In Vitro Activity of LY127935

ROBERT J. FASS

Division of Infectious Diseases, Department of Medicine, The Ohio State University College of Medicine,
Columbus, Ohio 43210

Received for publication 26 July 1979

LY127935 is a unique new β -lactam antibiotic. Its activity against 536 clinical isolates was studied by using microdilution methods of susceptibility testing and compared with the activities of cefamandole, cefoxitin, and cephalothin. The lowest concentrations required to inhibit at least 90% of strains tested (MIC_{90} s) of LY127935 for *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae* ranged from 2 to 8 μ g/ml. The MIC_{90} s for other staphylococci and streptococci were higher. The MIC_{90} s for *Enterobacteriaceae* and *Pseudomonas* species ranged from 0.12 to 8 μ g/ml and 8 to >32 μ g/ml, respectively. The MIC_{90} s for anaerobes ranged from 2 to >32 μ g/ml. As determined by MIC_{90} s, LY127935 was consistently the least active antibiotic against facultatively anaerobic gram-positive cocci and the most active against aerobic and facultatively anaerobic gram-negative bacilli. Its position with respect to activity against anaerobes varied from being the most active against *Bacteroides fragilis* and *Clostridium perfringens* to the least active against anaerobic cocci. In a population of multidrug-resistant isolates, concentrations of 8 μ g or less of LY127935 per ml inhibited 82% of *Enterobacteriaceae*; concentrations of 32 μ g or less per ml inhibited 100% of *Enterobacteriaceae* and 40% of *P. aeruginosa*. Increasing the inoculum size by 100-fold did not increase the minimal inhibitory concentrations of LY127935 or cefoxitin but did increase minimal inhibitory concentrations of cefamandole and cephalothin for some *Enterobacteriaceae*. All four drugs were bactericidal; minimal bactericidal concentrations were the same or one concentration higher than minimal inhibitory concentrations for 91 to 96% of strains tested. The broad spectrum and marked in vitro activity of LY127935 make it a promising new antibiotic.

LY127935 (compound 6059-S) is a new semi-synthetic β -lactam antibacterial agent which is highly active against a broad spectrum of micro-organisms. The replacement of sulfur with oxygen in the six-membered ring of the conventional cephem nucleus makes it unique among currently available β -lactam antibiotics (Lilly Research Laboratories, unpublished data).

This paper reports the in vitro susceptibilities of a broad spectrum of clinical isolates to LY127935, cefamandole, cefoxitin, and cephalothin.

MATERIALS AND METHODS

Organisms. The organisms studied included 498 randomly selected and 38 multidrug-resistant recent clinical isolates from patients hospitalized in The Ohio State University Hospitals, Columbus, Ohio. The randomly selected isolates included 86 strains of facultatively anaerobic gram-positive cocci (see Table 1), 271 strains of aerobic and facultatively anaerobic gram-negative bacilli (see Table 2), and 141 strains of anaerobes (see Table 4). Of the 357 aerobic and facultatively anaerobic strains, 60% were blood isolates; the

remainder were isolated from a variety of sources. The anaerobic bacteria were isolated primarily from wounds and abscesses. The multidrug-resistant isolates (see Table 3) included strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* which were resistant to 64 μ g of ampicillin, 128 μ g of carbenicillin, 4 μ g of gentamicin, and 8 μ g of tetracycline per ml.

Antibiotics. The antibiotics tested included LY127935, cefamandole, and cephalothin, which were obtained from Lilly Research Laboratories, Indianapolis, Ind., and cefoxitin, which was obtained from Merck, Sharp & Dohme, West Point, Pa. Laboratory standard powders were diluted as recommended by the manufacturers to stock concentrations of 1,000 μ g/ml and used immediately or frozen at -20°C , for up to 1 month, until used.

Susceptibility tests. Minimal inhibitory concentrations (MIC s) of the four study antibiotics were determined simultaneously and in duplicate for each isolate by microdilution methods. For aerobic and facultatively anaerobic organisms, antibiotics were diluted in broth and dispensed into microdilution plates with a Dynatech MIC-2000 (Dynatech Laboratories, Alexandria, Va.) dispensing instrument (3). Antibiotic-containing plates were stored at -20°C until used. For anaerobic organisms, a previously described method

(1) was used. Antibiotics were diluted in microdilution plates with a Medimixer (Flow Laboratories, Rockville, Md.) automatic diluter on the day of their use. Antibiotic concentrations consisted of log₂ dilutions which ranged from 32 to 0.01 µg/ml.

For aerobic and facultative gram-positive cocci and anaerobes, MICs were performed in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.); with *Bacteroides melaninogenicus*, *Fusobacterium*, and anaerobic cocci it was supplemented to contain 1% heat-inactivated (56°C for 30 min) horse serum. For aerobic and facultative gram-negative bacilli, MICs were performed in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with CaCl₂ and MgCl₂ to contain 5 mg of calcium and 2.5 mg of magnesium per dl, respectively.

Antibiotic-containing microdilution plates were inoculated with MIC-2000 disposable inoculators (Dynatech) so that the final inoculum was approximately 10⁶ to 5 × 10⁶ colony-forming units per ml. Inoculated plates were incubated at 37°C in either a room air incubator (aerobes and facultatives other than *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and

Streptococcus viridans) a 5% CO₂ incubator (*S. pyogenes*, *S. pneumoniae*, and *S. viridans*), or an anaerobic glove-box containing 85% N₂, 10% H₂, and 5% CO₂ (anaerobes). After 16 to 20 h (aerobes and facultatives) or 48 h (anaerobes) of incubation, plates were read with the aid of a magnifying mirror. The lowest concentrations of antibiotic which inhibited all visually apparent growth was considered the MIC. A control strain, *Escherichia coli* ATCC 25922 or *Bacteroides fragilis* ID 00170, was included with each group of organisms tested. For each antibiotic-organism combination, the MIC₅₀ was the lowest concentration of antibiotic which inhibited at least 50% of the strains tested, and the MIC₉₀ was the lowest concentration which inhibited at least 90 of strains tested.

To determine the effects of varying inoculum size on MICs, three strains each of nine organisms (see Table 5) were studied. MICs of the four study antibiotics were simultaneously determined with a standard inoculum, and inocula containing 100-fold higher and 100-fold lower colony-forming units per milliliter.

To determine minimal bactericidal concentrations (MBCs), wells from 116 MIC plates (10 strains of

TABLE 1. Comparative *in vitro* activity of LY127935, cefamandole, cefoxitin, and cephalothin against staphylococci and streptococci

Organism (no. of strains)	Antibiotic	MIC range (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
<i>Staphylococcus aureus</i> (24)	LY127935	4-8	8	8
	Cefamandole	0.12-1	0.5	0.5
	Cefoxitin	2-4	2	4
	Cephalothin	0.12-0.25	0.12	0.25
<i>Staphylococcus epidermidis</i> (10)	LY127935	16->32	32	32
	Cefamandole	0.25-1	0.5	0.5
	Cefoxitin	2-32	8	16
	Cephalothin	0.12->32	0.25	0.5
<i>Streptococcus pyogenes</i> (10)	LY127935	1-2	1	2
	Cefamandole	0.03-0.06	0.06	0.06
	Cefoxitin	0.12-0.5	0.5	0.5
	Cephalothin	0.06-0.12	0.12	0.12
<i>Streptococcus agalactiae</i> (10)	LY127935	4-8	4	8
	Cefamandole	0.03-0.12	0.06	0.06
	Cefoxitin	1-4	2	4
	Cephalothin	0.06-0.12	0.12	0.12
<i>Streptococcus pneumoniae</i> (11)	LY127935	0.03-4	0.5	2
	Cefamandole	≤0.01-0.12	≤0.01	0.06
	Cefoxitin	≤0.01-2	0.12	0.5
	Cephalothin	≤0.01-0.12	≤0.01	0.06
<i>Streptococcus viridans</i> (10)	LY127935	1->32	8	32
	Cefamandole	0.06-1	0.12	0.12
	Cefoxitin	0.06-8	2	8
	Cephalothin	0.06-0.12	0.12	0.12
<i>Streptococcus faecalis</i> (11)	LY127935	>32	>32	>32
	Cefamandole	16->32	32	32
	Cefoxitin	>32	>32	>32
	Cephalothin	8-32	16	32

TABLE 2. Comparative *in vitro* activity of LY127935, cefamandole, cefoxitin, and cephalothin against *Enterobacteriaceae* and *Pseudomonas*

Organism (no. of strains)	Antibiotic	MIC range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
<i>Escherichia coli</i> (25)	LY127935	0.03-0.25	0.12	0.12
	Cefamandole	0.06-4	0.25	1
	Cefoxitin	0.5-2	1	2
	Cephalothin	0.5-8	4	8
<i>Klebsiella pneumoniae</i> (25)	LY127935	0.06-0.5	0.12	0.25
	Cefamandole	0.12-8	0.5	1
	Cefoxitin	0.5-4	2	2
	Cephalothin	1-32	2	4
<i>Enterobacter aerogenes</i> (20)	LY127935	0.06-8	0.25	4
	Cefamandole	0.25->32	1	>32
	Cefoxitin	8->32	>32	>32
	Cephalothin	>32	>32	>32
<i>Enterobacter cloacae</i> (24)	LY127935	0.06-32	0.12	8
	Cefamandole	0.5->32	2	>32
	Cefoxitin	32->32	>32	>32
	Cephalothin	32->32	>32	>32
<i>Serratia marcescens</i> (25)	LY127935	0.25-16	0.5	2
	Cefamandole	8->32	>32	>32
	Cefoxitin	4->32	8	>32
	Cephalothin	>32	>32	>32
<i>Citrobacter diversus</i> (15)	LY127935	0.12-1	0.12	0.5
	Cefamandole	0.12->32	8	32
	Cefoxitin	0.5-16	2	8
	Cephalothin	1->32	2	>32
<i>Citrobacter freundii</i> (16)	LY127935	0.06-2	0.12	2
	Cefamandole	0.25->32	0.5	>32
	Cefoxitin	32->32	>32	>32
	Cephalothin	32->32	>32	>32
<i>Proteus mirabilis</i> (22)	LY127935	0.06-0.25	0.12	0.25
	Cefamandole	0.12-0.5	0.25	0.5
	Cefoxitin	1-2	2	2
	Cephalothin	1-2	2	2
<i>Proteus vulgaris</i> (16)	LY127935	0.12-1	0.25	0.5
	Cefamandole	1->32	>32	>32
	Cefoxitin	1-8	2	8
	Cephalothin	4->32	>32	>32
<i>Proteus rettgeri</i> (10)	LY127935	0.06-4	0.12	0.25
	Cefamandole	0.06->32	0.5	16
	Cefoxitin	0.5->32	2	>32
	Cephalothin	1->32	>32	>32
<i>Providencia stuartii</i> (18)	LY127935	0.06-1	0.12	0.5
	Cefamandole	0.12-32	0.5	8
	Cefoxitin	0.5->32	2	16
	Cephalothin	1->32	>32	>32
<i>Morganella morganii</i> (15)	LY127935	0.12-0.5	0.25	0.5
	Cefamandole	2->32	8	32
	Cefoxitin	4->32	8	32
	Cephalothin	>32	>32	>32

TABLE 2—Continued

Organism (no. of strains)	Antibiotic	MIC range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
<i>Pseudomonas aeruginosa</i> (20)	LY127935	4–>32	16	32
	Cefamandole	>32	>32	>32
	Cefoxitin	>32	>32	>32
	Cephalothin	>32	>32	>32
<i>Pseudomonas maltophilia</i> (10)	LY127935	4–32	8	8
	Cefamandole	>32	>32	>32
	Cefoxitin	>32	>32	>32
	Cephalothin	>32	>32	>32
<i>Pseudomonas cepacia</i> (10)	LY127935	32–>32	>32	>32
	Cefamandole	32–>32	>32	>32
	Cefoxitin	16–>32	>32	>32
	Cephalothin	32–>32	>32	>32

Staphylococcus aureus, 6 strains of *Staphylococcus epidermidis*, 75 strains of *Enterobacteriaceae*, 12 strains of *P. aeruginosa*, and 13 strains of *B. fragilis*) were subcultured to microdilution plates containing sterile broth with disposable inoculators. The absence of growth after incubation of the subculture from a given well of an MIC plate indicated a reduction in the original number of colony-forming units of 99.9% or greater in that well. MBCs were the lowest concentrations of antibiotic which yielded no growth in subculture after overnight incubation.

RESULTS

The comparative in vitro activities of the four study antibiotics against staphylococci and streptococci are shown in Table 1. Cephalothin and cefamandole were most active, cefoxitin was intermediate in activity, and LY127935 was the least active drug studied. Concentrations of 8 μg of LY127935 per ml inhibited 100% of strains of *S. aureus*, *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* but not *S. epidermidis*, *S. viridans*, or *S. faecalis*.

The comparative in vitro activities of the four study antibiotics against randomly selected *Enterobacteriaceae* and three species of *Pseudomonas* are shown in Table 2. LY127935 was the most active drug against all organisms tested. Concentrations of 8 $\mu\text{g/ml}$ inhibited 98.7% of *Enterobacteriaceae*. Concentrations of 32 $\mu\text{g/ml}$ inhibited 99.6% of *Enterobacteriaceae* and *Pseudomonas* other than *P. cepacia*.

The in vitro susceptibilities of multidrug-resistant *Enterobacteriaceae* and *P. aeruginosa* are shown in Table 3. In addition to resistance to ampicillin, carbenicillin, gentamicin, and tetracycline, most strains were also resistant to cephalothin, cefamandole, cefoxitin, tobramycin, and chloramphenicol, but 89% of the *Enterobac-*

teriaceae and 6 of the 10 strains of *P. aeruginosa* were inhibited by 16 μg of amikacin per ml. Concentrations of 8 μg of LY127935 per ml inhibited 82% of the *Enterobacteriaceae*. Concentrations of 32 $\mu\text{g/ml}$ inhibited 100% of *Enterobacteriaceae* and 4 of 10 strains of *P. aeruginosa*.

The comparative in vitro activities of the four study antibiotics against anaerobes are shown in Table 4. LY127935 was the most active drug tested against *Bacteroides* and *Clostridium perfringens*; 100% of strains of *B. fragilis* and *C. perfringens* tested were inhibited by 2 $\mu\text{g/ml}$. Although it was usually less active than the other drugs against other anaerobes, particularly the anaerobic cocci, 87% of all anaerobes tested were inhibited by 8 $\mu\text{g/ml}$ and 96% were inhibited by 32 $\mu\text{g/ml}$.

Lowering the inoculum size 100-fold had no effect on the MICs of the four study antibiotics. The effects of increasing the inoculum size 100-fold to approximately 10^7 to 5×10^7 colony-forming units per ml are shown in Table 5. With cephalothin and cefamandole, increases in MICs of more than two concentrations were observed with some susceptible organisms. Such increases were not observed with LY127935 or cefoxitin.

The four antibiotics studied were bactericidal against all 11 species of organisms studied. MBCs were the same or 1 log₂ dilution higher for 91 to 96% of the strains tested. MBCs were within 2 log₂ dilutions of MICs for 99% of strains with all four antibiotics.

DISCUSSION

LY127935 had a broad spectrum of activity against a variety of clinical isolates. Although it was less active than cefamandole, cefoxitin, and

TABLE 3. *In vitro* susceptibilities of multidrug-resistant^a *Enterobacteriaceae* and *P. aeruginosa*

Organism	MICs (μg/ml)						
	LY127935	Cefamandole	Cefoxitin	Cephalothin	Tobramycin	Amikacin	Chloramphenicol
<i>E. coli</i>	0.12	4	1	16	>16	16	8
	0.5	2	2	8	>16	>32	4
<i>K. pneumoniae</i>	0.25	4	8	16	>16	16	>32
	0.25	32	1	>32	>16	1	2
	2	16	32	>32	>16	2	>32
	4	16	32	>32	>16	2	>32
	32	>32	>32	>32	8	32	>32
<i>E. aerogenes</i>	8	>32	>32	>32	16	1	>32
	8	>32	>32	>32	>16	2	>32
	8	>32	>32	>32	>16	2	4
	16	>32	>32	>32	>16	4	>32
<i>E. cloacae</i>	0.25	>32	>32	>32	>16	2	8
	0.25	>32	>32	>32	>16	2	>32
	0.25	>32	>32	>32	>16	2	>32
	0.25	>32	>32	>32	>16	4	>32
	0.5	>32	>32	>32	>16	2	>32
<i>S. marcescens</i>	2	>32	>32	>32	>16	4	32
	8	>32	>32	>32	>16	4	32
	16	>32	>32	>32	>16	4	16
	16	>32	>32	>32	>16	>32	32
	32	>32	>32	>32	>16	4	16
<i>C. diversus</i>	0.5	32	8	>32	>16	8	16
	0.5	>32	8	>32	>16	2	16
<i>P. rettgeri</i>	0.12	1	2	>32	16	4	— ^b
	0.12	16	>32	>32	>16	2	—
	0.12	16	>32	>32	>16	16	—
<i>P. stuartii</i>	0.12	1	1	>32	8	8	—
	2	>32	>32	>32	8	2	16
<i>P. aeruginosa</i>	16	>32	>32	>32	4	32	>32
	32	>32	>32	>32	2	16	>32
	32	>32	>32	>32	4	16	>32
	32	>32	>32	>32	>16	16	>32
	>32	>32	>32	>32	4	16	>32
	>32	>32	>32	>32	4	32	>32
	>32	>32	>32	>32	>16	16	>32
	>32	>32	>32	>32	>16	16	>32
	>32	>32	>32	>32	>16	32	>32
	>32	>32	>32	>32	16	>32	>32
	>32	>32	>32	>32	16	>32	>32

^a Multidrug-resistant, MICs: ampicillin, >64 μg/ml; carbenicillin, >128 μg/ml; gentamicin, >4 μg/ml; and tetracycline, >8 μg/ml.

^b —, Not tested.

cephalothin against staphylococci, streptococci, and some anaerobes, concentrations of 8 μg or less per ml inhibited most strains tested excluding *S. epidermidis*, *S. viridans*, and *S. faecalis*. It was the most active drug tested against *Enterobacteriaceae*, *Pseudomonas*, *B. fragilis*, and *C. perfringens*. In a population of multidrug-

resistant, gram-negative bacilli, all *Enterobacteriaceae*, and 40% of strains of *P. aeruginosa* were inhibited by 32 μg or less per ml. MICs of LY127935 were not affected by increasing inoculum size, although MICs of cefamandole and cephalothin for some *Enterobacteriaceae* increased considerably in this study and others

TABLE 4. Comparative *in vitro* activity of LY127935, cefamandole, cefoxitin, and cephalothin against anaerobes

Organism (no. of strains)	Antibiotic	MIC range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
<i>Bacteroides fragilis</i> (15)	LY127935	0.12-2	1	2
	Cefamandole	16->32	16	32
	Cefoxitin	2-8	4	8
	Cephalothin	32->32	>32	>32
<i>Bacteroides fragilis</i> group ^a (14)	LY127935	0.5-16	4	16
	Cefamandole	16->32	32	>32
	Cefoxitin	2-32	16	16
	Cephalothin	16->32	>32	>32
<i>Bacteroides melaninogenicus</i> (15)	LY127935	0.03-4	0.5	2
	Cefamandole	0.03-4	0.25	4
	Cefoxitin	0.06-4	0.25	1
	Cephalothin	0.03-4	0.12	2
<i>Fusobacterium</i> (16)	LY127935	0.25-16	1	8
	Cefamandole	$\leq$ 0.01-32	0.06	8
	Cefoxitin	0.06-8	0.12	8
	Cephalothin	$\leq$ 0.01-32	0.12	4
<i>Peptococcus</i> (12)	LY127935	0.12-8	1	8
	Cefamandole	$\leq$ 0.01-2	1	1
	Cefoxitin	$\leq$ 0.01-4	0.12	0.25
	Cephalothin	$\leq$ 0.01-1	0.12	0.5
<i>Peptostreptococcus</i> (13)	LY127935	0.5->32	4	16
	Cefamandole	0.12-2	0.5	2
	Cefoxitin	0.25-8	1	8
	Cephalothin	$\leq$ 0.01-2	0.12	1
<i>Veillonella</i> (11)	LY127935	0.5-16	8	16
	Cefamandole	0.12-2	0.5	2
	Cefoxitin	0.25-8	1	4
	Cephalothin	0.25-4	2	2
<i>Clostridium perfringens</i> (15)	LY127935	0.25-2	0.5	2
	Cefamandole	0.5-4	2	4
	Cefoxitin	1-2	2	2
	Cephalothin	1-8	2	4
<i>Clostridium</i> sp. (other) (15)	LY127935	2->32	4	>32
	Cefamandole	0.25->32	1	>32
	Cefoxitin	0.25->32	1	>32
	Cephalothin	0.25->32	1	>32
Gram-positive, nonsporeforming bacilli ^b (15)	LY127935	0.25->32	1	32
	Cefamandole	$\leq$ 0.01-16	0.12	2
	Cefoxitin	0.12->32	0.25	16
	Cephalothin	$\leq$ 0.01-16	0.5	8

^a *B. fragilis* group includes *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*.^b Gram-positive nonsporeforming bacilli includes *Eubacterium*, *Bifidobacterium*, *Lactobacillus*, and *Propionibacterium*.

TABLE 5. Increases in MICs caused by increasing inoculum size 100-fold

Organism	Increases in MICs (no. of log ₂ dilutions)			
	LY127935	Cefamandole	Cefoxitin	Cephalothin
<i>S. aureus</i>	0, 1, 1 ^a	1, 2, 0	2, 0, 2	1, 2, 1
<i>E. coli</i>	0, 0, 1	1, 0, 1	0, 1, 0	4, 2, ≥2
<i>K. pneumoniae</i>	1, 0, 1	6, ≥5, 2	0, 0, 0	3, ≥3, 3
<i>E. aerogenes</i>	0, 1, 0	1, 2, 1	U, ≥2, U ^b	≥2, ≥2, ≥3
<i>E. cloacae</i>	0, 0, 0	≥7, ≥5, ≥4	U, U, U	U, U, U
<i>S. marcescens</i>	0, 0, 0	U, U, ≥1	0, 1, 1	U, U, U
<i>P. mirabilis</i>	2, 2, 1	1, 1, 0	1, 0, 1	1, 0, 0
<i>P. aeruginosa</i>	1, ≥1, 0	U, U, U	U, U, U	U, U, U
<i>B. fragilis</i>	0, 0, 0	≥2, ≥1, ≥1	0, 1, 1	U, U, U

^a Each number represents the increase for one of the three strains tested.

^b U, Undetermined: MIC > 32 µg/ml with an inoculum of 1×10^5 to 5×10^5 colony-forming units per ml.

(2). The antimicrobial activity of LY127935 was bactericidal, as it was for the comparative drugs.

The broad spectrum and marked activity of LY127935, particularly against aerobic, facultative, and anaerobic gram-negative enteric bacilli, make it a promising new antibiotic.

ACKNOWLEDGMENTS

I thank Valerie L. Helsel for technical assistance.

This work was supported by a grant from Lilly Research Laboratories, Indianapolis, Ind.

LITERATURE CITED

1. Fass, R. J. 1978. Antimicrobial susceptibility testing of anaerobic bacteria using microdilution techniques. Infect. Dis. Rev. 5:25-42.
2. Findell, C. M., and J. C. Sherries. 1976. Susceptibility of *Enterobacter* to cefamandole: evidence for a high mutation rate to resistance. Antimicrob. Agents Chemother. 9:970-974.
3. McMaster, P.R.B., E.A. Robertson, F.G. Witebsky, and J.D. MacLowry. 1978. Evaluation of a dispensing instrument (Dynatech MIC-2000) for preparing microtiter antibiotic plates and testing their potency during storage. Antimicrob. Agents Chemother. 13:842-844.