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Micellar electrokinetic chromatography as a generalized alternative to high-performance liquid chromatography for purity determination of a class of investigational antibacterial drugs

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Abstract

Micellar electrokinetic chromatography (MEKC) was successfully used to provide purity data for a number of oxazolidinone antibacterials. A run buffer of 100 m*M* SDS and 40 m*M* HEPES (pH 7.5, NaOH) separated fifteen different materials of neutral and cationic, anionic or zwitterionic character, usually with efficiencies ten-fold of those observed for HPLC. Different HPLC conditions were required for compounds with different structural characteristics. While the high efficiency and finite migration window of MEKC may allow observation of impurities not seen by HPLC, general use of this method for purity screening of combinatorial compounds will require micellar solutions tolerant of high amounts of organic, in order to accommodate materials of low aqueous solubility.

Keywords: Antibacterial drugs; Oxazolidinones

1. Introduction

Micellar electrokinetic chromatography (MEKC) has shown increasing popularity as a method for characterizing drug substances [1–4]. While HPLC has traditionally been the method of choice for purity determination of pharmaceutical substances, the advantages of MEKC (higher efficiency; speed; minimal sample requirement) may show it to be superior to HPLC for certain applications. While free-solution CE has been used for purity and affinity screening of combinatorial libraries [5–7], no references found in the literature mention the use of MEKC for purity screening of combinatorially produced drugs.

PNU-100 766 and PNU-100 592 (linezolid and eperezolid, I and V in Fig. 1) are antibacterial drugs presently in clinical trials for infections caused by multiply-resistant bacterial strains; preclinical efficacy has been established [8]. The objective of this work was to examine MEKC as an alternative to HPLC for purity determination of linezolid and some of its congeners (the oxazolidinones; other representative structures in Fig. 1), and to determine whether MEKC might provide a viable and superior alternative to HPLC for non-combinatorially produced drug candidates. Based on the results of this investigation, an assessment might be made regard-

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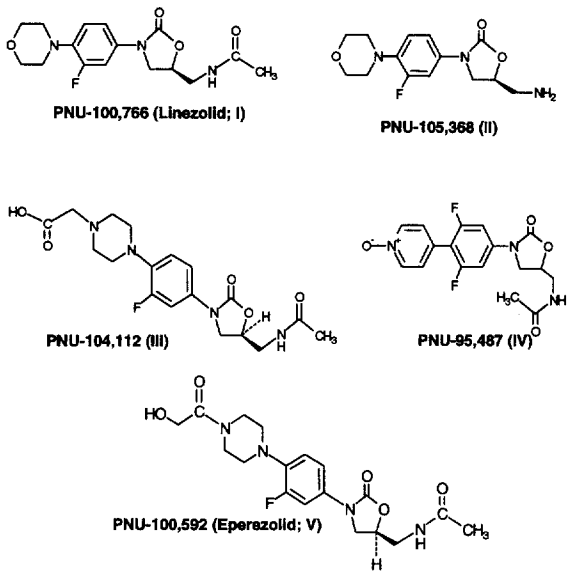

Fig. 1. Representative structures of oxazolidinone antibacterial drugs.

ing the prospects of MEKC for purity screening of combinatorial candidates.

2. Experimental

2.1. Materials

Sodium dodecyl sulfate (SDS) and sodium hydroxide were either J.T. Baker (Phillipsburg, NJ, USA) or Mallinckrodt (St. Louis, MO, USA) AR grade; HEPES (2-hydroxyethyl-1-piperazine-ethanesulfonic acid) and *n*-alkylphenones were from Aldrich (Milwaukee, WI, USA); solvents were EM Science HPLC Grade; water was 18 MΩ as delivered by a Milli-Q (Millipore, Bedford, MA, USA) water purifying system. Other chemicals (including HPLC mobile phase modifiers triethylamine, glacial acetic acid and trifluoroacetic acid) were AR grade or better from established suppliers. Materials assayed were from either the Pharmacia and Upjohn Laboratory Specialty Services collection or the Bulk Drug Profile Archive Sample collection.

Fused-silica capillary tubing was untreated, polyimide coated, 375 μm O.D.×50 μm I.D. from Polymicro Technologies (Phoenix, AZ, USA). Columns for HPLC comparison were either C_8 or C_{18} bonded silica from YMC (Wilmington, NC, USA), Mac-Mod (Chadds Ford, PA, USA), or Waters (Milford, MA, USA).

2.2. Apparatus

A Beckman P/ACE 2050 CE instrument was employed, with 254 nm filter detection and a 57 cm (50 cm to detector window)×50 μm I.D. capillary. Positive voltage, 21 kV (370 V/cm) was used for all MEKC separations for 25 min; the capillary cartridge was thermostatted at 30°C. Data collection was accomplished using an in-house chromatography data system, with Perkin-Elmer/Nelson (Cupertino, CA, USA) Model 941 Series interface boxes transferring the digitized signal to a Harris Computer Corp. (Fort Lauderdale, FL, USA) Nighthawk computer for processing. Microsoft EXCEL (Redmond, WA, USA) was used to normalize MEKC peak response to migration time, as is required for area-percent electrokinetic methods [9].

HPLC analysis was performed on a Hewlett-Packard 1090M liquid chromatograph, with diode array detection at 254 nm and identical data collection hardware and software.

2.3. Procedures

The run buffer was prepared by adjusting the pH of a 100 m*M* SDS solution containing 40 m*M* of HEPES to 7.5 with 1 *M* and 0.1 *M* NaOH. Samples were dissolved up to their solubility limit or a maximum of 2.5 mg/ml in a 10 m*M* SDS solution; the alkylphenone solution to determine the migration window was prepared from stock solutions (10 mg/ml) of *n*-alkylphenones in acetonitrile, dried down and reconstituted at 0.1 or 0.2 mg/ml in 10 m*M* SDS. A linearity study for PNU-100 766 was performed by using the 2.5 mg/ml solution as the top level of a series of halving dilutions down to 2.44 μg/ml in 10 m*M* SDS. The capillary was rinsed with run buffer for 4 min before a 5-s pressure injection of sample; post-run rinses were with 100 m*M* NaOH and 18 MΩ water, for 2 min each.

HPLC procedures for comparison employed YMC C-8 or ODS-AM; Waters Symmetry C-8; or Mac-Mod Zorbax SB-C18 or Rx-C8 columns. HPLC purity methods for comparison with MEKC ranged

from 37 to 60 min in length, and generally (except for chromatograms shown in Figs. 2B and 5B) allowed the major component to elute on an initial isocratic portion with water–methanol or water–acetonitrile, with a gradient to stronger solvent after major peak elution. Triethylamine, glacial acetic acid and/or trifluoroacetic acid were added when necessary to obtain good major component peak shape.

Both MEKC and HPLC determinations were performed in triplicate with appropriate blank correction to confirm the presence of minor components.

3. Results and discussion

3.1. Size of migration window

The elution profile for *n*-alkylphenones showed the EOF disturbance (t_{EOF}) at about 3 min. Near-co-migration of octanophenone with total co-migration (confirmed by separate injection) of decano- and dodecanophenone, suggested that the micellar migration time (t_{MC}) is at this co-migration (about 14.2 min). The latest time observed for any components of materials assayed in this 25-min method was about 15 min, lending further credence to the existence of t_{MC} here. This would mean that all neutral, and most charged, compounds, should migrate between about 3 and about 14 min.

3.2. MEKC of production-quality lots of linezolid (I)

The linearity of response for linezolid was described by the equation [area of linezolid peak]= (42 832±635)·[mg/ml linezolid]+(454.09±578) with $n=10$ and $r^2=0.9997$. The noticeable trend of the regression points below the least-squares fit line for the bottom concentrations suggests that there may be some adsorption of this material to the capillary wall; the estimated bias at these levels is near 0.1% of the top concentration, or about 2–3 μg/ml.

A total of sixteen lots of linezolid were compared using an area-percent purity method and the MEKC conditions described, with an RP-HPLC impurities method that sums all observed impurities above 0.05% by area. After also excluding all impurities of 0.05% or less in the MEKC method, the average value obtained for fourteen lots assaying at least 0.05% impurities was 0.22% by MEKC and 0.12% by HPLC. In three of the fourteen lots were there a greater number of impurities seen by MEKC than by HPLC; the reverse was true for two other lots (a total of 3 impurities with any peak area greater than 0.05% was the most ever observed in any single lot). The observation of roughly twice the average impurities values by MEKC could be due to a number of factors, including differential detector behavior, response linearity differences and consistent observation of a different, higher-responding impurity by MEKC. Impurity values for production lots of linezolid were low enough to allow one to say that this average difference is insignificant, but could be attributable to some as yet undetermined systematic difference in the separations.

3.3. Comparative separations of variously-charged analogues

Fig. 2 compares the MEKC migration pattern for a very crude sample of I with the HPLC method presently used for impurities quantitation for this material. Note that the observable impurities spectrum migrates between 7 and 12 min for MEKC (28 peaks observed); the HPLC method, while resolving 6 more components, requires a window from 5 to 31 min to observe all eluted impurities. At the pH of the run buffer (7.5), linezolid is expected to be neutral, as the piperazine amine nitrogen has a measured pK_a of about 1.8. This crude sample shows rather poor purity agreement between the two methodologies, with MEKC again giving a lower purity value than HPLC. This is possibly due to non-elution of material in the HPLC method, which uses only 75% acetonitrile at the end of the gradient elution. All solubilized material in the MEKC separation will probably be observed in the migration window of 3–15 min.

Figs. 3–5 compare separation methodologies for structures II–IV, which are expected to be cationic, anionic and zwitterionic compounds, respectively, at the pH of the MEKC run buffer. While MEKC elutes all neutral material during the migration window,

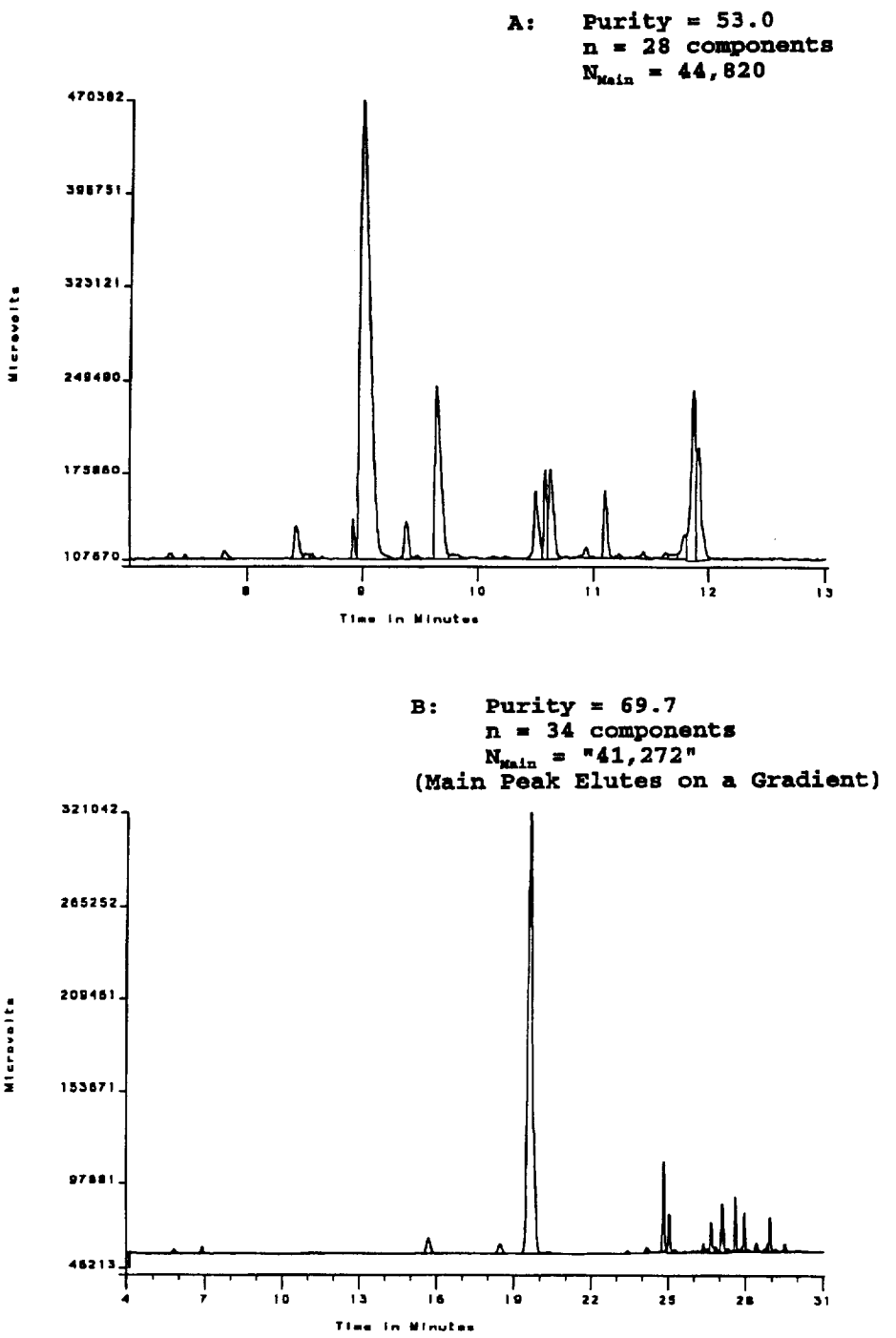

Fig. 2. Comparison of MEKC (A) and HPLC (B) separation of a crude sample of PNU-100 766 (linezolid, I). MEKC conditions: 57 (50 to detector) cm×50 μm I.D. bare silica; run buffer: 100 m*M* SDS, 40 m*M* HEPES, pH 7.5; +21 kV, 254 nm UV, 30°C, 5-s pressure injection of 2.5 mg/ml in 10 m*M* SDS. HPLC conditions: YMC ODS-AM 15×0.46 cm I.D. column; acetonitrile–water (10:90) with 0.1% trifluoroacetic acid for 2 min, linear gradient to acetonitrile–water (75:25) (0.1% TFA) at 35 min (2 min hold at 75% acetonitrile); 1.0 ml/min, 254 nm UV; 10-μl injection of 0.4 mg/ml in acetonitrile–water (10:90).

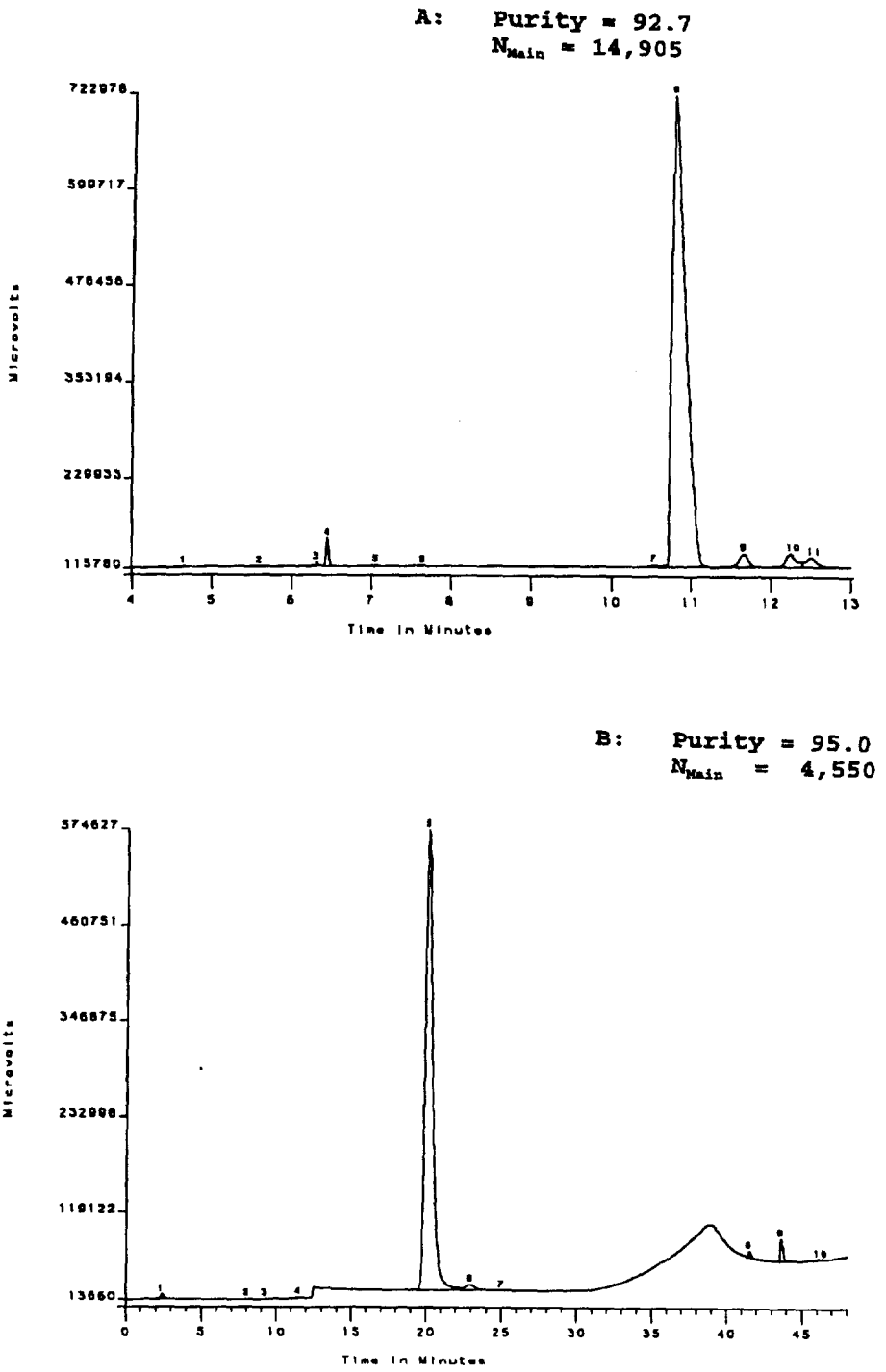

Fig. 3. Comparison of MEKC (A) and HPLC (B) separation of an impure sample of PNU-105 368 (II). MEKC conditions as in Fig. 2. HPLC conditions: YMC-C8 15×0.46 cm I.D. column; acetonitrile–water (15:85) with 0.2% triethylamine for 25 min, linear gradient to acetonitrile–TEA (99.8:0.2) at 55 min (5 min hold); 0.5 ml/min, 254 nm UV; 8-μl injection of 0.5 mg/ml in initial mobile phase.

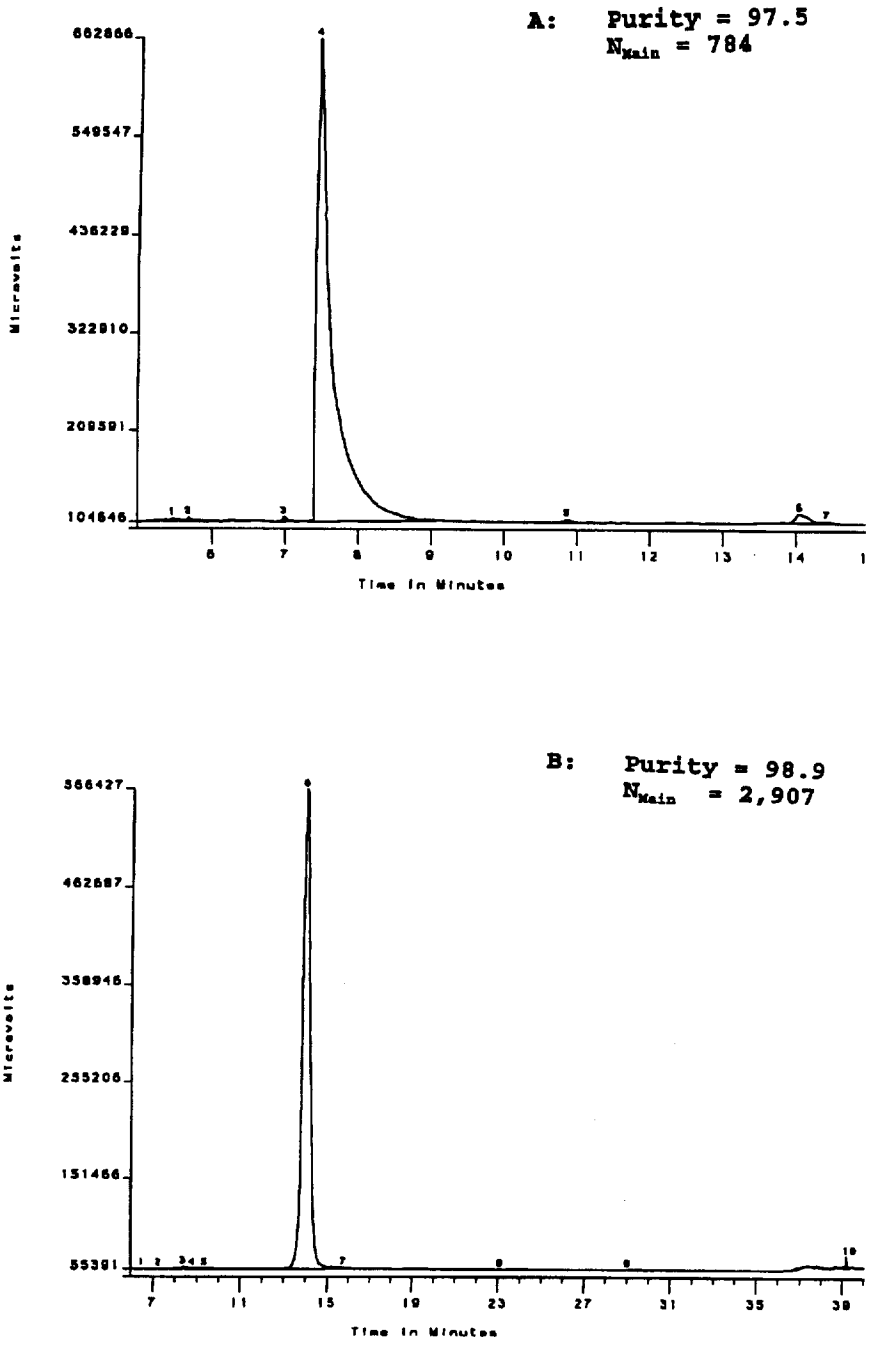

Fig. 4. Comparison of MEKC (A) and HPLC (B) separation of an impure sample of PNU-104 112 (III). MEKC conditions as in Fig. 2. HPLC conditions: Waters Symmetry C-8 15×0.39 cm I.D. column; acetonitrile–water (7.5:92.5) for 30 min, linear gradient to 100% acetonitrile at 50 min (5 min hold); 0.5 ml/min, 254 nm UV; 10-μl injection of 0.6 mg/ml in initial mobile phase.

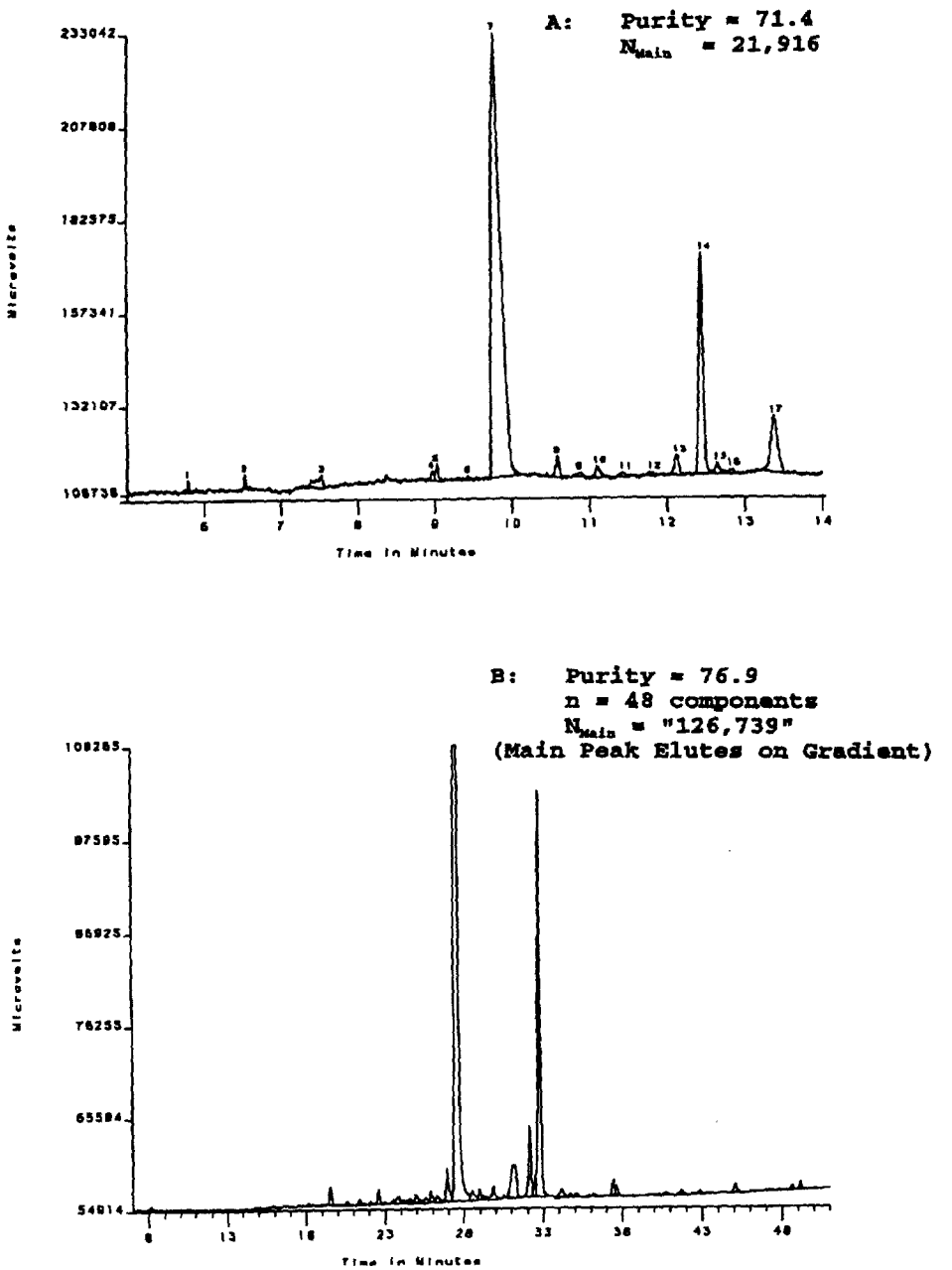

Fig. 5. Comparison of MEKC (A) and HPLC (B) separation of an impure sample of PNU-95 487 (IV). HPLC separation is a 7-fold expansion of the response. MEKC conditions as in Fig. 2. HPLC conditions: Zorbax Rx-C8 25×0.46 cm I.D. column; acetonitrile–water (7.5:92.5) for 5 min, linear gradient to acetonitrile–water (90:10) at 55 min (5 min hold); 0.5 ml/min, 254 nm UV; 10-μl injection of 0.8 mg/ml in initial mobile phase.

compounds with other charge states are also observed. There is good agreement for the two methods for these compounds, despite the poor efficiency for the main component III, which is anionic.

Table 1 gives comparative purity and impurities data for related oxazolidinones using the MEKC conditions described herein, and various sets of RP-HPLC conditions. While eight of the eleven tabulated materials show agreement between the two methods to within 2%, the two materials with the greatest disagreement give a lower purity when assayed by MEKC than when assayed by HPLC. Again, this discrepancy is unexplained but might be due to any of the factors discussed previously.

Fig. 6 is an electropherogram showing co-injection of eight compounds injected at 1–5% of the level expected for an oxazolidinone with full 2.5 mg/ml solubility and expected UV_{254} response. Linezolid co-migrates with both compounds E and F, which could account for the somewhat lower efficiency for this peak.

3.4. MEKC peak parameters for other oxazolidinones

In order to further assess the scope of this technique for oxazolidinones, a second set of twelve variants was assayed. The area percent purity for this set ranged between 81 and 100%; theoretical plate numbers ranged from 9100 to 113 500. A second anionic compound in this set gave very good peak shape and efficiency (N=41 000), in contrast to PNU-104 112 (Fig. 4). The lowest response observed for this second set of materials was 71 mV (7% of full scale). Low response was likely due to poor solubility, which was not encountered with most of the compounds of this class. Only one other material examined (of about 30) gave solubility so

Table 1
Comparison of numbers of impurities and area percent purity results for eleven oxazolidinones: MEKC vs. HPLC

Compound charge state at pH of MEKC run buffer		MEKC number of impurities	MEKC area perecnt purity	HPLC number of impurities	HPLC area % purity and (MEKC–HPLC) purity difference
A	Neutral	3	99.9	4	99.9 0.0
B	Cationic	9	98.5	13	96.2 +2.3
C	Neutral	7	97.1	8	96.8 +0.3
D	Cationic	0	100.0	4	99.3 +0.7
E	Neutral	1	99.9	4	99.7 +0.2
F	Neutral	8	98.9	8	99.2 −0.3
G	Cationic (possibly doubly charged)	9	90.2	8	97.5 −7.3
H	Neutral	6	97.9	1	99.3 −1.4
I	Zwitterionic	5	97.3	2	99.8 −1.5
J	Neutral	5	99.4	2	99.7 −0.3
K	Zwitterionic	5	95.2	3	99.3 −4.1

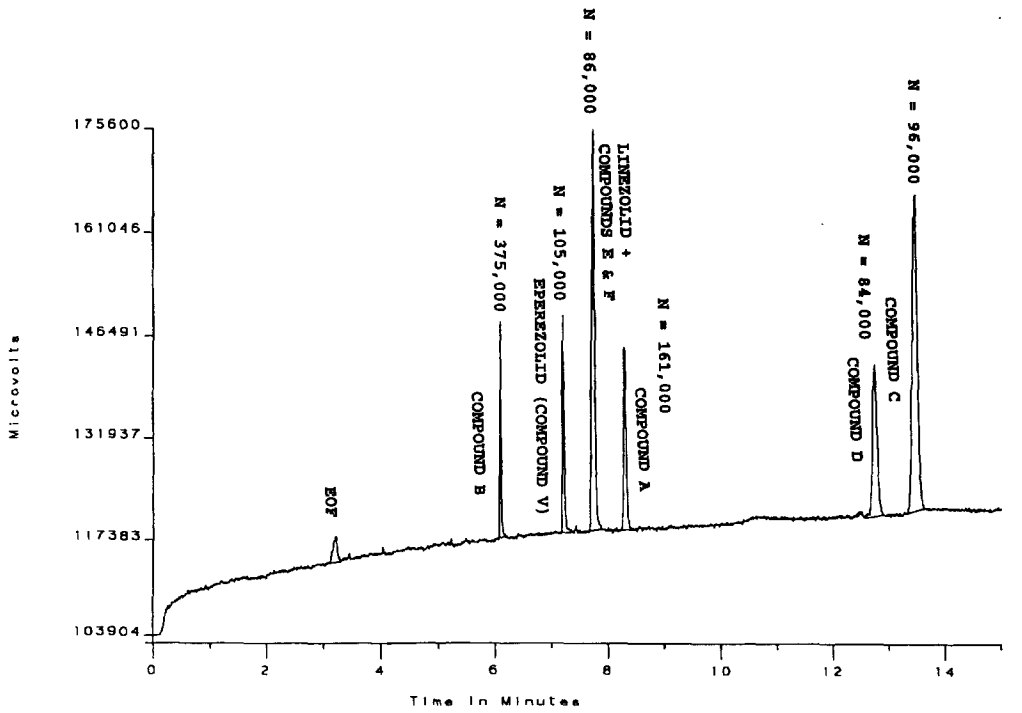

Fig. 6. Co-injection of eight oxazolidinones at 1–5% of the MEKC assay's target concentration. Conditions as in Fig. 2.

low as to preclude the use of 10 m*M* SDS as sample solvent.

3.5. Advantages and limitations of this method

The two main advantages of MEKC over HPLC for purity screening of compound families are (1) efficiencies often ten-fold that of isocratic HPLC methodology and (2) assurance of detection of all soluble material in a sample. With short (20 cm) capillaries, run times might be shortened to 3 min or less with little sacrifice in efficiency. Additional advantages include sample size (10–30 nl injected out of 5-μl volume) and fast run times, but HPLC is approaching MEKC with respect to these features.

The major limitation of MEKC is the incompatibility of micellar run solutions with organic solvents. Only about 20% acetonitrile was tolerated by the system described here, with respect to peak shape and retention; this level of organic was insufficient to adequately dissolve most compounds examined outside the oxazolidinone class. Use of a high-sensitivity flow-cell such as that supplied with the PE-ABI instrument [10] may overcome some of this solubility limitation, albeit with some loss in resolution and/or efficiency.

4. Conclusions

An aqueous, SDS-based run buffer provided purity data for a large number of oxazolidinone antibacterials using MEKC, in a run time one-quarter to one-third that used for HPLC assay of the same materials. Linezolid purity data for sixteen production lots agreed well with HPLC data for this high-purity material, with the average impurities levels by MEKC being about twice that shown by HPLC (0.2 versus 0.1%).

Of fifteen lower-purity oxazolidinones of various

structural types, nine showed agreement to within 2% between the two methodologies, with a single set of MEKC conditions and various conditions for HPLC. Of the six materials giving greater than 2% discrepancy between the methodologies, five gave lower purity results by MEKC than by HPLC. While this systematic difference could be due to differential linearity or detector response, it also could be due to the fact that the higher efficiency and finite migration window of MEKC is allowing observation of material not seen by HPLC.

Further development of MEKC for generalized combinatorial screening of families of compounds will depend upon the ability to use organic solvents. As micellar solutions are not maintained in totally non-aqueous environments, this restricts the use of this technique to compounds with appreciable water solubility. Ionic polymers [11,12] have much higher tolerance for organic solvents with respect to micelle structure, and these may prove to be an option for high-throughput purity screening of combinatorial molecules with limited water solubility.

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