A High-Performance Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Cethromycin (ABT-773) in Human Plasma, Bronchoalveolar Lavage Fluid, and Alveolar Cells

Qiulei Ren[1], John E. Conte, Jr.[2,*], Elisabeth Zurlinden[2], and **Emil T. Lin[1]**

[1]Department of Biopharmaceutical Sciences and [2]Department of Epidemiology and Biostatistics, Infectious Diseases Research Laboratory, University of California, San Francisco, San Francisco, CA 94117

Abstract

A method is developed for the specific and sensitive determination of cethromycin concentrations in plasma, bronchoalveolar lavage (BAL), and alveolar cells (AC), using a high-performance liquid chromatographic–tandem mass spectrometry (MS) method. The mobile phase consists of 50% acetonitrile–0.05% acetic acid–5mM ammonium acetate; the column used is a C_8 reversed-phase stationary phase. The preparation of samples requires a solvent extraction step. The retention times for cethromycin and the internal standard are approximately 2.0 and 2.7 min, respectively, with a total run time of 3.5 min. Detection is carried out using electrospray MS in a multiple reaction monitor mode. The detection limits for cethromycin are 1 ng/mL for plasma and 0.2 ng/mL for BAL supernatants and AC suspensions. The assay has excellent performance characteristics and has been used to support a study of the intrapulmonary pharmacokinetics of cethromycin in human subjects.

Introduction

The rapid emergence of drug-resistant bacteria has encouraged the development of new antibiotics (1,2). Cethromycin (shown in Figure 1) is an investigational agent under evaluation for its effectiveness against penicillin- and macrolide-resistant gram-positive bacteria. It is rapidly absorbed with maximum plasma concentrations occurring 1 to 3 h after oral administration (3). High-performance liquid chromatography (HPLC) has been used for the determination of cethromycin plasma using fluorescence detection (4). Liquid chromatography (LC)–tandem mass spectrometry (MS) techniques are widely utilized in the analysis of compounds in biological samples because of their high specificity and sensitivity. These assays usually do not require a derivatization procedure and have shorter analysis times than those provided by less specific methods, such as those that utilize UV detectors (5–8). In this report, a sensitive HPLC–MS method has been developed to measure cethromycin in plasma (detection limit, 1 ng/mL), bronchoalveolar fluid (detection limit, 0.2 ng/mL), and alveolar cell (AC) suspensions (detection limit, 0.2 ng/mL). Compared with the previously reported methods, the technique has the advantages of increased sensitivity and a capability to analyze small sample volumes. The specificity of HPLC–MS–MS detection also minimizes the risk of interference from other substances in the biological samples. This is especially important when analyzing specimens from patients such as those with AIDS who may be taking concomitant medications. Our assay is being used to support a Phase 1 study of the intrapulmonary pharmacokinetics of cethromycin in normal subjects.

Experimental

Chemicals

All solvents and chemicals were HPLC grade except acetic acid, which was reagent grade. A 1.0-mg/mL solution of cethromycin (Abbott Laboratories, Abbott Park, IL) was prepared in 50% acetonitile and stored in a refrigerator at 4°C. This solution was further diluted to produce working solutions of 0.1, 1.0, and 10 µg/mL of cethromycin for spiking of standard curves. The internal standard (Figure 1) used was a synthetic analog of the analyte (ABT-257). The internal standard stock solution of 1.0 mg/mL was prepared in 50% acetonitrile. It was then diluted to a concentration of 5 µg/mL for use as the internal standard in the plasma assay and 0.5 µg/mL for the bronchoalveolar lavage (BAL) supernatant and AC suspension assays.

* Author to whom correspondence should be addressed: email jconte@aids2.ucsf.edu.

Chromatography

Mobile phase containing 50% acetonitrile–0.05% acetic acid–5mM ammonium acetate was used with a BDS Hypersil C_8 column (4.6 mm × 50 mm, 3-µm particle size) (Keystone Scientific, Bellefonte, PA) at a flow rate of 0.8 mL/min using a Shimadzu LC-10AD pump (Shimadzu, Walnut Creek, CA) or equivalent. Extracts prepared from samples were injected onto the system with a Waters Intelligent Sample Processor 717 Plus (Waters, Milford, MA). The retention times for cethromycin and the internal standard were approximately 2.0 and 2.7 min, respectively, with a total run time of 3.5 min.

MS

A Micromass Quattro-LC system (system I) (Micromass, Manchester, U.K.) was utilized for the assay of cethromycin in plasma and a Micromass Quattro-Ultima (system II) (Micromass) was utilized for the assay in BAL and AC. The MS settings and conditions were as follow: the multiple reaction monitor scanning mode was set at *m/z* 776–168 for cethromycin and 853.5–168 for the internal standard. Electrospray with positive ionization was used. The desolvation gas temperature, the sample cone voltage, and the capillary were set at 400°C, 55 V, and 1.5 KV, respectively, for system I. The collision energy was set at 44 eV for both cethromycin and the internal standard. As for system II, the parameters were: desolvation temperature, 400°C; sample cone, 56 V; capillary voltage, 3.5 kV; and collision energy, 40 eV.

Sample preparation

Standard curves

Plasma standard curves were prepared by adding appropriate volumes of cethromycin working solutions into 0.5 mL of blank plasma to yield the following concentrations: 1, 2, 4, 8, 16, 32, 80, 160, 320, 640, and 1000 ng/mL of cethromycin. The standards for BAL supernatants and cell suspensions were spiked to yield concentrations of 0.2, 0.4, 0.8, 1.6, 4, 8, 20, 40, 80, and 200 ng/mL. The standard curves were constructed by 1/*y* weighted least-squares linear regression of the cethromycin to internal standard peak area ratios versus the spiked nominal concentration of cethromycin.

Preparation of plasma standards and samples

The sample preparation consisted of addition of the internal standard to an aliquot of 0.5 mL of plasma. This was followed by extraction with 3 mL of methyl *t*-butyl ether. The extract was evaporated to dryness and reconstituted with 50% of acetonitrile prior to the injection to LC–MS–MS.

Preparation of BAL supernatants and AC pellet standards and samples

In order to calculate the cell volume collected in the BAL (9,10), a white cell count and differential (identification of different cell types) were performed on the BAL fluid, then a 30-mL aliquot was centrifuged at 400 × g for 5 min, and the supernatant was immediately separated from the cells. The cell pellet was then resuspended volumetrically in distilled water and sonicated for 5 min to lyse the cells. Standards and controls for AC suspensions were prepared in a suspension of monocyte enriched human leukocytes (Biological Specialty Corp., Colma, PA), which were purified through a Ficoll-Hypaque at a density 1.077 g/mL (Histopaque 1077, Sigma, St. Louis, MO) separation and sonicated. An aliquot of 0.5 mL of blank BAL supernatant, monocyte enriched leukocytes, or AC suspensions were then taken, and the pH value of the sample was adjusted by adding 50 µL of a 0.1M NaOH solution. The sample was extracted with 3.0 mL of methyl *t*-butyl ether. The extract was evaporated to dryness and reconstituted with 50% acetonitrile prior to the injection onto the LC–MS–MS.

Preparation of controls for method validation

Two sets of stock solution were prepared, one used for spiking standards and the other for spiking controls. Measured amounts of plasma were spiked at three control levels: 2, 40, and 800 ng/mL in duplicate and frozen at –70°C for stability studies. The samples were removed from storage and analyzed at selected intervals up to 6 months. The control samples at three concentrations were also subjected to five cycles of freeze/thaw tests and benchtop stability (at ambient temperature) tests up to 6 h. To assess the interday reproducibility, standard curves with controls spiked at three concentrations of 2, 40, and 800 ng/mL were analyzed on six different days in duplicate. Intraday reproducibility was assessed by analyzing six preparations of each of the three concentrations on the same day. In addition, six samples representing the lower limit of quantitation (LLOQ) were prepared within the same run. The recoveries were obtained from the measurement of the peak response ratios (drug–internal standard) of

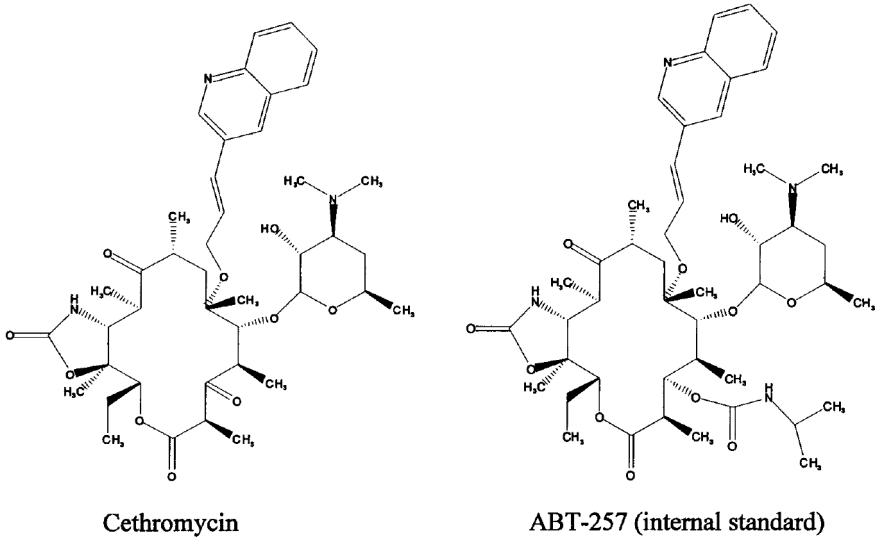

Figure 1. Structures of cethromycin and ABT-257 (internal standard).

processed samples to unprocessed samples, evaluated in triplicate at three concentrations. Similarly, the validation for BAL supernatant and AC suspension was conducted at the following concentrations: 0.4, 8, and 160 ng/mL. The long-term stability was evaluated up to three months.

Results and Discussion

LLOQ assessments

The daughter ion spectra of cethromycin and the proposed fragmentation of the compound are illustrated in Figure 2. The transition from *m/z* 766 to 168 was possibly attributable to the cleavage of the C–O bond that causes the formation of a quinolyl propenyl ion (*m/z* 168). This transition was monitored for the detection and quantitation of cethromycin. The fragmentation of the internal standard compound (spectra not shown) was indicative of the same cleavage at the C–O bond. A representative HPLC–MS–MS chromatogram of cethromycin and internal standard in BAL supernatant is shown in Figure 3. Chromatograms for plasma and AC are similar. The blank tests did not show significant interfering peaks because of endogenous compounds at the retention times of cethromycin or internal standard. Therefore, the specificity of the method was excellent. The linear range for the plasma assay was from 1 to 1000 ng/mL and the range was from 0.2 to 200 ng/mL for both BAL and AC assays. The mean (± SD) coefficient of determination (r^2) from 18 standard curves (6 in plasma, 6 in BAL, and 6 in ACs) was 0.986 ± 0.0160. The LLOQ was equivalent to the lowest point of the standard curve. The values were 1 ng/mL for plasma and 0.2 ng/mL for BAL supernatants and AC suspensions. The LLOQ in plasma had a mean value of 1.07 ng/mL with a CV of 8.76% for the intraday evaluation (n = 6). The interday evaluation was carried out by taking the six back-calculated concentrations of the lowest point of the standard curves for interday runs. The mean interday LLOQ in plasma was 0.966 ng/mL with a CV of 2.83%. In BAL the mean LLOQ was 0.199 ± 0.0121 ng/mL and 0.201 ± 0.00585 ng/mL for intraday and interday evaluation, respectively. The corresponding values in AC were 0.228 ± 0.0171 ng/mL and 0.196 ± 0.00509 ng/mL, respectively.

Assay precision, accuracy, and recovery

Results for assay precision and accuracy assessments (11) in plasma, BAL, and AC suspension are summarized in Tables I–III. The mean (± SD) coefficients of variation and ranges of the assay for intraday and interday determination together for

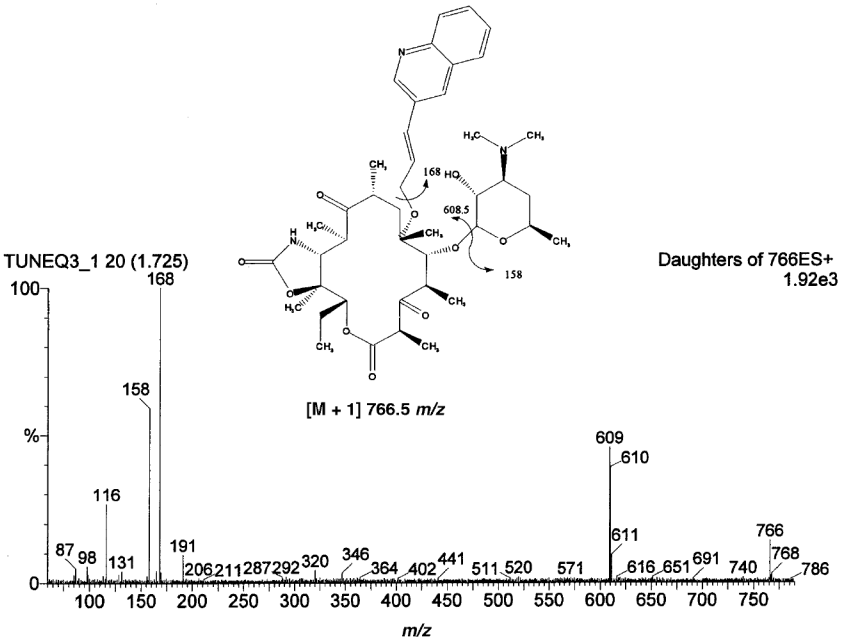

Figure 2. Daughter ion spectra and proposed fragmentation of cethromycin.

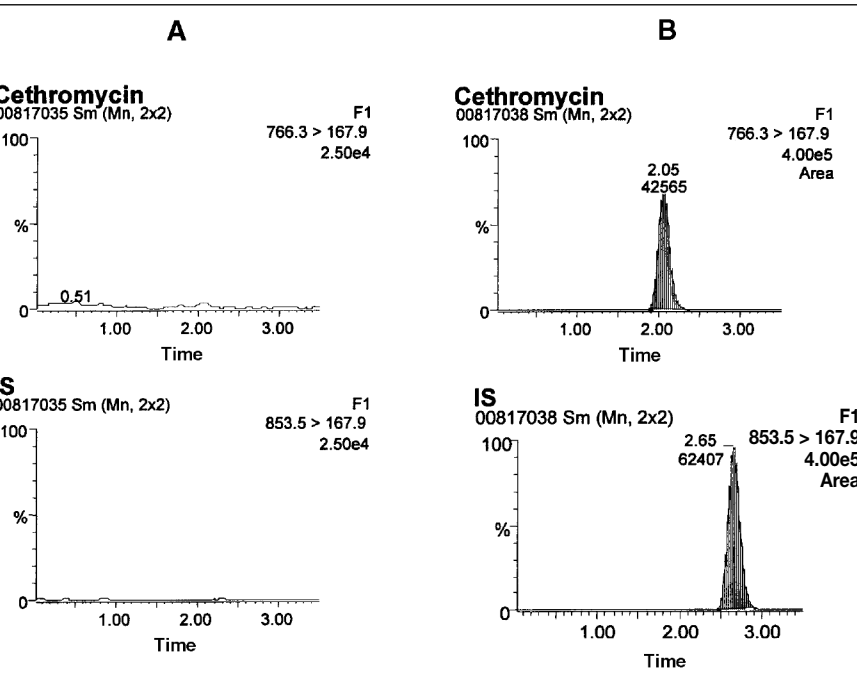

Figure 3. Blank BAL (A) and study subject's BAL obtained 4 h after the 5th dose of 300 mg of cethromycin administered once daily (14.8 ng/mL) (B). The top is cethromycin and the bottom is the internal standard.

plasma, BAL supernatants, and AC suspensions were 6.65 ± 3.16 (range 2.51–10.5%), 6.64 ± 2.43 (range 2.74–8.92%), and 7.14 ± 2.70 (range 2.08–9.58%), respectively. The accuracy ranges for all determinations in plasma, BAL supernatants, and AC suspensions were –8.89–12.0%, –5.67–11.6%, and –0.229–11.9%, respectively.

The absolute recovery of cethromycin, that is, the amount of drug recovered from the matrix during the sample preparation (11), was evaluated by comparing the processed spiked sample with the unprocessed spiked sample. The recovery results are shown in Table IV. The overall mean recoveries from all of the three concentration levels were 89.8% for the plasma assay, 81.8% for the BAL assay, and 92.4% for the AC assay. The internal standard recoveries were 86.4% for plasma, 97.2% for the BAL, and 101% for the AC (all experiments in triplicate).

Stability

The results of repeated determination of cethromycin in spiked plasma, BAL supernatants, and ACs stored at –70°C revealed no significant degradation of the drug. These determinations were performed over a period of six months for plasma and three months for BAL supernatants and AC suspensions. Experimental results also indicated that cethromycin was stable in the biological samples at ambient temperature for up to 6 h and was stable after 5 freeze/thaw cycles (data not shown).

Patient data

The concentrations of cethromycin in plasma, BAL supernatant, and ACs in 5 of 30 subjects who participated in a study of intrapulmonary pharmacokinetics of cethromycin are summarized in Table V. Drug concentrations in epithelial lining fluid were calculated using the urea diffusion method, and AC concentrations were calculated using cell counts in alveolar fluid as previously reported (9,10). Bronchoscopy was performed at 4 h and blood samples were obtained at 2 and 4 h following the last dose of cethromycin, 300 mg once daily, that had been administered orally for 5 days. The cethromycin concentration in plasma ranged from 0.22 to 0.67 µg/mL at 2 h and 0.18 to 0.75 µg/mL at 4 h after the last dose was administered. The con-

Table I. Assay Precision and Accuracy for Cethromycin in Plasma

Spiked concentration (ng/mL)	Measured concentration (mean ± SD)	Coefficient of variation (%)	Accuracy (%)*
Intraday† (*n* = 6)			
2	2.10 ± 0.182	8.66	5.08
40	42.2 ± 1.06	2.51	5.38
800	749 ± 23.6	3.15	–6.44
Interday‡ (*n* = 12)			
2	2.18 ± 0.229	10.5	8.96
40	44.8 ± 3.39	7.56	12.0
800	729 ± 54.9	7.53	–8.89

* (measured concentration – spiked concentration)/spiked concentration × 100.
† Six separately spiked samples at each of three concentrations.
‡ On six different days, spiked samples at three concentrations and analyzed in duplicate.

Table II. Assay Precision and Accuracy for Cethromycin in BAL

Spiked concentration (ng/mL)	Measured concentration (mean ± SD)	Coefficient of variation (%)	Accuracy (%)*
Intraday† (*n* = 6)			
0.4	0.377 ± 0.0170	4.52	–5.67
8	8.93 ± 0.718	8.04	11.6
160	1.67 ± 4.58	2.74	4.48
Interday‡ (*n* = 12)			
0.4	0.400 ± 0.0312	7.81	0.00
8	8.09 ± 0.631	7.80	1.10
160	161 ± 14.3	8.92	0.521

* (measured concentration – spiked concentration)/spiked concentration × 100.
† Six separately spiked samples at each of three concentrations.
‡ On six different days, spiked samples at three concentrations, and analyzed in duplicate.

Table III. Assay Precision and Accuracy for Cethromycin in AC

Spiked concentration (ng/mL)	Measured concentration (mean ± SD)	Coefficient of variation (%)	Accuracy (%)*
Intraday† (*n* = 6)			
0.4 (*n* = 5)	0.418 ± 0.0391	9.36	4.55
8	7.98 ± 0.166	2.08	–0.229
160	164± 12.0	7.35	2.29
Interday‡ (*n* = 12)			
0.4	0.417 ± 0.030	7.29	4.27
8 (*n* = 8)	8.95 ± 0.858	9.58	11.9
160	163 ± 11.8	7.20	2.14

* (measured concentration – spiked concentration)/spiked concentration × 100.
† Six separately spiked samples at each of three concentrations.
‡ On six different days, spiked samples at three concentrations, and analyzed in duplicate.

Table IV. Recovery of Cethromycin from Plasma, BAL, and AC (*n* = 3)

Matrix	Spiked concentration (ng/mL)	Mean recovery (%)
Plasma	2	82.3
	40	89.5
	800	97.7
BAL	0.4	71.4
	8	89.1
	160	85.0
AC	0.4	93.8
	8	88.9
	160	94.6

Table V. Cethromycin Concentrations in Plasma, ELF, and AC in Five Adult Volunteer Subjects

Subjects	1	2	3	4	5
Plasma (µg/mL)					
2 h post 5th dose	0.52	0.31	0.22	0.66	0.67
4 h post 5th dose	0.33	0.31	0.18	0.40	0.75
ELF (µg/mL) 4 h post 5th dose	1.74	1.53	0.94	5.82	3.81
AC (µg/mL) 4 h post 5th dose	26.47	44.80	29.90	106.3	35.14

centrations in epithelial lining fluid (ELF) and AC ranged from 0.94 to 5.82 and 26.47 to 106.3 µg/mL, respectively.

Conclusion

We have developed a sensitive HPLC–MS–MS assay that provides specific, rapid, and reliable determination for cethromycin in small volumes of plasma, BAL, and AC. Preparation of plasma, BAL supernatant, and AC suspension samples requires a solvent extraction process. The stability data indicated that no significant drug degradation occurred in plasma or BAL supernatant stored at –70°C over a period of six and three months, respectively. The linearity of the standard curve, in the range described, was excellent. Assay precision was high for plasma, BAL, and AC. The performance characteristics of the assay make the method suitable for clinical and pharmacological studies, particularly those that are designed to quantitate the intrapulmonary concentration of drugs. The method has been used to support a Phase 1 study of the pulmonary pharmacokinetics of cethromycin in normal volunteers. In the preliminary analysis, cethromycin concentrations were considerably greater in the AC than in plasma or ELF, indicating that this antibiotic was concentrated in AC. The preliminary results also suggest that the drug was concentrated in ELF, but to a lesser extent than in AC. These findings may be of importance in the treatment of intrapulmonary infection. A complete analysis of this pharmacokinetic study has been prepared for publication elsewhere.

References

1. H. Neu. The crisis in antibiotic resistance. *Science* **257:** 1064–72 (1992).
2. M.D. Cohen. Antimicrobial resistance: prognosis for public health. *Trends Microbiol.* **2:** 422–25 (1994).
3. R. Pradhan, L. Gustavson, D. Londo, Y. Zhang, and M. Paris. *Single Oral Dose Pharmacokinetics and Safety of ABT-773 in Healthy Subjects.* Presented to the Interscience Conference on Antimicrobial Agents and Chemotherapy. September 2000, Toronto, Canada.
4. D. Xuan, M. Ye, M. Kim, C.H. Nightingale, and D.P. Nicolau. Pharmacokinetics of ABT-773, a new semi-synthetic ketolide in neutropenic lung-infected mice: a population approach. *J. Pharm. Pharmacol.* **54(1):** 71–75 (2002).
5. G. Singh, V. Arora, P.T. Fenn, B. Mets, and I.A. Blair. A validated stable isotope dilution liquid chromatography tandem mass spectrometry assay for the trace analysis of cocaine and its major metabolites in plasma. *Anal. Chem.* **71:** 2021–27 (1999).
6. Y. Wu, J. Zhao, J. Henion, W.A. Korfmacher, A.P. Lapiguera, and C.C. Lin. Microsample determination of lovastatin and its hydroxy acid metabolite in mouse and rat plasma by liquid chromatography/ionspray tandem mass spectrometry. *J. Mass Spectrom.* **32:** 379–87 (1997).
7. J.E. Conte, Jr., E. Lin, Y. Zhao, and E. Zurlinden. A High-pressure liquid chromatographic–tandem mass spectrometric method for the determination of ethambutol in human plasma, bronchoalveolar lavage fluid and alveolar cells. *J. Chromatogr. Sci.* **40:** 113–18 (2002).
8. J.E. Conte, Jr., G. Wang, E.T. Lin, and E. Zurlinden. A High-performance liquid chromatographic–tandem mass spectrometric method for the determinatiohn of ethionamide in human plasma, bronchoalveolar lavage fluid and alveolar cells. *J. Chromatogr. B* **753:** 343–53 (2001).
9. J.E. Conte, Jr., J.A. Golden, S. Duncan, E. McKenna, and E. Zurlinden. Intrapulmonary concentration of pyrazinamide. *Antimicrob Agents Chemother.* **43:** 1329–33 (1999).
10. J.E. Conte, Jr., J.A. Golden, S. Duncan, E. McKenna, and E. Zurlinden. Intrapulmonary pharmacokinetics of clarithromycin and erythromycin. *Antimicrob. Agents Chemother.* **39:** 334–38 (1995).
11. R.V. Smith and J.T. Stewart. *Textbook of Biopharmaceutic Analysis*. Lea and Febiger, Philadelphia, PA, 1981, pp. 79,112.

Manuscript accepted August 18, 2003.