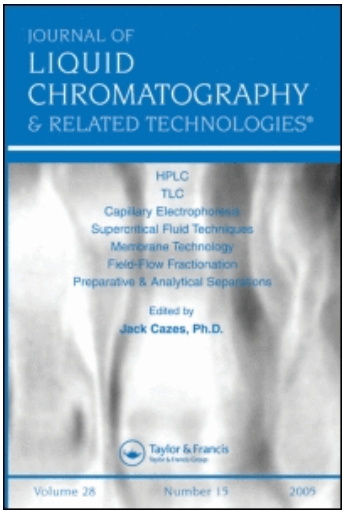


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# Evaluation of HPLC with Pulsed-Amperometric Detection for Analysis of an Aminosugar Drug Substance

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# EVALUATION OF HPLC WITH PULSED-AMPEROMETRIC DETECTION FOR ANALYSIS OF AN AMINOSUGAR DRUG SUBSTANCE

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**ABSTRACT**

The present report describes development of a reversed-phase, ion-pairing, high-performance liquid chromatographic method with pulsed amperometric detection for analysis of a new aminosugar drug substance, N-butyldeoxynojirimycin, which has been identified as an anti-viral agent. The method has been used to determine the purity of chemical lots prior to formulation for use in clinical trials. Elements of the method development and validation described include selectivity optimization, linearity of response, and precision.

## INTRODUCTION

Modern separation technology is an important part of the drug development process. Established separation methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) are used extensively in preclinical and clinical analysis of drug

substances. Drug development areas typically supported by HPLC and GC methodology include toxicology studies, pharmacokinetic studies, and formulation development, as well as the synthesis of the drug substance.

Pulsed amperometric detection (PAD) for HPLC is growing in popularity for several reasons (1). Most importantly, PAD is complementary to the most widely used HPLC detection mode, UV/VIS absorption. PAD has been shown to be applicable to the detection of several different classes of aliphatic molecules, which often do not have a chromophore or fluorophore for spectroscopic detection, or a DC-active electrophore for DC amperometric detection. Carbohydrates (2,3), aliphatic alcohols (4), amino acids (5), and various organosulfur compounds (6) have been analyzed by HPLC with PAD at noble metal electrodes. Rocklin and co-workers have indicated that simultaneous use of UV/VIS absorption and pulsed electrochemical detection provides universal detection for liquid chromatography (7).



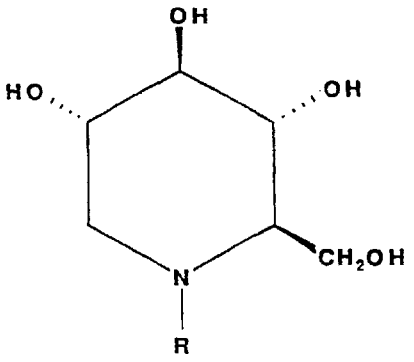
Previous reports have described the use of HPLC-PAD systems for the analysis of compounds with aminosugar and amino-alcohol functional groups. La Course et al. developed and characterized a reversed-phase, ion-pairing, HPLC-PAD system for the

determination of alkanolamines and used the system for the analysis of generic cold tablets, hand lotion, and hairspray samples (8). Polta et al utilized a reversed-phase, HPLC-PAD system for determination of aminoglycoside antibiotics in fermentation broths (9). The present report details the use of a reversed-phase, ion-pairing HPLC system with PAD for documentation of the synthesis and stability of an aminosugar derivative, N-butyldeoxynojirimycin (BuDNJ), which has been shown to be an anti-viral agent in vitro (10), and has recently undergone clinical trials for the treatment of AIDS. The structures of BuDNJ and related compounds are shown in Figure 1. Important aspects of the method development and validation are described, including selectivity optimization and statistical evaluation of method validation results. The method was formatted to assay the purity of chemical lots of BuDNJ prior to formulation for use in clinical trials and has been used to detect and quantitate levels of synthetic process impurities and degradation products. Validation results demonstrate that PAD can be used for the extended run times often required for preclinical chromatographic assays for drug substances.

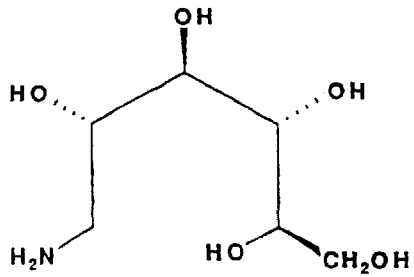


## EXPERIMENTAL

***Reagents and Materials.*** BuDNJ samples, reference standards, and related compounds were provided by the




| R | COMPOUND |
|---|---|
| H | DNJ |
| METHYL | MeDNJ |
| ETHYL | EtDNJ |
| PROPYL | PrDNJ |
| BUTYL | BuDNJ |




1-amino-1-deoxy-D-glucitol

Figure 1. Structures for BuDNJ and related compounds.



Chemical Development and Physical Methodology Departments of Searle Research and Development. The purity of BuDNJ reference standards was verified with nuclear magnetic resonance, differential scanning calorimetry, thin-layer chromatography, and HPLC. Mobile phase constituents were purchased from the following vendors: HPLC water, glacial acetic acid, Baker Chemical Co.; acetonitrile, Burdick and Jackson; sodium heptanesulfonate, Regis Chemical Comapny.

***Chromatographic System.*** The HPLC system consisted of the following components: a Beckman 421A Controller equipped with 114M solvent delivery modules, a Waters WISP 712 autosampler and a Waters Temperature Control Module. Three detection systems were used, a Kratos 783 variable wavelength detector, an ESA Coulochem Model 5100A detector equipped with a wall-jet glassy carbon cell, and a Dionex Pulsed Amperometric Detector equipped with a PAD-II cell. A Dionex DQP-1 post-column reactant delivery system was utilized for addition of sodium hydroxide solution to the eluant stream.



The mobile phase was 90% 0.005 M sodium heptanesulfonate adjusted to pH 3.0 with glacial acetic acid/ 10% acetonitrile (v/v). A Dupont Zorbax Rx C8 column (250 mm X 4.6 mm i.d.) was thermostatted at 35 degrees C and used for all experiments. The sample injection volume and concentration were 15 microliters and 1.0 mg/mL, respectively. A flow-rate of 1.0 mL/min. was used. Parameters for the PAD were as follows: E1 = +0.05 V, t1=300 msec, E2=+0.06 V, t2=120 msec; E3=-0.8 V, t3 = 300 msec; working electrode - gold, reference electrode - silver; counter electrode - stainless steel. The post-column reactant was 0.3 M sodium hydroxide, pumped at 0.5 mL/min.

***Sample Preparation and Quantitation.*** BuDNJ reference standards (STND) and sample (SAMP) solutions

were dissolved in mobile phase at 1.0 mg/mL. The impurity standard (IMPSTD) concentration was 0.005 mg/mL. Quantitative measurements were made with the following injection sequence: STND, SAMP, SAMP, IMPSTD, STND, SAMP, SAMP, IMPSTD, STND, etc. The following equation was used to calculate BuDNJ assay values: %BuDNJ = $(R_X/ R_{st})(C_{st}/C_X)X100$, where $R_{st}$ and $R_X$ represent the peak response for the previous BuDNJ reference standard and sample, respectively. $C_X$ and $C_{st}$ are the theoretical concentration of the sample and concentration of the standard, respectively. An analogous equation was used for calculation of known impurity levels. Statistical calculations were performed with Minitab release 6.1.1, VAX/VMS version. Equations for calculation of regression and precision parameters are in references 11 and 12, respectively.



***Cyclic Voltammetry.*** Cyclic voltammetry was performed with a Bioanalytical Systems CV-1B Cyclic Voltammograph and recorded with a Watanabe WX 1000 X/Y recorder. The cell volume was 25 mL. Glassy carbon and gold electrodes had diameters of 2.0 mm and 2.5 mm, respectively. A platinum wire auxiliary electrode and a silver/silver chloride reference were used. Glassy carbon experiments were performed in mobile phase. Gold electrode experiments were performed in mobile phase diluted 2:1 (v/v) with 0.3 M sodium hydroxide

(resulting media was pH 12.5). The BuDNJ concentration for the cyclic voltammetry experiments was 0.01 M.

## RESULTS AND DISCUSSION

***Cyclic Voltammetry.*** The basis of the use of electrochemical detection for BuDNJ is illustrated in Figure 2, which shows cyclic voltammograms for BuDNJ using glassy carbon and gold electrodes. As shown in Figure 2A, an oxidative response was observed with the glassy carbon electrode in the +1.0 V to +1.5 V range, presumably due to oxidation of the tertiary amine functional group (13). When a gold electrode was used with basic media (Figure 2B), an anodic wave was observed at +0.2 V, due to the oxidation of the alcohol functional group(s) (1).



***Method Development Studies.*** Method development studies concerned selectivity optimization for a mixture containing the compounds shown in Figure 1, which are potential synthetic process impurities and degradation products. An additional important aspect of the development studies was detector selection. The strategy for optimization of conditions for resolution of BuDNJ and related compounds was to use acidic conditions to protonate the amine functional groups, enabling retention to be controlled with an anionic

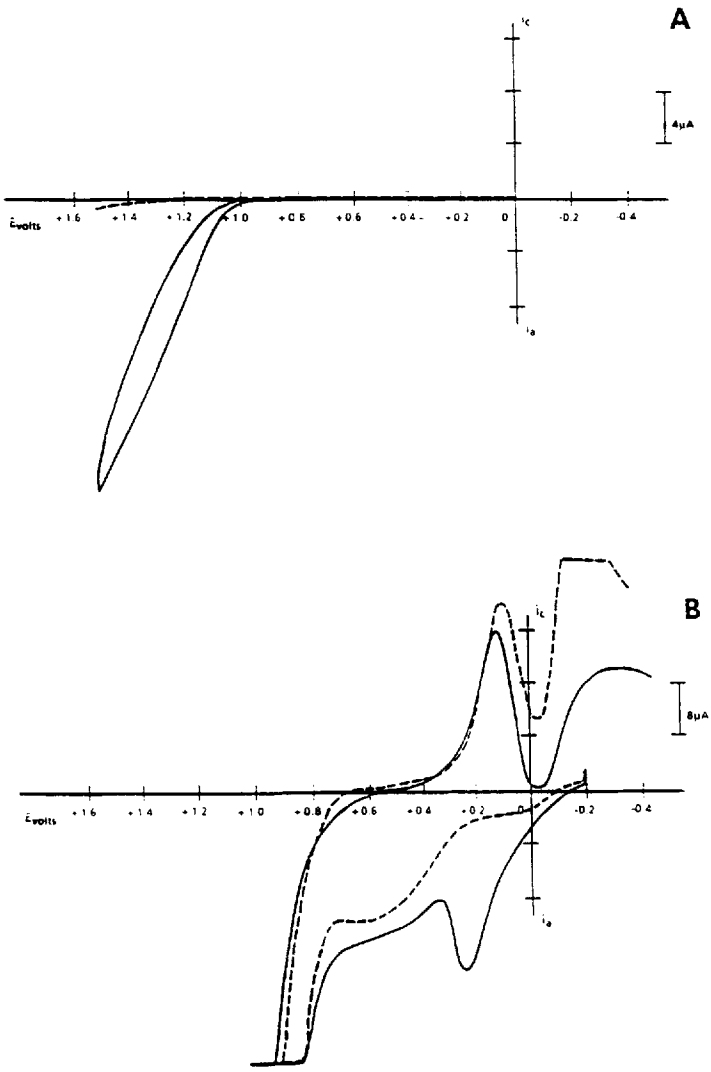



Figure 2. Cyclic voltammograms in a quiescent solution: (A) 90% 0.005 M sodium heptanesulfonate (pH 3.0 - acetic acid)/ 10% acetonitrile (v/v) with (-----) and without (- - -) 0.01 M BuDNJ at a glassy carbon electrode; (B) mobile phase as above diluted 2:1 (v/v) with 0.3 M sodium hydroxide (final pH 12.5) with (-----) and without (- - - -) 0.01 M BuDNJ at a gold electrode.

ion-pairing reagent. Figure 3A records a standard mixture chromatogram obtained with pH 3.0 water/acetonitrile/0.005 M sodium heptanesulfonate media, a Dupont Zorbax Rx C8 column, and PAD. Resolution for the compounds of interest was achieved.

The unique selectivity that PAD provides for BuDNJ and related compounds is illustrated by comparing the chromatograms in Figure 3A-B. PAD (Figure 3A) provides a response for all of the compounds of interest. Constant potential amperometric detection with a glassy carbon electrode yielded a signal for BuDNJ and potential impurities possessing a tertiary amine functionality, but did not generate signals for other compounds (Figure 3B). Utilization of conventional UV absorption detection was precluded because several compounds, including BuDNJ lacked chromophores that would yield reasonable sensitivity. Profiles of a recently synthesized lot of BuDNJ and a thermally degraded sample, which were recorded with the optimized chromatography conditions and PAD are shown in Figure 4A-B. The major synthetic process impurity in the lot was found to be deoxynojirmycin (DNJ) (Figure 1), present at a 0.2% level. Several peaks are evident in the chromatogram of the thermally degraded samples. One major degradation product was also found to be DNJ.

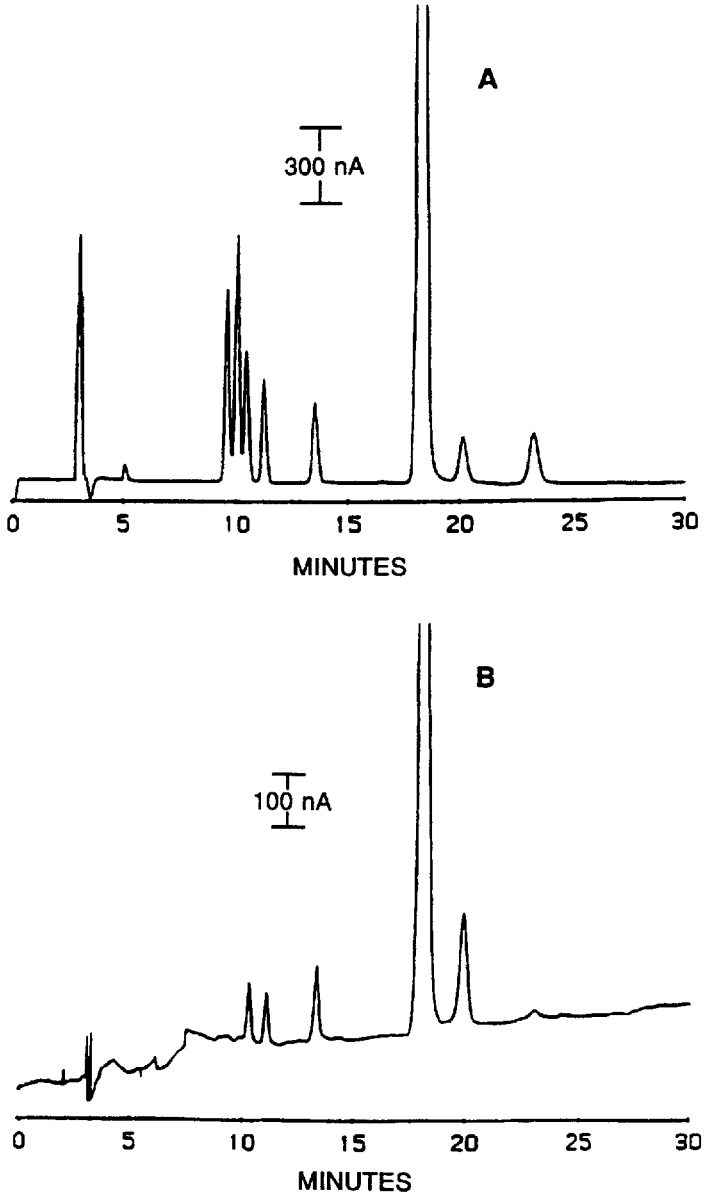



Figure 3. Standard mixture chromatograms for BuDNJ and related compounds: (A) Pulsed amperometric detection at a gold electrode; (B) DC amperometric detection at a glassy carbon (+1.0 V). Chromatographic conditions as given in text.

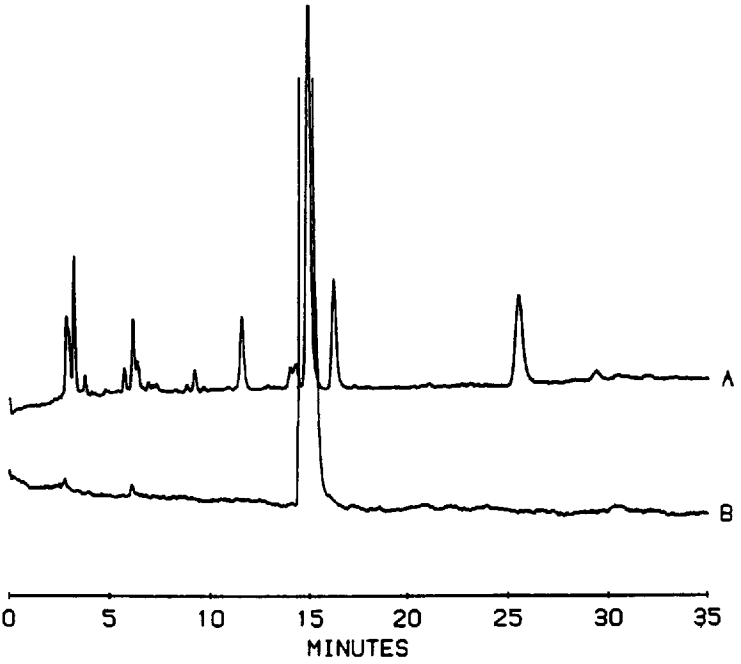



Figure 4. HPLC-PAD chromatograms for BuDNJ samples: (A) BuDNJ drug substance sample degraded at 120 degrees C for fifteen weeks; (B) typical BuDNJ drug substance sample.



***Method Validation.*** Method validation studies for the chromatography conditions used for Figures 3 and 4 addressed two areas: precision and linearity of response. Linearity studies evaluated the response of BuDNJ over a range representing 60% to 120% of the target assay concentration of 1.0 mg/mL. Results were generated on two days during two separate

**Table 1. Regression Data**

| compound | day/response | slope | intercept | correlation coefficient |
|---|---|---|---|---|
| BuDNJ | 1-areas | 0.27658 | 0.02515 | 0.9986 |
| | 2-areas | 0.23542 | 0.00824 | 0.9999 |
| | 1-heights | 0.46501 | 0.22345 | 0.9924 |
| | 2-heights | 0.43516 | 0.17648 | 0.9983 |
| DNJ | 1-areas | 0.49124 | 0.00019 | 0.9992 |
| | 2-areas | 0.36863 | 0.00001 | 0.9999 |
| | 1-heights | 3.65977 | 0.00109 | 0.9994 |
| | 2-heights | 2.30231 | -0.00021 | 0.9999 |



chromatographic "runs". Regression results are summarized in Table 1, for peak heights and areas. Linearity studies were also completed for DNJ, a synthetic process impurity and thermal degradation product of BuDNJ. Results are given in Table 1 for DNJ over a 0.0005 mg/mL to 0.05 mg/mL range (0.05% to 0.5% of the target concentration for BuDNJ).

It has been noted by Austin, et al (14) that anodic response at noble electrodes can be a non-linear function of analyte concentration, according to the Langmuir isotherm for adsorption. In our study, preliminary calibration experiments over an extended calibration range (40% to 200% of the target concentration range), yielded similar non-linear calibration plots; however, linearity studies over a limited range of 60% to 120% of the target concentration range yielded acceptable

correlation coefficients and minimal deviation from linearity. The approximation of direct proportionality between response and concentration permits the use of simplified, automated purity calculations. This approximation is even more appropriate for low level impurities, where the number of sites occupied by the analyte molecule is significantly less than the total number of "active" sites on the electrode-detector surface.



The day-to-day variation in slopes for both BuDNJ and DNJ responses by peak areas or heights is characteristic of a detection mode that depends on analyte adsorption. Despite observance of a strict protocol for electrode preparation prior to each run, day-to-day variations occur. The use of bracketing standards prevents the day-to-day variations from rendering the results unacceptable for routine use.

Precision studies were completed by generating assay values (percent purity) for a BuDNJ sample based on single-point external standard calibration with a BuDNJ reference standard. The BuDNJ and standard concentrations were 1.0 +/- 0.02 mg/mL. Precision was assessed by performing one analysis (six replicates) for two days. Percent purity data are summarized in Table 2. Values for RSD's for peak heights and areas

Table 2. Precision Data.

| compound | areas/1 | heights/1 | areas/2 | heights/2 |
|---|---|---|---|---|
| BuDNJ | 98.91 | 99.46 | 99.91 | 99.88 |
| | 98.52 | 98.67 | 99.88 | 99.54 |
| | 97.60 | 97.54 | 100.68 | 101.17 |
| | 100.67 | 100.82 | 98.50 | 97.82 |
| | 100.75 | 100.63 | 97.23 | 98.47 |
| | 99.74 | 99.65 | 100.84 | 100.74 |
| mean= | 99.4 | 99.5 | 99.5 | 99.6 |
| s.d.= | 1.25 | 1.23 | 1.39 | 1.29 |
| % RSD= | 1.3 | 1.2 | 1.3 | 1.3 |

| | areas | heights |
|---|---|---|
| assay | 99.40% | 99.60% |
| between-run RSD | 0 | 0 |
| within-run RSD | 1.33% | 1.26% |
| total RSD | 1.33% | 1.26% |
| 95% UCL for total RSD | 2.12% | 2.02% |

| compound | areas/1 | heights/1 | areas/2 | heights/2 |
|---|---|---|---|---|
| DNJ | 0.505 | 0.506 | 0.503 | 0.506 |
| | 0.505 | 0.506 | 0.509 | 0.510 |
| | 0.502 | 0.509 | 0.498 | 0.501 |
| | 0.503 | 0.504 | 0.498 | 0.498 |
| | 0.501 | 0.500 | 0.515 | 0.499 |
| | 0.505 | 0.508 | 0.492 | 0.498 |
| mean= | 0.503 | 0.505 | 0.502 | 0.502 |
| s.d.= | 0.02 | 0.03 | 0.08 | 0.05 |
| % RSD= | 0.36% | 0.65% | 1.60% | 1.00% |

| | areas | heights |
|---|---|---|
| between-run RSD | 0 | 0.37% |
| within-run RSD | 1.20% | 0.84% |
| total RSD | 1.20% | 0.92% |
| 95% UCL for total RSD | 1.90% | 1.60% |

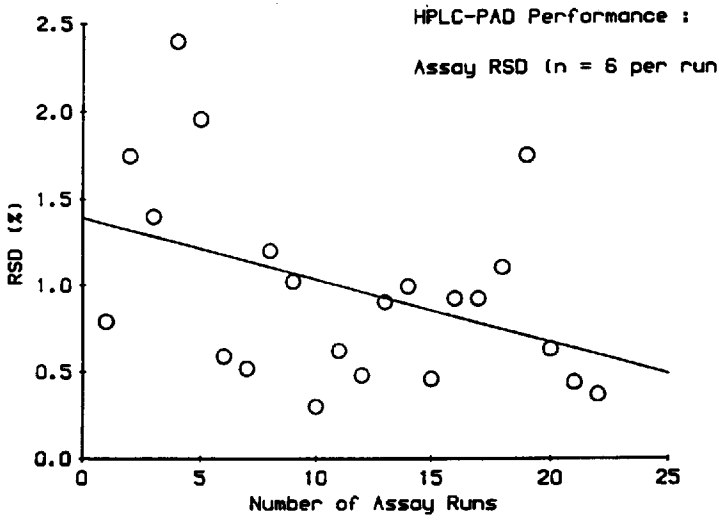



Figure 5. Long-term performance of the HPLC-PAD assay of BuDNJ drug substance samples.



were in the 1.2% to 1.3% range. Precision data for the quantitation of DNJ at the 0.5% level are also summarized in Table 2. The RSD values for twenty-two BuDNJ assays, observed during use of the HPLC-PAD system subsequent to validation studies, are shown in Figure 5.

Precision observed with the HPLC-PAD system used in the validation study and during subsequent use has been considered acceptable; however, the RSD values are larger than those typically generated during similar studies with UV absorption detection (15,16). The precision of BuDNJ assay results and DNJ level

**Table 3. Chromatographic Figures of Merit.**

| | |
|---|---|
| k' | 4.4 |
| N | 10,439 |
| RSD n=3 | 1.1% |
| $R_S$ | 1.02 |

determinations demonstrate that PAD detection for HPLC can be used to generate meaningful data when used over a several hour period (>6 hours). Electrode fouling with the subsequent decrease in signal can be a major limitation of electrochemical detectors when used during extended periods.

***OTHER AREAS.*** Table 3 summarizes typical chromatographic figures of merit observed during use of the BuDNJ assay, which can be used to establish system suitability. The resolution factor ($R_S$) was calculated for DNJ and 1-amino-1-deoxy-D-glucitol (note Figure 1), the second and third peaks, respectively, eluting in Figure 3A. For known impurities, the minimum quantitation level was set at 0.05% of the target concentration for BuDNJ (0.0005 mg/mL). Detection limits for impurities were estimated at 10% of the minimum quantitation level, which was the lowest level standard used during validation studies.

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