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Capillary electrophoretic purity method for the novel metal chelator TMT-NCS

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Abstract

A capillary electrophoretic method (CE) was developed for determining the purity of the novel metal chelator TMT-NCS. The separation of TMT-NCS from its degradation products, synthetic intermediates and by-products was accomplished using free solution CE in an aqueous–organic solvent system. This compound exhibits a complex impurity profile with the potential for over 30 degradants/impurities. The CE separation was optimized with respect to buffer type and concentration, pH, organic solvent and competitive chelator additive, allowing the resolution of all impurities in under 20 min. The specificity was established by examining stressed samples and evaluating peak purity using a diode-array detector. The sensitivity for low level impurities was optimized using sample stacking. Preliminary validation data were accumulated to support the utility of this method for estimating the purity of this drug intermediate.

Keywords: TMT-NCS; Metal chelator; Capillary electrophoresis; Purity determination; Reversed-phase liquid chromatography

1. Introduction

TMT-NCS (chemical name, 4-(3-isothiocyanate-4-methoxyphenyl)-6,6″-bis[*N*,*N*-di(carboxymethylaminomethyl]-2,2′:6′2″-terpyridine tetrasodium salt) is a novel macrocyclic nonadentate metal chelator (see Fig. 1). It exhibits especially high binding affinity for trivalent lanthanide metal ions [1,2]. Through the isothiocyanate moiety, TMT-NCS can be covalently conjugated to lysine residues on antibodies allowing the targeted delivery of certain radionuclides [3] for diagnostic as well as therapeutic applications.

TMT-NCS is produced as the tetrasodium salt via a 13-step synthesis and, therefore, has the potential to contain a number of intermediates or by-products. In addition, due in part to the high reactivity of the isothiocyanate moiety, TMT-NCS exhibits a complex degradation profile. As a result, the impurity profile of this chelator can involve well over 30 different compounds. Most of these compounds are close structural analogs of TMT-NCS and result from various reactions of the isothiocyanate group, different substitutions

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on the methoxyphenyl ring or a minor variation of the terpyridine system. A reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed to evaluate the impurity profile of TMT-NCS. Although capable of resolving impurities from the intact compound, several factors complicated the RP-HPLC analysis of TMT-NCS. These include the high number of potential impurities/degradants, the tendency of the chelator to interact with metal surfaces or trace metal ions in the chromatograph producing artifacts, the wide range of hydrophobicities of the impurities and the highly ionic character of the compound. For these reasons, capillary electrophoresis (CE) was evaluated as an alternative or complementary method for assessing the purity of this compound.

The available CE literature contains several documented applications involving the analysis of metal chelators [4–10]. In all of these cited examples, the analyses were conducted on different metal complexes of the respective chelators with the objective being the separation of the different metal species. In this work, the compound to be analyzed is the free chelator before the addition of metal ion, with the focus being the evaluation of the chemical purity of the chelator. This presents a different set of separation problems. In addition, the complexity of the impurity profile poses challenges even for a high resolution technique such as CE. The focus of this work is to review some of the developmental aspects of this CE method and report on some of the approaches used to achieve a suitable method for evaluating the purity profile of TMT-NCS which should be useful in analyzing other compounds in this class. In addition, some of the preliminary method validation data will be presented.

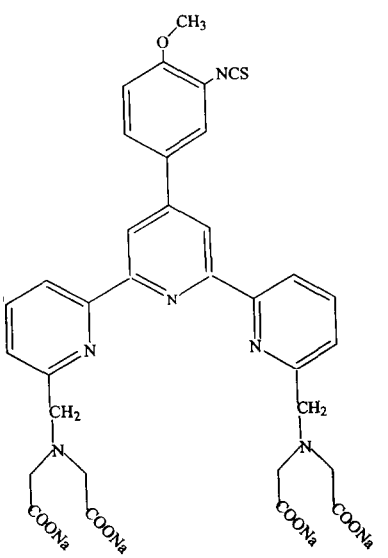

Fig. 1. Structure of TMT-NCS.

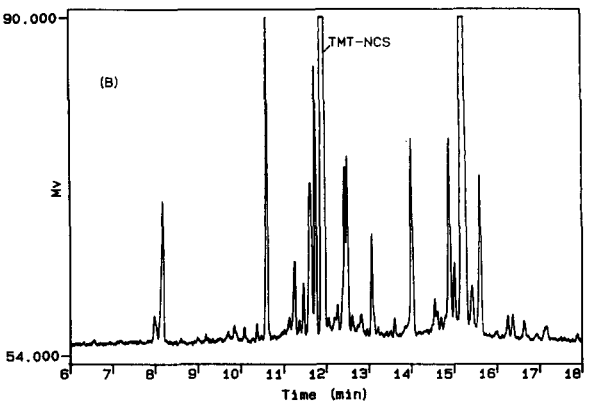

Fig. 2. Typical capillary electropherogram obtained on a stressed sample of TMT-NCS. CE conditions: capillary column, 50 μm i.d. × 67 cm; buffer, boric acid (100 mm)–tricine (25 mM)–EDTA (0.5 mM) (pH 8.05) in acetonitrile (33%)–water; temperature, 30°C, 30 kV; detection at 200 nm; injection time, 14 s. See the experimental section for additional details.

2. Experimental

2.1. Equipment

A Beckman PACE 2050 capillary electrophoresis system (Beckman, Palo Alto, CA) was used for most of the experiments. The instrument was controlled by an IBM model 50Z 80386 PC running PACE software. Data were collected and analyzed using PENelson Access*Chrom software, model 6000 (PENelson, Cupertino, CA) operated on a mainframe (VAX). Certain experiments were also run on a prototype HP CZE system equipped with a diode-array detector (Hewlett Packard, Wilmington, DE). Fused-silica

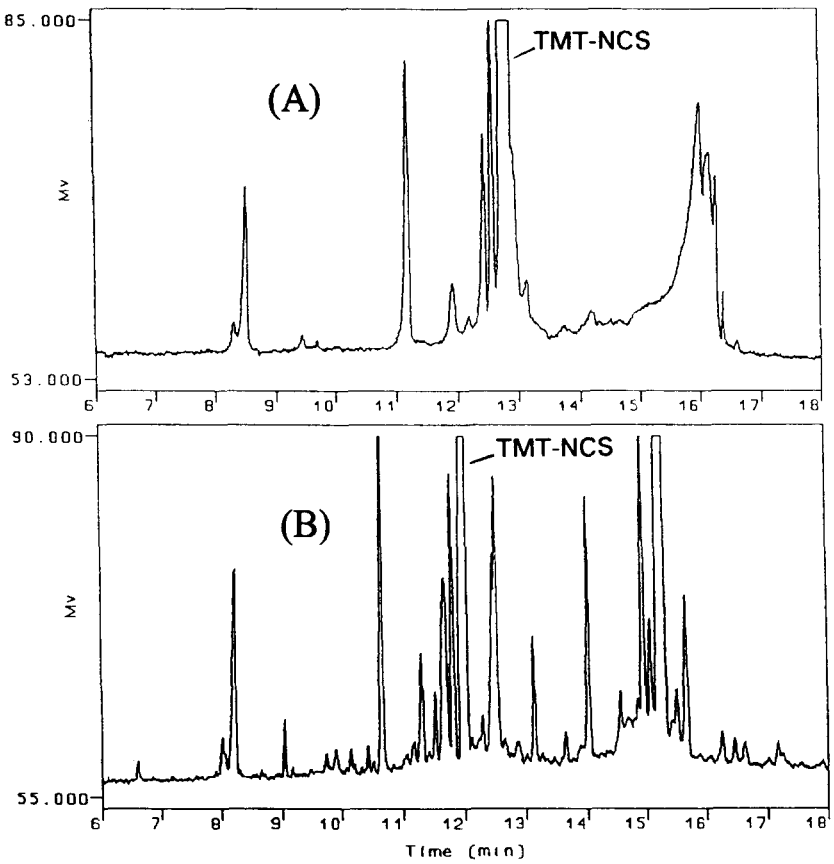

Fig. 3. Effectiveness of EDTA added as a competitive chelator in eliminating peak artifacts in the CE analysis of the TMT-NCS chelator. Conditions as given in the experimental section except that in (A) no EDTA was used in the buffer and in (B) 0.5 mM EDTA was incorporated into the buffer.

capillary tubing (50 μm i.d. × 67 cm; 60 cm to detector) was from Polymicro Technologies (Phoenix, AZ). The separation capillary was configured into the respective instrument cartridges and conditioned by rinsing with 1 N NaOH for 20 min prior to equilibrating with the run buffer. Data were collected at a sampling rate of 3.3 points s^{-1} with a range of 5 mAU mV^{-1} (PACE) or 2 mAU mV^{-1} (HP) using a PENelson model 941 A/D.

2.2. Materials

Boric acid, sodium hydroxide, EDTA disodium dihydrate, acetic acid, sodium phosphate monobasic monohydrate and HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ). Tricine was obtained from Aldrich (Milwaukee, WI). Samples of the TMT-NCS chelator were synthesized in the Department of Chemical Development at Sterling-Winthrop. Stressed samples of TMT-NCS were produced by subjecting the solid to heat (70°C) and light (900 ft candles) and solutions of TMT-NCS in a pH 7 phosphate and pH 10 borate buffer at room temperature for several days. Solution stressing was also conducted in an acetate (50%)–isopropanol (50%) buffer matrix at room temperature. Aqueous solutions of TMT-NCS in these different buffers were also exposed to light.

2.3. CE operating conditions

The separation buffer consisted of boric acid

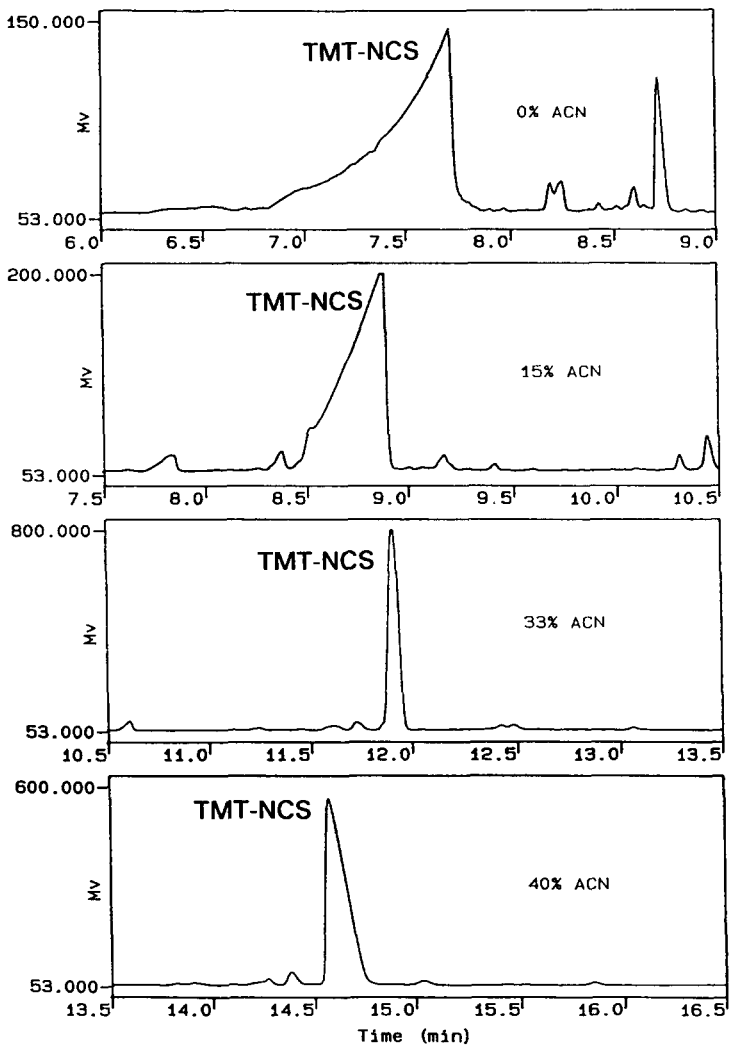

Fig. 4. Influence of different levels of acetonitrile in the separation buffer on the peak symmetry for TMT-NCS and resolution of related impurities. Separation conditions were as given in the experimental section except for (A) no acetonitrile; (B) 15% (v/v) acetonitrile; (C) 33% (v/v) acetonitrile and (D) 40% (v/v) acetonitrile in the separation buffer.

(100 mM)–tricine (25 mM)–EDTA (0.5 mM) (pH 8.05) in acetonitrile (33%, v/v) (note the pH is adjusted to 8.05 with 5 N NaOH prior to adding acetonitrile). The operating voltage is 30 kV and the capillary is thermostatted at 25°C. Detection is accomplished at 280 nm. Samples of TMT-NCS are dissolved in a matrix of acetic acid (20 mM)–EDTA (0.5 mM) (pH 5.6) in acetonitrile (33%). Samples are injected by pressure (0.5 psi) for 14 s. The run time is 20 min.

RP-HPLC analysis was performed using a Waters 600E gradient solvent system and model 712 WISP autosampler (Milford, MA) with an ABI (Foster City, CA) model 783 UV detector. A Hypersil BDS C-18, 5 μm, 4.6 mm $\times$ 150 mm column was used. Solvent (A) was NaH_2PO_4 (50 mM)–$Na_2EDTA \cdot 2H_2O$ (10 mM) adjusted to pH 7.0 with NaOH. Solvent (B) was methanol. The gradient program was from 10% B to 70% B in 30 min, and 100% B at 40 min. The flow rate was 1.0 ml min^{-1} and the detection wavelength was 287

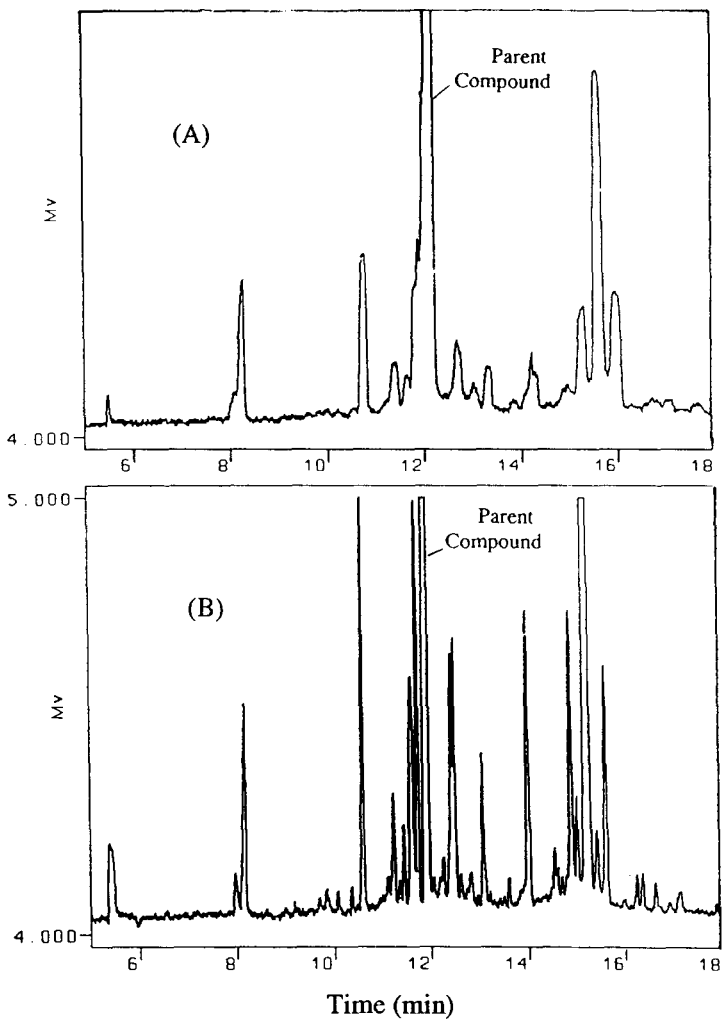

Fig. 5. Improvement in sensitivity for low level impurities of TMT-NCS using a pH stacking injection process. Separation conditions were as given in the experimental section using a 14 s injection except that in (A) the sample was injected out of the separation buffer and in (B) the sample was injected out of a lower pH, lower ionic strength acetate buffer.

nm. TMT-NCS was prepared at a concentration of 1 mg ml^{-1} in solvent (A).

3. Results and discussion

Fig. 2 shows an electropherogram obtained on a stressed sample of TMT-NCS which displays most of the potential degradants and impurities that may be encountered in performing purity analysis of this compound. Preliminary development experiments had shown that the pH 8–9 range was the most suitable for separating impurities from the intact TMT-NCS. A combination of a borate and a tricine buffer was found to give the best overall selectivity, and provided good buffering capacity and buffering range for further optimization. The buffer concentration was maximized for the optimal resolution of the components as well as to provide the potential for sample stacking for improved sensitivity. It was observed that the selectivity was highly dependent on the buffer pH with changes of 0.1 pH unit resulting in significant changes in the selectivity

Table 1
Precision of CE migration times for TMT-NCS and related impurities for six replicate injections

Precision parameter	Peak identity					
	Impurity 1	Impurity 2	TMT-NCS	Impurity 3	Impurity 4	Impurity 5
Mean (min)	8.14	10.66	11.56	11.84	13.47	15.22
SD (min)	0.00983	0.0147	0.00753	0.0150	0.0133	0.0225
RSD (%)	0.121	0.138	0.0651	0.127	0.0987	0.147

resolution. However, by careful optimization of the buffer composition and control of its preparation, highly reproducible separations were possible (see the precision data below).

In the course of developing a suitable method, several separation problems specific to TMT-NCS needed to be addressed to achieve a suitable separation and detection of all of the impurities in addition to the typical method optimization procedures. Based on the author's work with this metal chelator as well as other metal chelators, it was observed that different types of peak artifacts were possible when analyzing these free chelators by CE. Typically, this was manifested as peak tailing or a shoulder peak and resulted in decreased resolution, poorer sensitivity and poor reproducibility. The cause of these effects is believed to be due to trace metals, possibly emanating from the buffers or solvents, adhering to the surface of the capillary presenting adsorptive sites for the chelator (subsequent to this work, a paper [11] appeared which implicated trace iron and calcium adhering to the surface of the capillary as the cause of similar peak tailing for anionic dibasic and tribasic benzoates in free solution CE). For this application involving TMT-NCS, a low level of a competitive chelator (EDTA) was added to the running buffer to sequester these trace metals and circumvent the appearance of any peak artifacts. Fig. 3 demonstrates the effectiveness of this approach. Both separations in this figure were obtained on an identical sample using the same capillary, the difference being that in the top (A) electropherogram no EDTA was used while in the bottom (B) electropherogram, 0.5 mM EDTA was added to the run buffer.

Another problem that needed to be addressed in the course of developing this method was the significant electromigration dispersion for the main TMT-NCS peak which resulted from the high sample concentration used to enhance the sensitivity for low-level components. The high sample concentration in conjunction with the high charge state (between 2^- and 3^{-1}) for TMT-NCS when operating in the pH range investigated for this method resulted in significant fronting of the TMT-NCS peak with the borate–tricine buffer matrix. Other anionic buffers with mobilities closer to that of TMT-NCS were evaluated but none was found to provide the desired peak symmetry along with the necessary selectivity. A different approach for controlling peak asymmetry, which was found to be quite useful in this application, was to add an organic cosolvent to the separation buffer. Fig. 4 displays a series of electropherograms obtained on the same sample of TMT-NCS using the same buffer composition (buffer species, concentration and pH) except for the level of acetonitrile cosolvent. It was observed that the peak symmetry for the TMT-NCS peak could be controlled from a situation of significant peak fronting with no acetonitrile to a situation of

Table 2
Precision of CE migration times and peak area for six replicate injections of an impurity standard prepared at 1.5% of the nominal TMT-NCS concentration

Precision parameter	Migration time (min)		Peak area	
	Day 1	Day 2	Day 1	Day 2
Mean	11.78	12.07	680	722
RSD (%)	0.424	0.177	1.44	1.43

Table 3
Peak area normalization precision data for six replicate injections of a sample of TMT-NCS

Precision parameter	Peak identity				
	Impurity 1	Impurity 2	TMT-NCS	Impurity 3	Impurity 4
Mean (area %)	0.49	2.71	92.4	0.65	0.16
RSD (%)	8.2	0.873	0.403	5.5	11

peak tailing with 40% (v/v) acetonitrile. At 33% (v/v) acetonitrile, the TMT-NCS peak is symmetrical. The combination of improved peak symmetry and slower electro-osmotic flow with the addition of acetonitrile had the effect of improving the resolution of several minor impurity peaks from the main TMT-NCS peak.

The improvement in peak symmetry observed here with the addition of acetonitrile is not believed to be due to the effect of any shift in the apparent pH on the ionization/mobility of TMT-NCS. The ionization constants of TMT-NCS are such that there is no significant change in the ionization/mobility of the compound in the pH region of the buffer (7–9) that would be able to account for the improved peak shape. The ionization/mobility of the buffer co-ion(s) will change with small changes in the apparent pH. However, in the absence of acetonitrile, no improvement in peak shape could be achieved by varying the buffer pH from 7.5 to 9.5. Instead it is believed that there is some sort of preferential solvation of TMT-NCS by the acetonitrile which in some way effects the mobility of TMT-NCS relative to the buffer co-anions in a favorable manner.

In order to maximize the sensitivity for low-level impurities at or below the 0.1% level, a sample stacking procedure was developed. Initially, sample stacking from a more dilute (1:10) solution of the separation buffer was attempted. However, the use of this procedure was found to be ineffective for two reasons. Firstly, the TMT-NCS was not stable in the borate–tricine buffer matrix at pH 8.6 for long periods. Secondly, not enough sample stacking could be achieved using this approach to attain the desired sensitivity. Instead, a combination of a lower pH and a lower ionic strength acetate buffer matrix was used (see experimental section for details). This matrix gave good focusing by virtue of the higher mobility of TMT-NCS and its impurities at lower pHs in addition to a concentration-induced focusing. This sample diluent also provided better sample stability. Fig. 5 demonstrates the improvement in sensitivity achievable by this stacking mechanism relative to that obtained using a sample dissolved directly in the separation buffer. Using this approach, there was no significant change in resolution using injection times ranging from 2 to 16 s. An injection time of 14 s is used in the method.

The reproducibility of the separation in terms of migration times is summarized in Table 1. This table represents migration time reproducibility for several impurities in addition to the intact TMT-NCS for six replicate injections. RSDs for individual peaks were all in the 0.1% range. It should be noted that with this optimized buffer matrix, no reconditioning of the capillary with strong acid or base was needed in between injections. Typically, the capillary would be refreshed with buffer for 30 s between injections although this was not necessary.

To assess the peak response reproducibility of the method, a reference impurity standard was prepared at 1.5% of the nominal TMT-NCS sample concentration. Table 2 contains the migration time and peak area response precision data for six replicate injections made on each of two separate days. RSDs for the peak area were 1.44 and 1.43% for the two separate days.

The linearity of the method was evaluated over a concentration range from 0.002 to 1.00 mg ml^{-1} corresponding to 0.2–100% of the nominal sample concentration. Good linearity in terms of peak area response (11 standards; slope, 3873 (standard error, 25.4); y intercept, −3.55 (stan-

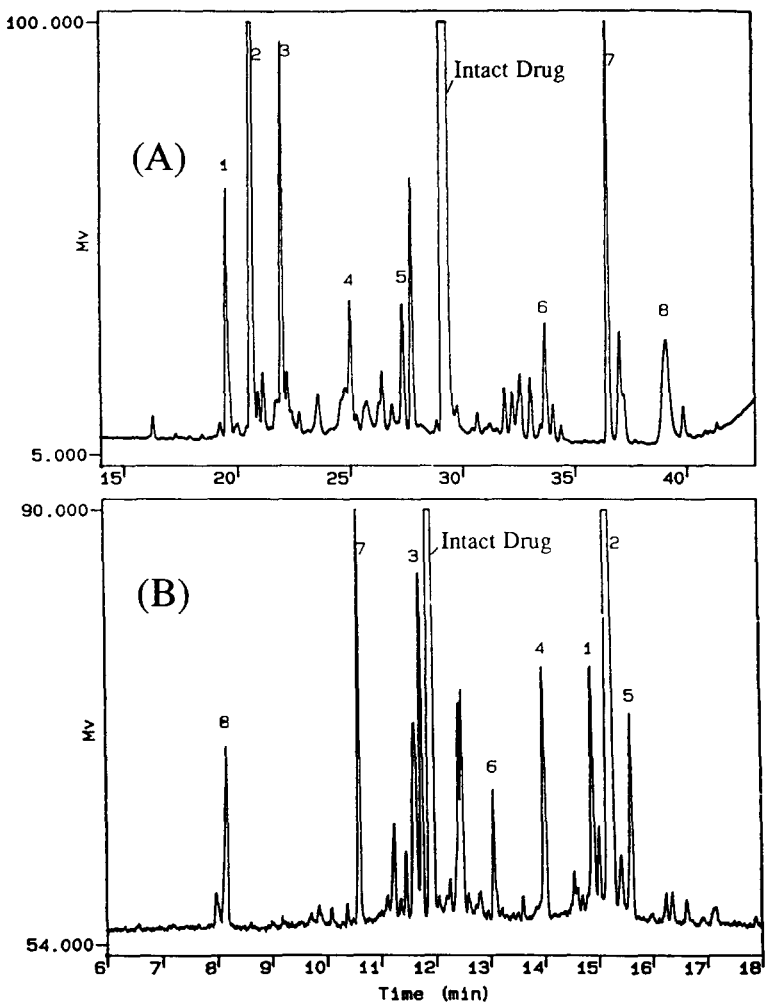

Fig. 6. Comparison of the selectivity of (A) RP-LC and (B) free solution CE for the purity analysis of TMT-NCS. Conditions were as given in the experimental section.

dard error, 8.8); $R^2 = 0.9996$) was obtained. The estimated limit of detection was 0.0007 mg ml^{-1} (3 × S/N) or 0.07% (w/w) of the sample. It should be noted that the limit of detection achievable varied significantly from instrument to instrument. For instance, with another instrument (Hewlett Packard) in the author's laboratory, a limit of detection that is nearly 10 times lower could be achieved. While the limit varied widely on different instruments, a limit below 0.1% was always achieved.

Given the fairly wide linear range for the method and the similarities in the UV spectral profiles of most impurities (data not shown), estimates of purity were obtained by peak area normalization (corrected for migration times). The precision of the peak area normalization quantitation for six replicate injections of a typical sample is shown in Table 3. This table contains peak area percentages for five of the impurities and for the intact TMT-NCS peak. RSDs for the impurities ranged from 0.87% for an impurity present at the 2.7% level to 11% for an impurity at the 0.16% level. The RSD for the overall purity estimate for TMT-NCS was 0.40%. It should be noted that there was a slight downward trend with each

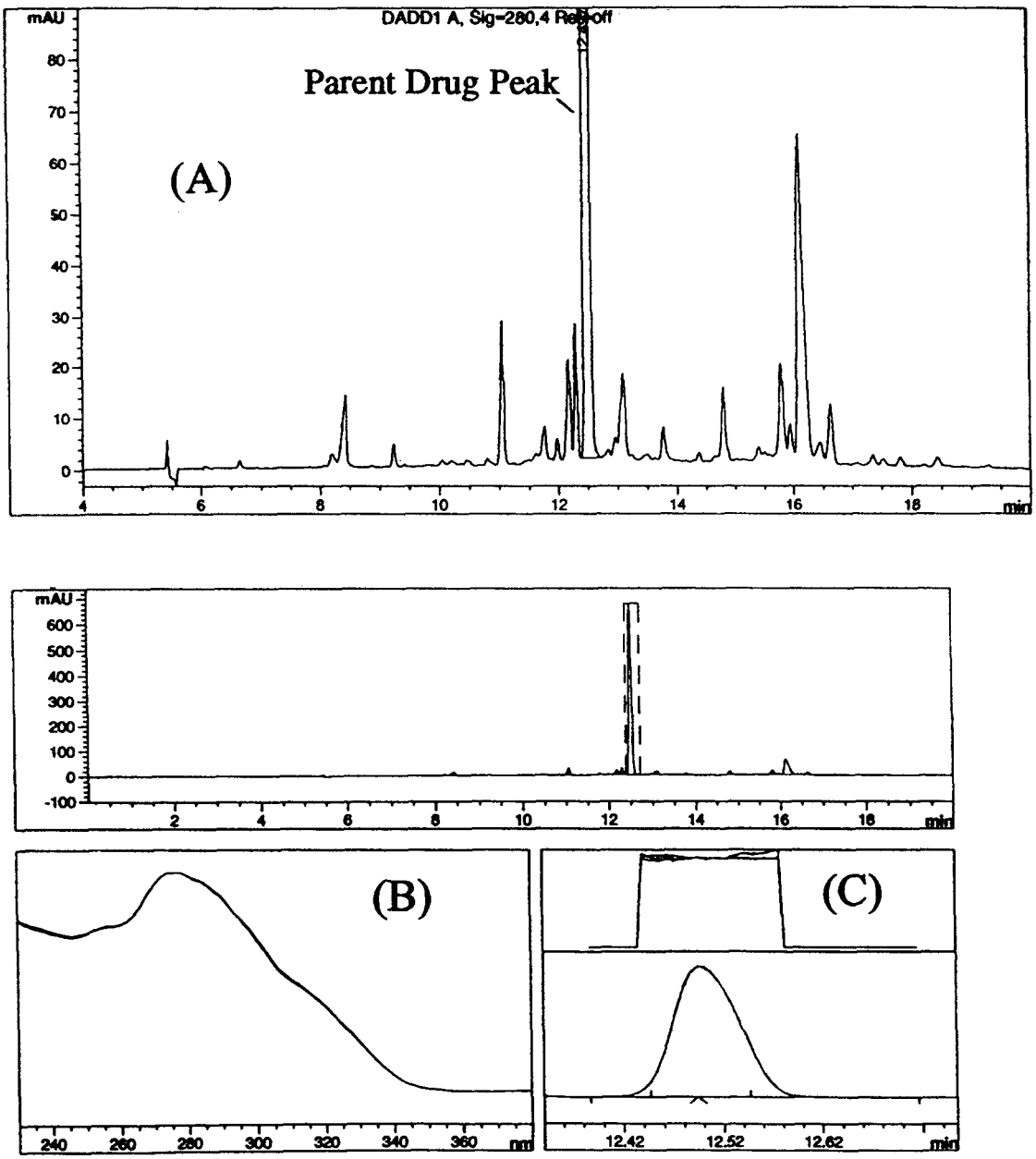

Fig. 7. Assessment of the peak purity of the TMT-NCS peak in a stressed sample as determined using a diode-array detector. Conditions were as given in the experimental section using the HP CZE instrument. (A) Sample electropherogram; (B) overlay of 7 spectra across the main peak (purity level, 999.7); and (C) wavelength ratio plot.

subsequent injection in the average purity results for TMT-NCS due to the slow but measurable degradation of the sample over the 2.5 h needed to complete the six analyses. Therefore, the true precision in the absence of any degradation is probably better than that documented here.

The precision of the method was evaluated by performing six assays (six separate sample preparations) on a typical sample. The RSD for the overall purity estimate of TMT-NCS was 0.25%.

RSDs for individual impurities ranged from 0.65% (impurity at 2.4%) to 8.6% (impurity at 0.13%).

Fig. 6 shows a comparison of the CE electropherogram with the RP-HPLC chromatogram of the stressed sample of TMT-NCS shown in Fig. 2. The numbers identify the identical components in each separation. This correlation of peaks was achieved by isolating the HPLC peaks and analyzing them by CE. In this experiment, none of the impurity peaks isolated by HPLC were found to coelute with TMT-NCS in the CE separation. In addition, this sample along with other stressed samples of TMT-NCS were evaluated for purity using the diode-array peak purity function of the prototype HP system. Fig. 7 summarizes the results from this experiment. The peak purity results indicated a homogeneous TMT-NCS peak supporting the specificity of the method.

4. Conclusion

A CE purity method was developed for the novel TMT-NCS chelator. The use of EDTA and acetonitrile as buffer additives was found to be useful in eliminating peak artifacts and improving peak symmetry and the resolution of impurities from the intact compound. Sample stacking using a pH focusing mechanism was effective in increasing sensitivity for low concentration impurities down below the 0.1% level. The preliminary validation data demonstrate that the method has the requisite selectivity, linearity, sensitivity and reproducibility to be used for the purity determination of TMT-NCS.

Acknowledgment

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