of clinical trials evaluating the potential utility of combining an as yet undefined optimal dose and schedule of

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novobiocin with a variety of anticancer drugs [6,7]. Proper interpretation of ongoing and planned clinical trials will require the measurement of the novobiocin concentrations achieved in serum and, possibly, other biological fluids. Other assays of novobiocin have been reported in the literature [8,9]. Although these previously published methods were quite sensitive, with limits of detection of 0.1 μg/ml and 0.01 ppm, respectively, we found them to be too time-consuming and involved for application to multiple human samples. We therefore developed a new HPLC method for quantifying novobiocin in human serum. This assay appears ideally suited for use in clinical trials as it requires minimal amounts of serum, is highly sensitive and reproducible, is performed with a minimal amount of sample preparation, and involves a short run time.

2. Experimental

2.1. Materials

Novobiocin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Novobiocin acid (U-7586), novenamine hydrochloride (U-33918A), and ring B hydrochloride (U-7587) [4] were graciously provided by Dr. Charles W. Ford of the Upjohn Co. (Kalamazoo, MI, USA). Mitomycin C was provided by Bristol-Myers Squibb (Wallingford, CT, USA). Acetonitrile and phosphoric acid were purchased from EM Industries (Gibbstown, NJ, USA) and Mallinckrodt (Paris, KY, USA), respectively.

2.2. Procedure

Triplicate 100-μl samples of serum, containing 1, 5, 25, 50, 100, 200, 300, 400, 500, 750, or 1000 μg/ml novobiocin or patient specimens, were placed into 1.5-ml microcentrifuge tubes. A 5-μl volume of 4 m*M* mitomycin C (in acetonitrile) internal standard was added with a Hamilton syringe (Hamilton Co., Reno, NV, USA), and the tubes were mixed. Acetonitrile (400 μl) was added to each tube, and the mixtures were then vortex-mixed and centrifuged at 12 000 *g* for 2 min. The resulting supernatants were transferred to autosampler vials, and 50-μl aliquots were injected onto the HPLC system. The HPLC system consisted of a Hewlett-Packard Model 1050 autosampler (Hewlett-Packard, Palo Alto, CA, USA) and a Waters (Milford, MA, USA) Model 510 pump fitted with a Brownlee RP18 NewGuard (7 μm, 15 × 3.2 mm I.D.) guard column and Brownlee Spheri-5 RP18 (5 μm, 220 × 4.6 mm I.D.) analytical column (Applied Biosystems, San Jose, CA, USA). The isocratic mobile phase, consisting of acetonitrile–0.01 *M* phosphoric acid (80:20, v/v), was pumped at a flow-rate of 0.8 ml/min. Column eluate was monitored at 340 nm with a Spectroflow 757 absorbance detector (Kratos Division, ABI Analytical, Foster City, CA, USA), and the detector signal was recorded and integrated with a Hewlett-Packard 3396 integrator. Concentrations of novobiocin were calculated by comparing the area of the novobiocin peak with that of the internal standard peak in each sample and relating the resulting ratio to a concomitantly performed standard curve. Standard curves were fit by linear regression without weighting.

In an additional set of experiments, either triplicate 100-μl samples of serum containing the novobiocin concentrations noted above or patient samples did not have the mitomycin C internal standard added before being deproteinized with acetonitrile and analyzed with the HPLC system described. In these cases, concentrations of novobiocin were determined from a concomitantly performed standard curve which related the novobiocin concentration to the area under the novobiocin peak.

Prior to analyzing samples from patients receiving novobiocin, the method described was applied to serum samples from 10 patients who were hospitalized for chemotherapy at the University of Maryland Cancer Center and who did not receive novobiocin.

To demonstrate the applicability of this HPLC method to clinical samples, it was used to analyze serum obtained from a 61-year-old patient with extensive small cell carcinoma of the lung who had been treated with 1 g of novobiocin by

mouth every 8 h for 4 d while also receiving 80 mg/m^2 of cisplatin i.v. on day 2 and 100 mg/m^2 of etoposide i.v. on days 2, 3 and 4. Venous blood was collected before the start of novobiocin therapy, and at 24, 26, 27, 28, 30, 48, and 72 h after initiation of novobiocin therapy. Each sample was allowed to clot for 30 min and then centrifuged at 1000 *g* for 10 min. The resulting serum was separated from clot and stored at −70°C until analyzed.

In order to assess the degree of protein binding of novobiocin in human serum, 50, 200, 500, and 750 μg/ml solutions of novobiocin were prepared in distilled water and human serum. These samples were then placed into Centrifree micropartition systems (Amicon Division, W.R. Grace, Beverley, MA, USA) and centrifuged at 1500 *g* for 20 min. The resulting protein-free ultrafiltrates were then subjected to HPLC analysis as described

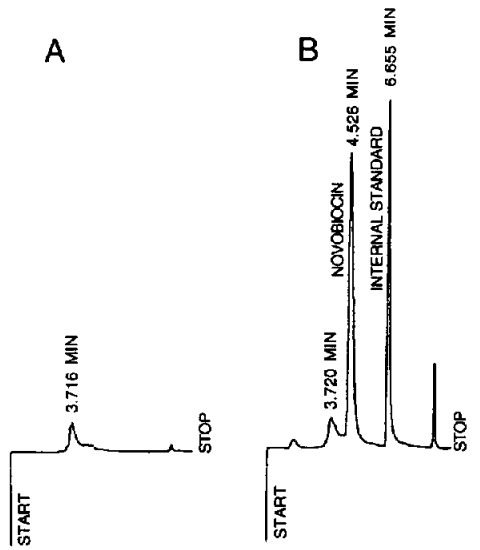

Fig. 1. Chromatograms of (A) control serum, and (B) control serum spiked with added 25 μg/ml novobiocin and mitomycin C internal standard. The ordinate uses an AUFS of 1.

3. Results

With the sample processing and chromatographic conditions described, novobiocin and internal standard were well resolved from each other, with retention times of 4.53 and 6.66 min, respectively (Fig. 1). There were no materials in serum of patients not receiving novobiocin which interfered with determination of either novobiocin or internal standard. In addition, neither novobiocin acid, novenamine hydrochloride, nor ring B hydrochloride interfered with the determination of either novobiocin or internal standard. With the method described, the lower limit of quantitation for novobiocin at a signal-to-noise ratio of 3, as defined by the lowest point on the standard curve, was 1 μg/ml, and the assay proved linear over the concentration range of 1–1000 μg/ml. There was no apparent effect of the serum matrix from which the samples were analyzed as recovery was >99% when compared to recovery of novobiocin from water, and simultaneously analyzed standard curves from distilled water and serum were indistinguishable. The correlation coefficients for three successive, triplicate standard curves using the internal standard were 0.9996, 0.9993, and 0.9960. For standard curves without internal standard, these values were 0.9998, 0.9981, and 0.9974. The within-day variation in triplicate samples containing internal standard was always <10% at every concentration studied. In the 3 triplicate curves not using an internal standard, coefficients of variation of 18.5%, 31.8%, and 3.1% were calculated for the 1 μg/ml standard. Between-day variation in three successive triplicate standard curves was also minimal. For curves using internal standard, coefficients of variation of 6.7%, 3.7%, and 3.3% were calculated for novobiocin:internal standard area ratios associated with concentrations of 1, 100, and 1000 μg/ml, respectively. Day-to-day reproducibility for other concentrations was similar. Coefficients of variation for 1, 100, and 1000 μg/ml were 26.5, 13.8, and 2.8%, respectively, in standard curves not using internal standard. Analysis of sets of 10 matched serum samples containing novobiocin at either 200 or 750 μg/ml was unbiased with mean errors of −1.65% and 1.15%, respectively. Novobiocin in serum was not decomposed when stored in serum for 18–24 h at room temperature (22–24°C). Similarly,

there was no loss of novobiocin or internal standard when extracted from serum and stored in acetonitrile for up to 24 h at room temperature.

Novobiocin was easily detected in the serum samples obtained during the 72 h after initiating therapy with 1 g of novobiocin orally every 8 h. After 24 h of therapy, *i.e.* just prior to ingestion of the fourth dose of drug, the concentration of novobiocin in serum was 151 μg/ml (Fig. 2). After ingesting the fourth dose, novobiocin concentrations in serum increased for 3–4 h and began to decline after 6 h. Concentrations of novobiocin in serum obtained just prior to the seventh and tenth doses of drug were 291 and 210 μg/ml, respectively. Similar values were calculated when standard curves with or without internal standard were used.

Recovery of novobiocin in the ultrafiltrate of distilled water solutions was 100%, implying no binding of novobiocin to the ultrafiltration membrane used in the Centrifree system (Fig. 3). In contrast, the concentrations of novobiocin in the ultrafiltrates prepared from serum containing 50, 200, 500, and 750 μg/ml were only 1, 1.6, 8, and 17 μg/ml, respectively, indicating that only 1–2% of the total novobiocin present in the serum was not protein-bound.

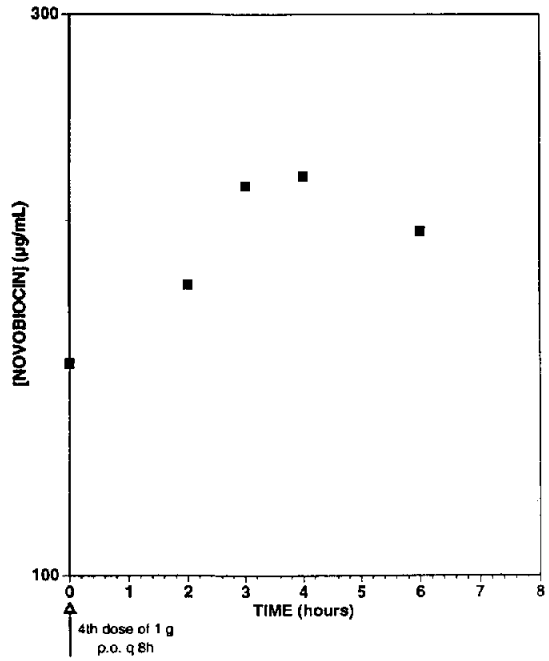

Fig. 2. Concentrations of novobiocin in the serum of a patient ingesting 1 g of novobiocin every 8 h for 96 h. Samples illustrated were obtained just prior to the fourth dose of novobiocin and at 2, 3, 4, and 6 h after ingestion of that dose. Concentrations of novobiocin are displayed on a logarithmic scale.

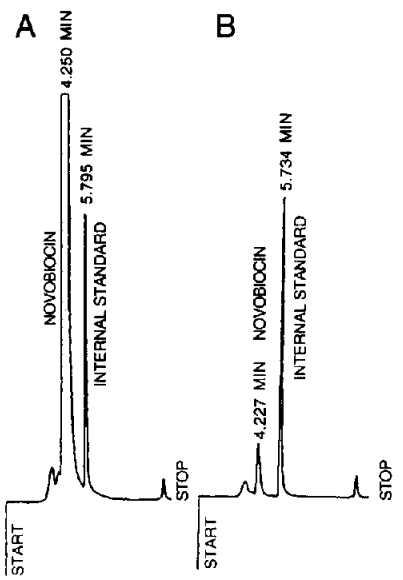

Fig. 3. Chromatograms of (A) 500 μg/ml solution of novobiocin in human serum, and (B) the protein-free ultrafiltrate prepared from that serum. The ordinate uses an AUFS of 1.

4. Discussion

Resistance, either *de novo* or acquired, represents a major problem in the effective chemotherapy of bacterial as well as neoplastic diseases [10]. Strategies employed to overcome resistance include the use of combinations of agents with different mechanisms of action, and the use of resistance modulating agents which can inhibit the mechanism(s) of resistance felt to be operative in a given system.

The emergence of methicillin-resistant *Staphylococcus aureus* in recent years [2–4] has caused concern over the continued ability to treat seri-

ous *Staphylococcus aureus* infections in hospitalized patients. The use of novobiocin, in combination with rifampinin, to eradicate the methicillin-resistant *Staphylococcus aureus* carrier state [5] may limit this condition and reduce the incidence of methicillin-resistant *Staphylococcus aureus* positive patients on hospital wards.

The antibacterial activity of novobiocin is thought to be based on its inhibition of bacterial DNA gyrase. DNA gyrase is a member of the superfamily of DNA unwinding proteins termed topoisomerases, members of which are also crucial to the replication of human cells [11]. *In vitro*, novobiocin can inhibit human topoisomerase II [12,13] and has been shown to inhibit repair of potentially lethal damage caused by irradiation and alkylating agents [14–17]. Novobiocin has also been reported to overcome etoposide resistance based on the active extrusion of etoposide from the cell [18]. These effects are seen at novobiocin concentrations >100 μg/ml [19]. A phase II trial in which patients with non-small cell lung cancer were treated with a combination of novobiocin and cisplatin [6] failed to demonstrate any improvement in outcome when compared to historical controls obtained by the same research group. However, the failure to demonstrate therapeutic benefit may reflect the fact that serum novobiocin concentrations, measured in 3 patients, were less than those associated with modulation of alkylator resistance *in vitro*. A phase I trial on the combination of novobiocin and cyclophosphamide subsequently reported that serum novobiocin concentrations that are effective at reversing drug resistance *in vitro* can be achieved in patients and described 2 partial responses in a group of 6 patients who had previously been refractory to cyclophosphamide [20]. Based on the observation that novobiocin can inhibit resistance to both cisplatin and etoposide *in vitro*, we are currently conducting a phase I trial in which novobiocin is combined with these agents. Other clinical trials are evaluating the utility and safety of combining novobiocin with a variety of antineoplastic chemotherapeutic agents. Proper interpretation of the results of these clinical trials will require measurement of the novobiocin concentrations achieved in serum and, possibly, other biological fluids. While other assays of novobiocin have been reported in the literature, we found them to be too time-consuming and involved for application to multiple human samples. We therefore developed a new HPLC method for quantifying novobiocin in human serum. This assay is ideally suited for use in human trials as it requires minimal amounts of serum, is highly sensitive and reproducible, is performed with a minimal amount of sample preparation, and involves a short run time.

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