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Limitations of Monastral Blue as a Vascular Label: Rapid Rate of Clearance Is Age-Dependent, and Interactions With Anesthetics Depress Arterial Blood Pressure in Rats

WENDY T. DRAKE, MICHAEL CREIGHAN, AND DAVID E. SIMS
Infection and Immunology Research Laboratory (W.T.D.), Isaac W. Killam Children's Hospital, Halifax, Nova Scotia, B3J 3G9, and Department of Anatomy and Physiology (M.C., D.E.S.), Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, C1A 4P3, Canada

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ABSTRACT Monastral blue (MB) has been described as an inexpensive, nontoxic vascular label. Discrepancies as to its rate of removal from circulation and physiological side effects prompted this study in which retention time of MB in the vascular system and effects of MB upon arterial blood pressure with different anesthetics (halothane, isoflurane, and pentobarbital) were measured in rats. Arterial pressure was monitored during intravenous infusion of MB with or without Evans blue, an albumin label. Localized areas of leakage were created by injecting 30 μL of 10^{-4} M histamine into abdominal dermis at −2, 0, 5, 7, 10, and 15 minutes from infusion of MB. Mean arterial pressure decreased by 25–30% after MB infusion when halothane or isoflurane was used, but not with pentobarbital. Sites which leaked at 10 and 15 minutes did not usefully label with MB, although Evans blue-labelled albumin appeared in the interstitium. Younger, lighter rats (125–200 vs. 200–250 gm) retained MB longer in circulation, and had a shorter duration of MB-induced hypotension. Spectrophotometric analysis of rat serum showed rapid elimination of MB from the vascular system, with a half-life of 3.5 ± 1.9 minutes. While MB remains a useful vascular label, its rapid removal from the circulation and its hypotensive effect must be recognized.

INTRODUCTION

Vascular labelling is a technique of light and electron microscopy that permits identification of leaky microvessels (Majno and Palade, 1961; Majno et al., 1961). The procedure requires intravascular infusion of colloidal particles which become trapped in walls of vessels if their endothelial linings have been interrupted, but basement membranes remain intact (Cotran et al., 1965, 1967).

When the original vascular labels, colloidal carbon and mercuric sulfide, became difficult to obtain, substitutes were sought. Monastral blue (MB) was subsequently introduced as a potential replacement. Joris et al. (1982) reported that following intravenous injection of MB, rat lungs returned to a normal pink color, there was no sign of respiratory distress, and hepatocytes were not damaged. They concluded that MB, a proprietary 3% suspension of copper phthalocyanine particles in a nonionic surfactant with biocide, was a stable, nontoxic vascular label when injected at 0.1 ml/100 gm body weight (BW), that would remain in the vascular system of a rat for up to an hour.

More recent investigations have shown that MB is effectively removed from the rat circulation within 15 minutes (Takagi et al., 1987), or in even less time, with a half-life in rats of only 3.4 minutes (MacDonald et al., 1988). Albertine and Staub (1986) observed that MB caused systemic hypotension, pulmonary hypertension, and bronchoconstriction in sheep. They concluded that the vascular tracer is not biologically inert in sheep. While species differences alone might account for the different responses to MB (Winkler, 1988), Albertine and Staub also used a different anesthetic (halothane) than the other researchers, who used pentobarbital.

Such discrepancies in the literature prompted a study to reevaluate MB as a vascular label in the rat. Goals of this present study were to 1) determine how long MB could effectively label microvessels, 2) measure MB concentration in serum as a function of time, and 3) characterize effects of MB upon arterial blood pressure with different anesthetics.

The anesthetics selected were halothane, isoflurane, and pentobarbital. Halothane is a halogenated hydrocarbon that is nonflammable and nonexplosive, easy to vaporize, potent, nontoxic, nonirritating, and effective in all species (Paddleford, 1988). Induction and recovery are very rapid (Green, 1982). During administration it is very easy to control the amount being given to the patient. Excessive monitoring is not required. Halothane is also economical. Halothane acts as a cardiovascular depressant, causing arteriolar vasodilation (Paddleford, 1988; Short, 1987). Anesthetic levels can

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Address reprint requests to David E. Sims, Department of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, C1A 4P3, Canada.

cause impaired metabolism in coronary microvasculature (Hatano et al., 1990; Paddleford, 1988; Trulson and Ulissey, 1987). There are also indications of a dose-dependent respiratory depression causing a reduction in tidal volume (Rock et al., 1990).

Isoflurane, a halogenated methyl ethyl ether, is the least soluble of the volatile inhalation anesthetics both in blood and body tissues, providing fast induction and recovery (Eger, 1981; Paddleford, 1988). It is almost completely exhaled unchanged, undergoing little or no biotransformation (Short, 1987). The most expensive of the anesthetics studied, isoflurane has the highest cardiovascular margin of safety (Paddleford, 1988).

Pentobarbital is an oxybarbiturate. Surgical anesthesia is obtained only at doses close to those which cause respiratory failure (Short, 1987). Pentobarbital causes excitement during induction and recovery and the two processes also take a comparatively long time (Paddleford, 1988). It provides poor analgesia unless the patient is under deep anesthesia, which in rats may be challenging to control and maintain. Pentobarbital may cause severe cardiovascular and respiratory distress (Green, 1982; Short, 1987). Cardiac output is decreased and circulatory collapse is common in deep anesthetic states (Paddleford, 1988).

Recent comparisons of these anesthetics suggest that their physiological effects are obtained by different mechanisms (Hatano et al., 1990; Weeks et al., 1990). Therefore, varying interactive effects with substances such as MB could be anticipated. Use of MB in quantitative studies requires that its rate of clearance and any cardiovascular effects be well understood, thus prompting this study. Many physiological parameters are age or size dependent, particularly in small rodents (Hinds and McNelly, 1981; Mio et al., 1989; Neve et al., 1981; Rao and Krinke, 1983). Therefore, as an additional variable, two sizes of rats were compared for removal times of MB.

MATERIALS AND METHODS

Experimental Animals and Anesthetization Procedure

Male Sprague Dawley rats (Charles River Canada Inc.), 200–250 gm (at 7 weeks of age) for the initial clearance studies, and 125–200 gm (at 5–6 weeks of age) for additional clearance studies and a comparison of anesthetics, were used in this study. In all of the experiments described below except the comparison of anesthetics, rats were anesthetized with halothane. Anesthetization (initially in a bell jar) was accomplished with a nonbreathing anesthetic circuit (Coaxial Bain Circuit, Hoechst) at a rate of 2.5 volume percent with fresh gas flow of oxygen at 0.7 L/min. Rectal temperature was monitored with a thermal probe and maintained at 35 ± 2°C with a warming pad.

Removal Time of MB From Circulation

Twenty-four 200–250 gm rats were used to determine the clearance time and useful circulating time of MB. A PE-50 polyethylene catheter (Intramedic, Becton Dickinson) was placed in the right femoral vein for MB infusion. The left femoral artery was cannulated for pressure monitoring. On the midventral surface of the abdomen six points were selected for injection sites. Monastral blue (Sigma Chemical Co., St. Louis, MO) was administered as a bolus over about 1 minute at 0.1 ml/100 gm of body weight (Joris et al., 1982). Localized areas of leakage from post capillary venules were then created by injecting 30 μL of 10^{-4} M histamine dihydrochloride (Sigma) into the abdominal dermis at −2, 0, 5, 7, 10, and 15 minutes with respect to intravenous infusion of MB. An additional site was injected with lactated Ringer's solution (Travenol Canada Inc.) at 5 minutes post-MB to show the injection procedure itself did not create any leaky vessels. Ten minutes after the last injection of histamine a 1.0 ml blood sample was drawn from the right femoral artery. Blood samples were immediately analyzed with a Nova Biomedical Stat Profile 4 Blood Gas Analyzer to verify that pH, pCO_2, PO_2, and K^+ were within normal ranges at the end of each experiment.

Four rats were pretreated with the H_1-antihistamine, mepyramine maleate B.P. (Poulenc), as controls to demonstrate leaky venules were due to the direct action of histamine. The H_1 blocker was given by intravenous infusion of 2 mg/kg in lactated Ringer's solution 10 minutes before the injection protocol (Black et al., 1975; Flynn and Owen, 1975).

Light Microscopy

The abdominal skin was removed, gently stretched, and pinned on cardboard. Injection sites were examined for labelled venules using a binocular dissecting microscope. After initial observations the skin was fixed in 10% buffered neutral formalin (BNF) for 48 hours, then cleared with two changes of glycerol over 2 weeks and reexamined.

Samples of liver and spleen were removed at the end of each experiment, immersion fixed in 10% BNF for 48 hours, then dehydrated in graded alcohols, and embedded in paraffin. Hematoxylin and eosin (H&E) stained tissue sections were examined with a light microscope for MB deposits.

Confirmation of Increased Permeability

Five rats were treated similarly except for an intravenous infusion of Evans blue (Sigma Co.) (0.2 gm/kg BW of a 5% solution in lactated Ringer's) 10 minutes before the regular injection schedule began. Evans blue marks plasma extravasation by binding to plasma albumin. Based on studies of the binding capacity of plasma albumin of other species (Freedman and Johnson, 1969; Levik and Michel, 1973), this dose of Evans blue presumably did not exceed the binding capacity of circulating plasma albumin in the rat.

MB Concentration in Serum Over Time

Four rats were used to analyze concentrations of MB in serum over time, to assess rate of MB removal by the mononuclear phagocytic system. PE-50 catheters were placed in the right femoral vein for MB and Ringer's solution infusion and the left femoral artery for blood withdrawal. Blood samples (0.5 ml aliquots) were drawn at 0 (control), 1, 3, 5, 7, 10, 15, 20, and 30 minutes with respect to the infusion of MB. For each unit

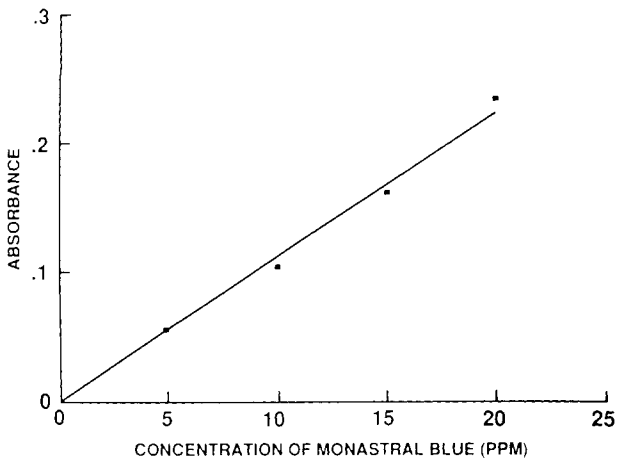

Fig. 1. Standard curve for Monastral blue in rat serum at 604 nm.

of blood removed an equal volume of lactated Ringer's solution was added.

Spectrophotometry and Elimination Kinetics

Serum concentrations of MB were measured by diode array spectrophotometry at 604 nm (Hewlett Packard model 8452A, HP ChemStation analysis software). Standards were prepared by adding MB to serum from untreated rats (Fig. 1). The linear standard curve of absorbance vs. concentration was best described by the equation: Absorbance = $-0.01 + 0.0119$[MB], with $r = 0.995$.

The zero time (pre-MB control) serum sample was used as a blank for each rat.

Interactions of MB and Anesthetics

Twenty-four rats (125–200 gm) were utilized in this portion of the study, eight for each of the three anesthetics. Rats anesthetized using isoflurane and halothane (MTC Pharmaceuticals) were induced first in an induction chamber, then at 3.0% and 2.5% concentrations per liter, respectively, at a flow rate of 3.0 L/min for both anesthetics. Animals were maintained at a flow rate of 0.8 L/min at concentrations of 2.5% and 2.0% for isoflurane and halothane, respectively. Rats anesthetized using pentobarbital (MTC Pharmaceuticals) were given a dosage of 0.10 mg/100 gm to induce anesthesia and maintained with injections of 0.10 mg as needed. Pentobarbital was administered to the animals via intraperitoneal injection, accompanied with oxygen at a flow rate of 0.8 L/min.

In a manner similar to the first clearance study, intradermal injections of 30 μL of 10^{-4} M histamine were given at 2, 5, 7, 10, 12, and 15 minutes. The slightly altered times enabled more critical evaluation of the 10–15 minute time period after administration of MB. Upon completing the six injections, the animal was left for 10 additional minutes without any further disturbance prior to euthanasia.

Arterial Blood Pressure Monitoring

The left femoral artery catheter in all the rats except the four used for serum collection was connected to a Gould P23 series pressure transducer. Blood pressure was monitored continuously during the experiments and recorded on a linear chart recorder (Gould, Cleveland, OH). A Blood Pressure Systems Calibrator (Bio-Tek Instruments Inc., Winooski, VT) was used to calibrate the transducer to a range of 0–200 mmHg.

Statistical Analysis

Student T-tests were performed with the MINITAB^R statistics package. Unless otherwise indicated, a probability value of 0.05 was used to determine statistical significance. Data are expressed as mean ± SD.

RESULTS

Removal Time of MB

MB labelling of venules was apparent in the −2, 0, 5, and 7 minute injection sites in all the rats tested. Labelling was not seen in the 10 and 15 minute sites in any of the rats. Analysis of skins immediately after removal and later after clearing showed essentially the same results, MB being easier to detect in cleared skins. Labelling with MB at the 10 minute sites became barely evident after clearing (Fig. 2), although the amount of labelling was reduced to the point of being ineffective. Control rats pretreated with mepyramine maleate, and sites given Ringer's solution injections in place of histamine showed no labelling or edema. At the conclusion of each experiment, the liver and spleen were darkly stained with MB. Lungs and other tissues not injected with histamine had no blue coloration.

Evans blue leakage into the interstitium occurred in all six injection sites of all five animals tested (Fig. 3). Blood gases and electrolytes remained within normal ranges, ruling out complications due to alterations in acid/base balance or electrolytes.

Examination of the H&E stained tissue samples revealed abundant MB particles within the Kupffer cells of the liver and the splenic macrophages, the constituents of the mononuclear phagocytic system responsible for removing 90% of intravenous injected particle suspensions (Halpern et al., 1965).

Elimination Kinetics of MB

Elimination of MB from the vascular system was rapid, showing first order kinetics (Fig. 4). The half-life was 3.54 ± 1.9 minutes. The apparent mean volume of distribution, calculated from a ratio of dose given to the Y-intercept concentration at T = 0, was 0.9 L/kg. Data were best fitted by a one compartment model.

Effect of MB on Blood Pressure

Infusion of MB into halothane-anesthetized rats caused a significant drop in arterial blood pressure (Fig. 5). The mean arterial pressure dropped from a preinfusion value of 82 ± 14 to 58 ± 18 mmHg ($N = 19$). This represents a 30 ± 13% (mean ± SD, $P < .0001$) decrease in 200–250 gm rats, reaching the low-

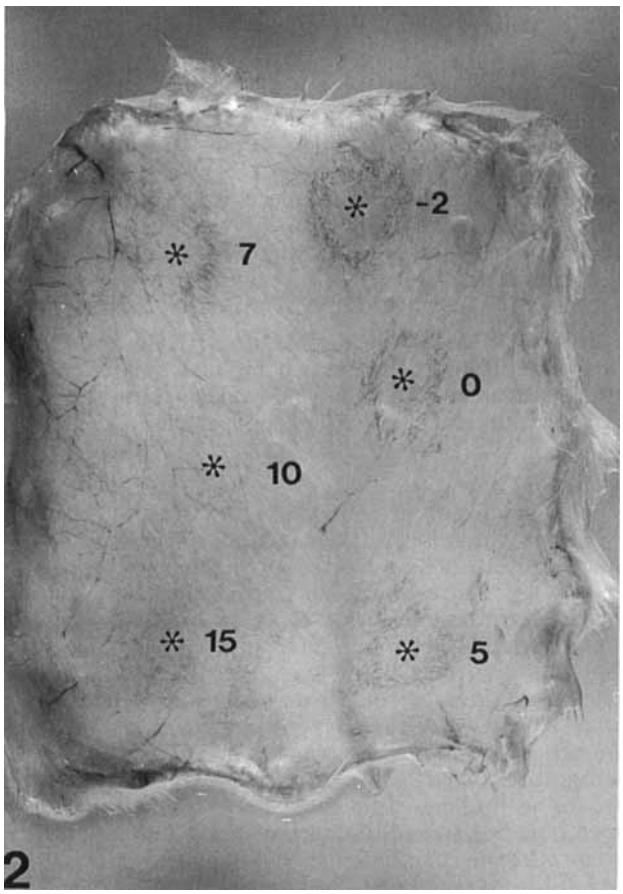

Fig. 2. Glycerin-cleared 250 gm rat abdominal skin showing the six sites of intradermal injection of 30 μL of 10^{-4} M histamine (*). The leaky venules labelled at the −2, 0, 5, and 7 minute post-Monastral blue infusion sites. No useful labelling was evident at the 15 minute sites, while weak labelling occurred at 10 minutes. ×3.

est value by 1.08 ± 0.3 minutes postinfusion. Blood pressures recovered to pre-MB values by 10 minutes in all of the experiments. Evans blue infusion had no effect on blood pressure. Pretreatment with mepyramine maleate did not block the hypotensive effect.

After determining that MB transiently reduced arterial blood pressure, the anesthetic used, halothane, was compared to isoflurane and pentobarbital, to determine if interactions between MB and anesthetics were occurring. The results are summarized in Table 1. The depression of arterial blood pressure that occurred with halothane and 200–250 gm rats was again observed with 125–200 gm rats. The hypotensive response also occurred with isoflurane, but not with pentobarbital. The initial blood pressures of rats anesthetized with halothane and isoflurane were also significantly lower than in the pentobarbital group; the duration of time until maximal depression of blood pressure was also increased with halothane and isoflurane (Table 1). The younger rats had useful concentrations of MB in circulation for longer periods of time (10–15 vs. 7–10 minutes).

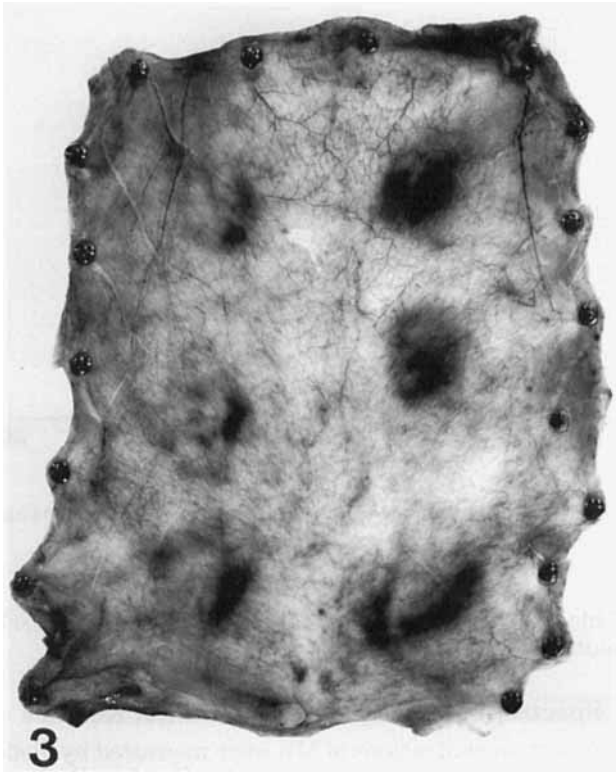

Fig. 3. Rat abdominal skin (250 gm) showing the six sites of intradermal injection of 30 μL of 10^{-4} M histamine with Evans blue-labelled albumin leakage into the interstitium.

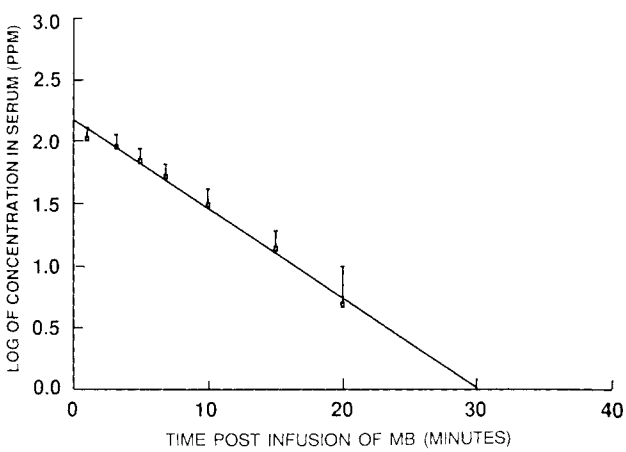

Fig. 4. Concentration of Monastral blue in rat serum over time.

DISCUSSION

By virtue of its size (50–300 nm) MB can leave the blood through gaps between endothelial cells, but cannot traverse intact basal laminae of the endothelial cells and pericytes. It is trapped within vessel walls, thereby labelling sites of extravasation (Joris et al., 1982). MB can be detected in venule walls with the naked eye, light microscopy, and electron microscopy.

TABLE 1. Comparison of MB-initiated changes to mean arterial blood pressure (±SD) in rats with different anesthetics

	Isoflurane (n = 8)	Halothane (n = 8)	Pentobarbital (n = 8)
Mean arterial blood pressure before injection of MB (mmHg)	79.4 ± 14.4 *	70.6 ± 12.1 *	103.0 ± 18.8[1]
Mean arterial blood pressure at maximum decrease after injection of MB (mmHg)	60.1 ± 13.4 *	49.4 ± 4.7 *	93.7 ± 17.8[1]
Decrease in mean arterial blood pressure (%)	25.0 ± 6.1	28.9 ± 7.7	8.7 ± 5.6[1]
Time from injection of MB to maximum decrease in blood pressure (sec)	75.1 ± 28.7	74.3 ± 11.1	60.3 ± 21.6[1]
Time from maximum decrease in blood pressure to full recovery (sec)	124.3 ± 126.6	210.0 ± 85.8	115.4 ± 48.1

*Significant difference of post- vs. pre-MB pressures, $P < 0.05$.
[1]Significant difference of values within rows between pentobarbital and isoflurane/halothane, $P < 0.05$.

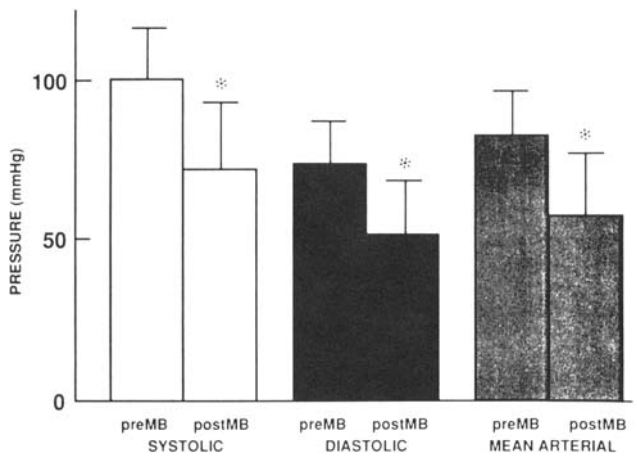

Fig. 5. Effect of Monastral blue infusion in rats on arterial blood pressure when halothane was used as anesthetic. An intravenous infusion of Monastral blue consistently caused a significant (*, $P < .0001$) decrease in blood pressure.

In 200–250 gm rats, sites of histamine injections given at 10 and 15 minutes after infusion did not accumulate enough MB to be usefully labelled, even though Evans blue-albumin complexes in the interstitium confirmed plasma leakage had occurred. These results indicate that MB's effectiveness as a label was limited to about 7 minutes (half-life 3.5 ± 1.9 minutes). The half-life of circulating MB may be somewhat longer in smaller rats, but even with a slightly greater time frame, quantitative comparisons of dye accumulation would be difficult unless substances being compared could be simultaneously injected. The one compartment model of kinetics indicated a very rapid distribution phase that presumably occurred before the first post-MB sample was taken. The apparent mean volume of distribution supported the one compartment model; 0.9 L/kg corresponded to calculated blood volumes for rats (Levik and Michel, 1973).

MB infusion into rats caused a significant decrease in arterial blood pressure when halothane and isoflurane were used as anesthetic. These results are similar to the findings of Albertine and Staub (1986), who, using sheep, were the first to suggest that MB is not biologically inert. Histamine release in response to vascular tracers has been a concern of investigators. Histamine liberation did not appear to be an important factor in this study, since an H_1 antagonist did not block the decrease in pressure. Albertine and Staub (1986) reported that indomethacin, a cyclooxygenase inhibitor, partially blocked the pathophysiological effects of MB.

MB has useful properties for permeability studies. Tracers, such as Evans blue, do not reveal the precise locations of permeability changes, because the extravasated dye quickly diffuses away from the sites of leakage. While MB meets the basic needs for a vascular label, it has two restrictions: MB may, depending upon the anesthetic used, decrease blood pressure, and is effectively removed from the vascular system within 7–15 minutes by the mononuclear phagocytic system. If two or more permeability agents are to be tested in a quantitative manner using MB, their injections should be simultaneous, to avoid complications of the rapid clearance rate. Our results also indicate that age of the animals used with MB should also be closely controlled. Yet to be determined are comparisons between the clearance characteristics of MB and established colloids, the sites of cardiovascular alterations, and the substances within commercially supplied MB that are not biologically inert. Enough evidence is now available, however, to design experiments in ways that incorporate the more useful properties of MB.

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