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STERILIZATION

Sterilizing Combination Products Using Oxides of Nitrogen

A new sterilization method offers an alternative to device manufacturers—particularly those developing combination devices.

James Kulla, Robert Reich, and Sheldon Broedel Jr.

A new sterilization process under development relies on oxides of nitrogen, and principally, nitrogen dioxide. For decades medical device manufacturers have sterilized their products using ethylene oxide (EtO), radiation (gamma or e-beam), or heat (steam or dry) processes. Many articles have been published regarding each of these processes and their respective advantages and disadvantages. Over time, there have been many technological improvements made to the equipment used to deliver these sterilization processes as well as refinements made to the processes themselves.

However, despite these improvements and refinements, inherent limitations regarding material compatibility, high equipment costs, long sterilization cycles, sterilant residues, high temperatures, and operator risk still persist and limit the options available to manufacturers for processing their products. More recently, low-temperature processes utilizing vaporized hydrogen peroxide, hydrogen peroxide gas plasma, or ozone have been developed and have gained varying degrees of acceptance in the hospital market for the sterilization of reusable devices. None of these processes has been widely accepted for production use by medical device manufacturers nor are they broadly suitable for terminal sterilization of combination products and biomolecules.

Sterilization using nitrogen oxides shows great promise and may be the first new process in many years to offer manufacturers an alternative, not only for many devices, but also for the rapidly developing array of combination products and novel drug-delivery platforms. This article presents an overview of the process of sterilization with oxides of nitrogen and provides a description of sterilant characteristics, preliminary microbial efficacy, and biological and material compatibility data as well as potential process applications.

Nitrogen Oxide in Biological Systems

Oxides of nitrogen are a family of compounds that are ubiquitous in nature. Within living systems, the oxides of nitrogen are referred to as reactive nitrogen species (RNS) and include nitrogen dioxide (NO_2). These RNS are involved in diverse roles, eliciting both positive and negative effects on biological molecules and systems. Nitric oxide (NO) has a dual role, being both beneficial and detrimental to organisms. NO is one of the few known gaseous-signaling molecules. In the literature, the dual role is widely recognized, and NO is referred to as a key biological messenger, playing a role in a variety of biological processes both beneficial and deleterious (see Stamler et al., 1997, and Hess et al., 2005, for reviews).[1,2]

As an example of a beneficial role, NO is known as endothelium-derived relaxing factor, or EDRF, which is synthesized from arginine and oxygen by nitric oxide synthase (NOS) enzymes and by the reduction of inorganic nitrate. The endothelium lining of blood vessels use NO to signal the surrounding smooth muscles to relax, thus dilating the artery and increasing blood flow. A second example of a beneficial role is that the production of nitric oxide is elevated in populations living at high altitudes, which helps these people avoid hypoxia.

NO has a negative effect on microorganisms, with one of the many results being the inhibition of germination. NO is generated by macrophages and neutrophils as part of the mammalian immune response to pathogens. Nitric oxide has been shown to be toxic to a broad spectrum of microbes including bacteria, fungi, viruses, and parasites. NO chemistry, in combination with reactions with superoxide, forms the basis for the antimicrobial activity observed in phagocytes. These reactions are varied, involving a complex array of different molecular targets to effect the antimicrobial activity.[3–5]

Numerous studies have reported the antimicrobial activity of NO and RNS (see Fang 2004, Akaike and Maeda 2000, and Mannick 2006 for reviews).The breadth of organisms inhibited by NO and RNS spans all genera of pathogenic and nonpathogenic microbes. The use of NO gas as a therapeutic agent against pulmonary tuberculosis and the potential of NO-releasing compounds as antiinfective agents has been examined. Examples of evidence for the direct application of NO and RNS as biocides includes studies with *Bacillus* spores, *Escherichia coli*, and Venezuelan equine encephalomyelitis virus.[4–12]

The antimicrobial activity of RNS has been less well studied than NO; however, direct exposure of Venezuelan equine encephalomyelitis virus to NO_2 at 5 ppm was sufficient to reduce the viability of the virus, and peroxynitrite was effective in killing *Bacillus subtilis* spores.[10,12]

Nitrogen Oxide—Reactions in Air

Figure 1. (click to enlarge) Model of NO/NO_2 sterilization.

NO is a highly reactive gas formed by natural chemical and physical reactions in the atmosphere. It is also produced by certain animal and plant cells from the amino acid L-arginine. Because it is so small and diffusible, NO passes through cell membranes. Figure 1 depicts the reactions of nitrogen oxide and RNS in the presence of air and water and biochemical targets present in living organisms.

In the presence of oxygen (air), nitric oxide reacts to form nitrogen dioxide (NO_2) and, to a much lesser concentration, its dimer, dinitrogen tetroxide (N_2O_4). Other transient species may be present at low concentrations (<1 ppm), including: nitrogen trioxide (NO_3), dinitrogen trioxide (N_2O_3), dinitrogen pentoxide (N_2O_5), and nitrous oxide (N_2O). In a sterilization chamber where NO is mixed with air, most of the NO reacts to form NO_2. The only other oxide of nitrogen that forms under these circumstances and is stable at concentrations higher than 1 ppm is N_2O_4, which exists in equilibrium with NO_2 and the concentration of which is determined by the NO_2 vapor pressure. If the air is humidified, NO_2 can be converted into nitric acid ($HONO_2$) at trace levels.

The Sterilization Process

A recently developed sterilization process uses low concentrations (<21 mg/L) of nitrogen dioxide gas in the presence of air and water vapor. The process is typically delivered at or near room temperature and consists of evacuation of air from the chamber, the introduction of the sterilant, and the addition of humidified air to a preset pressure, which is typically at or near ambient pressure. Depending on the physical design and packaging of the device to be sterilized, the sequence of

vacuum → sterilant injection → humid air injection

may be repeated several times or the sequence can be changed. At the nitrogen dioxide concentrations used, and considering the operating temperature and pressure of the process, the NO_2 remains in the gas phase and acts as an ideal gas throughout the sterilization cycle.

Sterilant Characterization. It has been determined that in the gas sterilization process NO_2 is the key sterilizing agent. Other RNS may contribute in less-significant ways. Although the literature cites many other potential reactions, the specific environment established with the sterilization system limits the chemical species formed and the breadth of biological response. This limit allows the process to be focused and controlled.

This controlled reaction was demonstrated by evaluating biological indicators (BIs) exposed under a variety of controlled circumstances (microbiological aspects of the process are discussed later in this article). For example, when NO is injected into the sterilization chamber and combined with humid air, the NO reacts with the oxygen at a predictable rate to form NO_2, which is lethal to the BIs. When NO is injected into a sterilization chamber with no atmospheric oxygen present, the NO does not form NO_2, and BIs are not sterilized (although other macromolecular processes are inhibited, as described later).

Gas reactions that occur in the sterilization chamber are predictable and have been determined by calculation, computer modeling, and empirically. The sterilant gas concentration mixtures over time are predictable. The reaction rates and resulting concentrations of NO_2 and other oxides of nitrogen that result from the reaction of air and NO have been calculated. The starting concentration was set at 0.1% NO in air. NO

reacts with oxygen in the air to create NO_2. The constant sum of NO and NO_2 indicates that these two molecules account for almost all of the nitrogen present. The calculations predict a rapid conversion of NO to NO_2 with only trace amounts of N_2O_4 due to the low NO_2 concentration (see the upper graph in Figure 2).

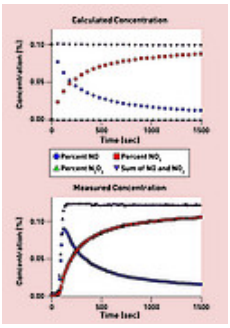

Figure 2. (click to enlarge) Sterilant gas characterization.

Gas concentrations in prototype chambers have been measured via a gas recirculating loop connected to an FTIR or UV spectrophotometer. The result of spectrophotometric measurements is shown in the lower graph in Figure 2. The graph tracks the concentrations of NO and NO_2 and also shows the sum of the two concentrations. The sum of the concentrations of NO and NO_2 remains fairly constant, indicating few other RNS molecules being formed. The NO concentration rises from zero as the NO is injected into the chamber with air. The NO concentration quickly drops as the NO_2 is formed. The sum of the NO and NO_2 in the chamber is conserved, confirming that few other nitrogenous species are formed.

ORGANIZATION	NO_2 EXPOSURE LIMITS
Occupational Safety and Health Administration	PEL: 5 ppm (9 mg/m³) (ceiling) STEL: 1 ppm (1.8 mg/m³)
National Institute for Occupational Safety and Health (part of CDC)	REL: ST 1 ppm (1.8 mg/m³) IDLH: 20 ppm (38 mg/m³)
American Conference of Governmental Industrial Hygienists	STEL: 1 ppm TWA: 3 ppm TWA

Table I. (click to enlarge) Published NO_2 exposure limits.

Sterilant Safety. Table I shows the published limits for exposure to NO_2. Although NO_2 is toxic in high concentrations, there is little risk to personnel or the environment when using the Noxilizer process for the following reasons:

- NO_2 is an effective sterilant at low concentrations, often between 8 and 10 mg/L and typically less than 21mg/L, depending on application. Therefore, relatively small containers of the NO_2 are required.
- NO_2 is nonexplosive and nonflammable.
- NO_2-exposed materials do not require lengthy aeration since no significant residues remain after sterilization.
- NO_2 is effectively removed by passing the exhaust gases through a proprietary scrubber.

As with any toxic gas, precautions must be taken to protect workers from accidental exposure. However, NO_2 presents few risks, especially when compared with EtO.

Microbiological Aspects. Preliminary studies in our laboratories indicate that *Geobacillus stearothermophilus* is a suitable challenge organism for assessing the lethality of the Noxilizer process. The resistance of the spores of this species exceeds that observed to date with other bacterial and fungal species, including *Bacillus atropheus, Bacillus pumilus, Aspergillus brasiliensis* (formerly *niger*), and *Bacillus subtilis*. Additional studies using a variety of aerobic and anaerobic bacteria, fungi, and viruses are pending.

Figure 3 shows the survivor curves of *Geobacillus stearothermophilus* spores for varying concentrations of NO and NO_2. The slower reaction rate with NO is the result of the need to convert the gas to NO_2 before lethality occurs. Although presented as a straight line, the NO data may likely be a convex line at the shorter exposure times. At 5.12 mg/L NO_2, the D-value is only 0.3 minutes. At 3 mg/L, the D value is approximately 1.9 minutes. The D-value at projected operating concentrations of 8–10 mg/L is so rapid that it cannot practically be measured.

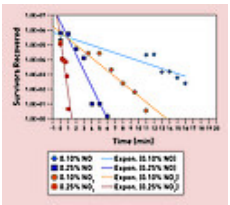

Figure 3. (click to enlarge) Survivor curves for NO and NO_2.

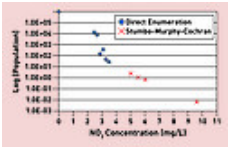

Figure 4. (click to enlarge) D-value versus NO_2 concentration. Exposure time = 1.5 minutes.

An inactivation curve as a function of concentration was evaluated to better understand the kinetics of process-delivered lethality and is presented in Figure 4. In this study, the exposure time was held constant at 1.5 minutes. Studies have shown that an NO_2 concentration below 2 mg/L has marginal lethality. The D_{conc} value determined by fraction negative testing (Stumbo-Murphy-Cochran method) was 0.93 mg/L.

OXIDANT	OXIDATION POTENTIAL, V
Fluorine	3.0
Hydroxyl radical	2.8
Ozone	2.1
Hydrogen peroxide	1.8
Nonsilver's sterilant	~0.8

Table II. (click to enlarge) Comparative oxidation potentials.

Mode of Action. RNS react with a range of biomolecules that includes: nucleic acids, proteins, carbohydrates, and lipids. Many molecules susceptible to oxidation can be nitrated by RNS. The main mode of RNS is nitration. The oxidation potential of NO_2 is significantly lower than that of ozone or hydrogen peroxide (see Table II). Nitration sites in biomolecules are less numerous than oxidation and alkylation sites, and as a result, the reaction of NO_2 with biomolecules is much more selective than the reactions caused by heat, EtO, hydrogen peroxide, and radiation. This selectivity is important in the application of the sterilization technology.

Exposure of bacterial spores to NO_2 interferes with several cellular activities. For example, ATP production is inhibited, the germination process is disrupted, and reproduction ceases. Most notable is the observation that NO_2 causes single-strand DNA breaks, which accumulate rapidly and immediately on exposure to sterilant. As the number of single-strand DNA breaks increases, double-strand breaks begin to accumulate resulting in the loss of DNA integrity. The accumulation of strand breaks correlates with the loss of cell viability.

Interestingly, there is less than a twofold increase in mutation rates in spores or plasmid DNA recovered after exposure to sublethal conditions relative to nonexposed DNA. DNA sequence analysis of the recovered mutants revealed only random point mutations and no deletions, insertions or inversions. These data suggest that the strand breaks are not repairable. If chromosomal repair were taking place during germination, late outgrowth would be expected. However, data from over 5000 biological indicators (approximately 5 billion spores) tested from more than 450 sterilization cycles showed no evidence of outgrowth past 4 days of incubation. The absence of late growth supports the conclusion that recovery and repair of the DNA strand breakage does not occur.

Compatibility with Common Device Materials, Designs

MATERIAL	RESULT
Insulin	Some change at certain concentrations, needs optimization
Protein, no Zn or Fe sites	Slight change
Protein, Zn or Fe sites	Not compatible
Antibodies	Slight loss of activity (10%)
Sirolimus	Less compatible than EtO, more compatible than radiation
Paclitaxel	Less compatible than EtO, more compatible than radiation
Trypsin	Slight change
Shape-memory polymer	Compatible
Poly-DL-lactide-co-glycolide	No significant changes
Poly-DL-lactide-COOH	No significant changes
Poly-L-lactide	No significant changes
Poly-ε-caprolactone	No significant changes

Table III. (click to enlarge) Materials tested for compatibility with the NO_2 sterilization process

Many medical device materials tolerate sterilization with NO_2 quite well and show no toxicity after exposure (using cytotoxicity as a screening for biocompatibility). Table III shows the results of cytotoxicity testing of a number of materials commonly utilized in medical device construction, using both the ISO 10993-5 MEM [Minimum Essential Medium] Elution procedure and a rat hepatocyte assay.[13] With the exception of polyacetal, no material showed an increase in toxicity greater than that seen in untreated control material. Most materials show no detectable cytotoxicity.

Cellulose-based materials, brass, copper containing alloys, and some polyurethanes are not compatible with NO_2 sterilization. Polyurethanes show slight color change (yellowing) after one exposure, but no other change that would affect functionality. Copper-containing alloys discolor due to the formation of $CuNO_3$, and brass showed evidence of change after a single exposure. Studies have shown that material interactions are affected by varying cycle conditions so that it may be possible to optimize conditions for specific device sterilization processes.

Studies using *G. stearothermophilus* have shown that NO_2 is capable of sterilizing mated instrument surfaces, tubing lumens of lengths up to 1 m, and devices with electrical contacts. Additional studies are ongoing to further explore the capabilities and boundaries of the technology.

Compatibility with Packaging

Biological indicators were packaged in Tyvek/Tyvek pouches, Tyvek/Mylar pouches, and Tyvek/PET pouches, and were also wrapped in polypropylene surgical wrap. There was no difference in delivered lethality when compared with unpackaged control BIs. Vacuum used with the process is typically in the 0.3–3-in. HgA range, so heat seals capable of withstanding EtO sterilization can withstand the NO_2 sterilization process. NO_2 does not readily penetrate plastic films. The sterilization process is not compatible with paper, corrugated cartons, or other cellulosic packaging and labeling materials; therefore, this would preclude sterilization in final shipping cartons. The use of this technology for terminal sterilization would require exposing bulk sealed packages followed by secondary packaging for shelf-packs and shipping after sterilization.

Unique Applications of the NO_2 Sterilization Process

As mentioned earlier, RNS react with a range of biomolecules such as nucleic acids, proteins, carbohydrates, and lipids. In essence, any molecule susceptible to oxidation can be nitrated by RNS, although the oxidation potential of NO_2 is significantly lower than that of ozone or hydrogen peroxide. In addition, nitration sites in biomolecules are less numerous than oxidation and alkylation sites. As a result, the reaction of NO_2 with biomolecules is much more selective than the reactions caused by heat, EtO, hydrogen peroxide, and radiation. This selectivity is important in the application of the sterilization technology for certain combination products.

Combination products have posed new challenges in delivering a sterile product, especially in developing and validating terminal sterilization processes. Combination products are not new to the sterilization industry. Early examples include alcohol- or PVP-saturated pads and swabs, prefilled syringes, and antimicrobial- or anticoagulant-coated catheters. Today, much more sophisticated combination products are being produced, including combining biologicals, antibiotics, enzymes, and oncological molecules with unique delivery systems, using complex manufacturing processes.

Devices or polymers sterilized by EtO or radiation are combined with drugs or other therapeutic agents that have been aseptically prepared. The process of aseptically combining the products is performed using barrier technology (isolators or RABS [restricted access barrier systems]) or aseptic assembly in an ISO 5 cleanroom. Such manufacturing processes are both time-consuming and costly in time, equipment, and facilities. Additionally, from a regulatory viewpoint, aseptic assembly is not considered to guarantee the same product sterility assurance level as a terminal process. It has, therefore, always been FDA's position that if possible, a terminal sterilization process should be used.

NO_2 sterilization may be more compatible for sterilizing therapeutic biomolecules. The number of reaction sites in biomolecules susceptible to nitration is far lower than those available to be oxidized or alkylated. For example, NO_2 reacts primarily with tyrosine residues in proteins and under the conditions used with no other amino acids. Statistically there are fewer tyrosine amino acids than amino acids susceptible to oxidation or alkylation; therefore, there should be fewer nitration events than oxidation or alkylation events.

Consequently, the NO_2 sterilization process by nitration is more selective than other medical device sterilization processes such as hydrogen peroxide (oxidative process), EtO (alkylation process), or radiation (ionization). This selectivity makes possible a terminal sterilization process with oxides of nitrogen. This process can be used for devices consisting of novel polymers and therapeutic molecules and drugs that might not be feasible with the more-aggressive processes. Table IV summarizes the results of testing with a number of types of therapeutic molecules.

NO CYTOTOXIC EFFECTS	NO INCREASED CYTOTOXIC EFFECTS OVER CONTROLS	INCREASED CYTOTOXIC EFFECTS
Aluminum	Brass	Polyacetal
Bionate (Urethane)	Latex (Glove)	—
Elasthane (Urethane)	Latex (Pertrose)	—
EDPE	Neoprene	—
Mylar	Nylon	—
Nylon MG	—	—
PMMA	—	—
Silicone	—	—
Stainless Steel	—	—
Tyvek	—	—
UHMWPE	—	—

Table IV. (click to enlarge) Results of cytotoxicity testing by ISO 10993-5 MEM elution method and by hepatocyte cytotoxicity testing.[13]

This concept was investigated by examining the effects of NO_2 sterilization on 20 different proteins. The proteins used are shown in Table III. Of the 20 proteins assayed, only four were significantly inhibited; most of the proteins were either not inhibited or only partially inhibited by either of the gases and showed a dose dependence with the higher gas concentrations giving greater levels of inhibition.

Further, it has been shown that the protein inhibition could be reduced by optimization of the cycle parameters. All of the proteins tested have either free cysteine (reactive with NO) or tyrosine (reactive with NO_2) residues or both. Some of the proteins tested are sensitive to oxidation or have divalent cations as part of the reactive site. However, the presence of any of these potential reactive sites did not predict inhibition by the sterilant gases. Therefore, the context in which the RNS reactive site is found likely determines the sensitivity of a given protein to NO or NO_2. Oxidation did not appear to be occurring; however, the role of oxidation needs to be investigated in more depth.

Bioresorbable polymers compatible with the NO_2 sterilization process are shown in Table IV and are used as implant materials and for drug delivery. A difficulty in utilizing these moieties is the ability to sterilize them in a nondestructive fashion. Four commonly used polyester-based bioresorbable polymers, including Lactel DLPLG (poly-DL-lactide-co-glycolide, 50/50), DLPLA (poly-DL-lactide-COOH), LPLA (poly-L-lactide), and PCL (poly-ε-caprolactone) have been evaluated with the NO_2 sterilization process. Samples were treated using a very high concentration of nitrogen oxide (3%). Treated samples were evaluated using mechanical testing, GPC molecular weight analysis, and dissolution testing. Testing occurred at different time points including immediately after treatment (Day 0), after Day 60, and after 6 months of desiccated storage. Treated samples displayed no significant differences in molecular weight profile or change in mechanical properties from control samples when evaluated up to 6 months poststerilization.

A model has not yet been developed that is predictive of the relative compatibility of biopharmaceutical compounds and proteins. However, the above studies suggest that optimization of the sterilizing parameters, when the activity of a biomolecule or protein is inhibited, is often successful by defining a cycle that is compatible with the material. In general, each material would need to be individually tested for compatibility and the parameters optimized accordingly.

Conclusion

A sterilization process utilizing oxides of nitrogen offers an attractive option to medical device manufacturers and companies involved in the manufacture or development of combination products and novel therapeutic delivery systems. The sterilization process is effective at or near ambient temperatures and allows for the sterilization of temperature-sensitive materials. The process utilizes very low concentrations of sterilant that leaves no measurable residue on products being sterilized.

A large variety of commonly used medical device materials are not adversely affected by the process. Following exposure to the sterilant, materials do not require aeration or degassing. Once sterilizing conditions are achieved, the sterilization process is very rapid. The kinetics of the process as well as the critical processing variables are understood, thus allowing customization of the technology to unique applications. Of particular interest is the compatibility of the process with many drugs and other biomolecules. This compatibility

offers the possibility of terminal sterilization of many of the new and developing combination products and delivery systems that cannot be sterilized by conventional modalities. This new technology may provide another sterilization option for medical device, combination product, and biotherapeutic manufacturers. Studies are ongoing to provide a complete understanding of the capabilities and boundaries of the technology.

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James Kulla is vice president, business development for LexaMed (Toledo, OH), and Robert Reich is president of the company. They can be contacted at jkulla@lexamed.net and rreich@lexamed.net. Sheldon Broedel Jr., PhD, is founder, CEO, and chief science officer of Athena Environmental Sciences Inc. (Baltimore). He can be contacted at corporate@athenaes.com.

References

1. JS Stamler et al., "(S)NO Signals: Translocation, Regulation, and a Consensus Motif," *Neuron* 18 (1997): 691–696.

2. DT Hess et al., "Protein S-Nitrosylation: Purview and Parameters," *National Review of Molecular and Cellular Biology* 6 (2005): 150–166.

3. PG Jorens et al., "Human and Rat Macrophages Mediate Fungistatic Activity against Rhizopus Species Differently: In Vitro and Ex Vivo Studies," *Infection and Immunity* 63 (1995): 4489–4494.

4. JB Mannick, "Immunoregulatory and Antimicrobial Effects of Nitrogen Oxides," *Proceedings of the American Thoracic Society* 3 (2006): 161–165.

5. FC Fang, "Antimicrobial Reactive Oxygen and Nitrogen Species: Concepts and Controversies," *Nature Reviews: Microbiology* 2 (2004): 820–832.

6. T Akaike and H Maeda, "Nitric Oxide and Virus Infection," *Immunology* 101 (2000): 300–308.

7. R Long, B Light, and JA Talbot, "Mycobacteriocidal Action of Exogenous Metric Oxide," *Antimicrobial Agents and Chemotherapy* 43 (1999): 403–405.

8. R Long et al., "Inhaled Nitric Oxide Treatment of Patients with Pulmonary Tuberculosis Evidenced by Positive Sputum Smears," *Antimicrobial Agents and Chemotherapy* 49 (2005): 1209–1212.

9. R Ciccone et al., "Inhibitory Effect of NO-Releasing Ciprofloxacin (NCX 976) on Mycobacterium Tuberculosis Survival," *Antimicrobial Agents and Chemotherapy* 47 (2003): 2299–2302.

10. PC Genest et al., "Killing of Spores by *Bacillus subtilis* by Peroxynitrite Appears to Be Caused by Membrane Damage," *Microbiology* 148: 307–314.

11. JK Hurst and SV Lymar, "Toxicity of Peroxynitrite and Related Reactive Nitrogen Species toward *Escherichia coli*," *Chemical Research in Toxicology* 10 (1997): 802–810.

12. R Ehrlich and S Miller, "Effect of NO_2 on Airborne Venezuelan Equine Encephalomyelitis Virus," *Applied Microbiology* 23 (1972): 481–484.

13. AP Li, "Primary Hepatocyte Cultures as an In Vitro Experimental Model for the Evaluation of Pharmacokinetic Drug-Drug Interactions," *Advances in Pharmacology,* Series 43 (1997): 103–130.