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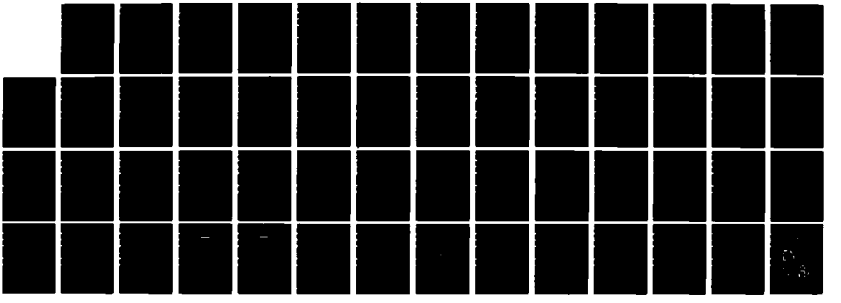

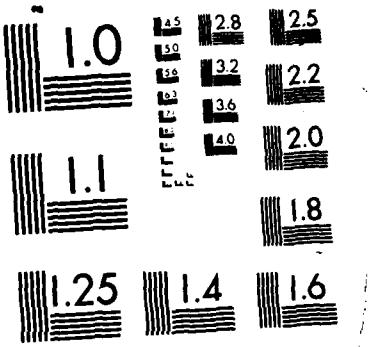

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BLOOD SUBSTITUTES: EFFECTS ON DRUG PHARMACOKINETICS

FINAL REPORT

Glenn R. Hodges, M.D.
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MARCH 1984

Support by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The effect of exchange transfusion with Fluosol DA 20% (FDA) or stroma-free hemoglobin (SFH) on the effectiveness of penicillin therapy of pneumococcal infection and on the pharmacokinetics of antipyrine, diazepam, morphine, penicillin, and sulfamethazine was studied. Penicillin only suppresses pneumococcal infection in FDA transfused rats but cures pneumococcal infection in SFH transfused rats. Transfusion with FDA or SFH does not alter the pharmacokinetics of antipyrine, diazepam, or penicillin. In FDA transfused animals given morphine, the $t_{\frac{1}{2}}$ and AUC were increased and the Cl was...

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decreased. In FDA transfused animals given sulfamethazine, there was an increase in $t_{\frac{1}{2}}$ and Vd. In SFH tranfused animals given morphine, the $t_{\frac{1}{2}}$ and Vd were increased. In SFH transfused animals given sulfamethazine, the AUC was decreased and Cl and Vd were increased. The oxygen-carrying resuscitation fluids alter the pharmacokinetics of morphine and sulfamethazine, but not antipyrine, diazepam, and penicillin. These observations may have important clinical implications. Keywords: Perfluorocarbons

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SUMMARY

The effect of exchange transfusion with FDA or SFH on the effectiveness or penicillin therapy of pneumococcal infection and on the pharmacokinetics of antipyrine, diazepam, morphine, penicillin, and sulfamethazine in rats was determined. Rats were sham transfused or exchanged transfused with 25 ml of Fluosol DA or stroma-free hemoglobin. They were subsequently, intraperitoneally challenged with type 3 pneumococcus and treated with penicillin for 120 h. Twelve of 15 (80%) sham transfused rats were alive at 281 h; only 3 of 13 (23%) Fluosol DA transfused rats were alive at 281 h ($p = 0.005$). Seven of 10 (70%) sham transfused rats were alive at 281 h compared to 9 of 10 (90%) stroma-free hemoglobin transfused rats ($p = 0.24$). Penicillin therapy only suppressed pneumococcal infection in Fluosol DA transfused rats and relapse occurred after therapy was stopped. In contrast, pneumococcal infection in stroma-free hemoglobin transfused rats was cured with penicillin therapy. These data suggest that Fluosol DA alters the ability of rats to respond to pneumococcal infection.

Because FDA and SFH may be used to treat trauma victims, the pharmacokinetics of morphine was studied in rats after transfusion with only one of these fluids. During development of HPLC assay for morphine in plasma, an in vitro interaction between plasma, FDA, or SFH and morphine was observed at pH > 10.5. This interaction was pH dependent and specific for morphine compared to codeine. The interaction between SFH and morphine appeared to be covalent in nature. The $t_{\frac{1}{2}}$ of morphine was significantly prolonged from 1.02 ± 0.50 hr (mean ± SD) to 2.46 ± 2.68 hr ($p = 0.03$) after transfusion with SFH and to 2.05 ± 0.95 hr ($p = 0.006$) after transfusion with FDA. The Vd was increased from 1.35 ± 0.81 $L \cdot Kg^{-1}$ to 2.99 ± 1.45 $L \cdot Kg^{-1}$ ($p = 0.004$) after transfusion with SFH; no difference was observed after transfusion with FDA ($p = 0.86$). The AUC was increased from 2.37 ± 1.78 $mg \cdot hr \cdot L^{-1}$ to 6.02 ± 6.61 $mg \cdot hr \cdot L^{-1}$ ($p = 0.02$) and Cl was decreased from 1.02 ± 0.53 $L \cdot hr^{-1} \cdot Kg^{-1}$ to 0.55 ± 0.36 $L \cdot hr^{-1} \cdot Kg^{-1}$ ($p = 0.01$) after transfusion with FDA; no significant differences were observed after transfusion with SFH ($p = 0.48$ and $p = 0.81$, respectively). These data show that SFH prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Vd. In contrast, FDA prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Cl. These data may have important therapeutic implications.

After transfusion with FDA or SFH, the pharmacokinetics of antipyrine, diazepam, and penicillin were unchanged when compared to control animals. After transfusion with FDA, the $t_{\frac{1}{2}}$ of sulfamethazine was increased from 3.15 ± 0.56 to 7.65 ± 2.41 hr ($p < 0.05$) and the Vd was increased from 60.7 ± 17.5 to 152 ± 16 ml ($p < 0.05$). In contrast, after transfusion with SFH, the AUC of sulfamethazine was decreased from 129 ± 28 to 80.5 ± 27.7 $\mu g \cdot h \cdot ml^{-1}$ ($p < 0.05$) and there was an increase in Cl from 12.2 ± 3.4 to 20.2 ± 6.0 ml h^{-1} ($p < 0.05$) and Vd from 60.1 ± 11.8 to 132 ± 49 ml ($p < 0.05$). The reason for these alterations is not clear. FDA and SFH may alter the acetylation of sulfamethazine.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-32, Revised 1978].

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VI. BODY OF REPORT

A. EFFECT OF EXCHANGE TRANFUSION WITH FLUOSOL DA OR STROMA-FREE HEMOGLOBIN ON OUTCOME OF PNEUMOCOCCAL INFECTION IN RATS

1. Introduction

Oxygen-carrying resuscitation fluids include a variety of substances such as buffers (1-3), plasma (4,5), perfluorocarbon emulsions (5-9), and stroma-free hemoglobin (SFH) have been the most extensively studied (5,11,14). One perfluorocarbon emulsion, Fluosol-DA 20% (FDA; Alpha Therapeutics, Los Angeles, CA) has shown enough promise that clinical trials have been initiated (14,15). The major use of the oxygen-carrying resuscitation fluids will be to treat refractory anemias, blood loss, and vascular occlusions, and for organ perfusion and preservation (16). An important use of these fluids will be to resuscitate trauma victims while waiting for blood or blood products to reach them. Because trauma victims are at high-risk for developing infection, the effect of exchange transfusion with FDA or SFH on recovery from experimentally induced pneumococcal infection, previously described by us (12), was studied. The results indicate that (i) penicillin therapy only suppresses pneumococcal infection in FDA transfused rats, (ii) pneumococcal infection in SFH transfused rats is cured with penicillin therapy, and (iii) FDA may alter the ability of rats to respond to pneumococcal infection.

2. Materials and Methods

a. Animals

Young, adult, male Sprague-Dawley rats (Sasco, Omaha, NE) weighing 250 to 350 g were used for these studies.

b. Fluosol DA 20%

Fluosol DA 20% with the following composition was kindly supplied by Alpha Therapeutics, Los Angeles, CA, for these studies: perfluorodecalin (14.0 w/v%), perfluorotripropylamine (6.0 w/v%), pluronic F-68 (2.7 w/v%), yolk phospholipids (0.4 w/v%), potassium oleate (0.032 w/v%), glycerol (0.8 w/v%), NaCl (0.6 w/v%), KCl (0.034 w/v%), $MgCl_2$ (0.02 w/v%), $CaCl_2$ (0.028 w/v%), $NaHCO_3$ (0.21 w/v%), glucose (0.18 w/v%), and hydroxyethylstarch (3.0 w/v%)

c. Stroma-Free Hemoglobin

A solution of stroma-free hemoglobin, 72 mg/ml, in 0.9% NaCl was generously supplied by the Letterman Army Institute of Research, The Presidio of San Francisco, CA, for these studies.

d. Exchange Transfusion

Under methoxyflurane anesthesia, the left external jugular vein of the rat was exposed and isolated. A polyethylene cannula (PE-50, Clay Adams, Persippany, NJ) was inserted cephalad and anchored with a suture. A 25 gauge scalp vein needle was inserted into a lateral tail vein. The cannula and needle patency were maintained by flushing with heparinized saline solution (25 units/ml). Isovolumentric exchange transfusion was then done by infusing 25 ml of FDA or SFH into the tail

vein by infusion pump while simultaneously withdrawing blood from the jugular cannula. The transfusion took 25 minutes and resulted in a 72.5 ± 1.0% (mean ± standard error of the mean) exchange for FDA and a 74.7 ± 0.9% exchange for SFH. After completion of the transfusion, the tail vein needle was removed. The external jugular vein cannula was left in place for serial blood sampling for pharmacokinetic studies. In animals that were to be infected subsequently, the external jugular vein was ligated above and below the venotomy and the cannula was removed. The wound was closed with autoclips and the animal placed in a cage with supplemental oxygen for 24 hours.

e. Sham Transfusion

Under methoxyflurane anesthesia, the left external jugular vein of the rat was exposed and isolated. A polyethylene cannula was inserted cephalad and anchored with a suture. The jugular vein cannula was used for serial blood sampling for pharmacokinetic studies. In animals that were to be infected subsequently, the left external vein was only ligated in two places. The wound was closed with autoclips and the animal was placed in a cage with supplemental oxygen for 24 hours.

f. Pharmacokinetic Studies

1750 U of penicillin G was administered by intraperitoneal (IP) injection. Plasma samples were obtained for determination of penicillin concentrations before and 5, 15, 30, 60, 120, and 180 minutes after injection. All samples were stored at -70°C until assayed.

g. Penicillin Assay

The concentrations of penicillin were determined using a disk diffusion method. Blank, 6.4 mm antibiotic susceptibility disks were saturated with either the test samples or reference standards diluted in pooled, normal rat plasma. Then, they were placed on the surface of antibiotic medium A, pre-seeded with Staphylococcus aureus (ATCC 6538-P) in 150 mm petri dishes. All test and reference samples were incubated at 37°C for 18 hours. Zones of inhibition around the disks were measured with a vernier caliper. Antibiotic concentrations were determinted by comparing the mean zone of inhibition of each sample tested in triplicate with a curve constructed from the mean zones of inhibition around the reference standards. The sensitivity of this assay was 0.05 U/ml.

h. Organism for Infection Studies

The organism used was Streptococcus pneumoniae type 3 (ATCC 6303). The S. pneumoniae was incubated on blood agar overnight at 37°C in 5% CO_2. Then two or three colonies were inoculated into 100 ml of Todd-Hewitt broth and incubated overnight at 37°C in 5% CO_2. The organisms were washed three times in saline and suspended in saline to a final concentration of 10^4 to 10^5 CFU for inoculation into the rats. The minimal concentration of penicillin to inhibit the growth of the S. pneumoniae type 3 was 0.04 U/ml.

i. Experimental Design

Rats were sham transfused or exchange transfused with 25 ml of FDA or SFH. Twenty-four hours later, the rats were challenged intraperitoneally with 10^4 to 10^5 colony forming units (CFU) of type 3 pneumococcus. Twenty-two hours later,

therapy was initiated with 1750 U of penicillin or 0.5 ml of saline by IP injection every six hours for twenty doses. All surviving rats were obtained for culture for pneumococcus from animals that died while receiving or after receiving penicillin therapy.

j. Data Analysis

Survival curves were constructed using the method of Kaplan Meier (17). Comparisons between survival curves were made using the generalized Kruskal-Wallis statistic (18).

3. Results

All FDA transfused, uninfected rats (n = 5) lived for the 281 hour test period (Fig. 1). All FDA transfused and infected but saline treated rats (n = 8) died by 73.5 hours after challenge. Similarly, all sham transfused and infected but saline treated rats (n = 6), except one, died by 61.5 hours after challenge (p = 0.74). Conversely, 12 of 15 (80%) sham transfused and infected but penicillin treated rats were alive at 281 hours. In contrast, only 3 of 13 (23%) FDA transfused and infected but penicillin treated rats were alive at 281 hours (p = 0.005). Of the 13 infected and penicillin treated rats from either sham or FDA transfused groups that died, 12 died after therapy was stopped. Pneumococcus was found in the peritoneal fluid, pleural fluid, and/or blood cultures of 8 of 10 rats that died and were examined (Table 1).

All SFH transfused, uninfected rats (n = 5) lived for the 281 hour test period (Fig. 2). All SFH transfused and infected but saline treated rats (n = 5) died by 55 hours after challenge. All sham transfused and infected but saline treated rats (n = 10) died by 37 hours after challenge (p = 0.43). In contrast, 7 of 10 (70%) sham transfused and infected but pencillin treated rats were alive at 281 hours. Similarly, 9 of 10 (90%) SFH transfused and infected but penicillin treated rats were alive at 281 hours (p = 0.24). Of the four penicillin treated rats from both groups that died, all died within 27 hours of the start of penicillin therapy. Pneumococcus was found in the peritoneal fluid, pleural fluid, and/or blood cultures of two of the four that died.

When penicillin concentrations were determined after IP injection immediately after transfusion with FDA, the penicillin concentrations were higher with sustained elevation compared to SFH and sham transfused rats (Fig. 3). However, 48 hours and 168 hours after transfusion, the concentrations of penicillin were similar in sham, FDA, and SFH transfused rats.

4. Discussion

One use for oxygen-carrying resuscitation fluids will be for the resuscitation of trauma victims prior to the availability of blood or blood products. These patients may be susceptible to infection because of the nature of the injury. To determine if transfusion with FDA or SFH could influence the outcome of infection, a well characterized model of pneumococcal sepsis after intraperitoneal inoculation of type 3 S. pneumoniae was used (19).

Penicillin G therapy was found to control type 3 pneumococcal infection in rats after exchange transfusion with FDA or SFH. However, after cessation of therapy with penicillin G, the majority of the FDA transfused animals succumbed

to pneumococcal infection. In contrast, delayed death caused by pneumococcal infection was not observed among SFH transfused rats. Thus, pneumococcal infection was only suppressed in FDA transfused rats, but cured in SFH transfused rats.

The observed results were not due to the inability of the administered penicillin G to be absorbed from the peritoneal cavity into the systemic circulation. In uninfected animals, penicillin G plasma levels were sustained immediately after transfusion with FDA. However, the plasma levels two and seven days after transfusion, comparable to the first and fifth day of therapy of infected rats, were similar to those observed in uninfected sham transfused rats and in uninfected SFH tranfused rats. Although penicillin G levels were not determined in infected animals, it is anticipated that because of the peritoneal inflammation induced by the infection the absorption of penicillin G would be improved.

Although the exchange transfusion procedure per se may alter the host's ability to respond to infection, the same exchange transfusion procedure was used for rats transfused with either FDA or SFH. These data suggest that the exchange transfusion procedure is not responsible for the results observed with FDA. Instead, the inability to cure pneumococcal infection in FDA transfused rats is related specifically to transfusion with FDA.

Perfluorocarbon compounds are cleared from the circulation by phagocytosis by fixed tissue macrophages (20). After transfusion with FDA, clearance of colloidal carbon by the reticuloendothelial system of rats is impaired, sensitivity of mice to the lethal effects of endotoxin is increased, and the ability of mice to withstand an injection of Enterobacter species is impaired (21). FDA has also been reported to inhibit the phagocytosis of latex beads by human and rabbit monocytes and neutrophils (22). Thus, in our studies, the rats may have been unable to clear their bodies of pneumococci in spite of treatment with penicillin because of FDA associated defects in their immunocompetence. Because FDA contains a number of components, the component(s) responsible for the observed results is unknown.

SFH, also, is cleared from the circulation by phagocytosis but other mechanisms, such as glomerular filtration, are operative (11). The apparent lack of effect of SFH on penicillin G therapy of pneumococcal infection may be related to the magnitude of the effect rather than the lack of effect on the immunocompetence of rats.

5. Conclusions

   FDA alters the ability of rats to respond to pneumococcal infection to a greater extent than SFH.

6. Recommendations

   a. Further characterization of pneumococcal infection in FDA and SFH transfused rats.

   b. Characterization of immunologic responsiveness of FDA and SFH transfused rats.

B. INTERACTION OF OXYGEN-CARRYING RESUSCITATION FLUIDS WITH MORPHINE

1. Introduction

Perfluorocarbon emulsions and hemoglobin solutions have been the most extensively studied oxygen-carrying-resuscitation fluids (5,11,14). One perfluorocarbon emulsion, FDA, has shown enough promise that clinical trials have been initiated (14,15). The major uses of these fluids will be to treat refractory anemias, as well as for organ perfusion and perservation (16). One important use of a blood substitute will be to resuscitate trauma victims while waiting for blood or blood products to reach them. Patients who receive blood substitutes under these circumstances usually also will receive drug therapy, including analgesics such as morphine. When coadministered with a blood substitute, the pharmacokinetics of these drugs may be altered to such an extent that they are toxic or ineffective.

Because of these considerations, this study was done to determine the pharmacokinetics of morphine in rats transfused with either FDA or SFH. In doing so, an unexpected in vitro interaction between morphine and plasma, FDA, or SFH was observed. Morphine, but not codeine, was found to interact with plasma, FDA, or SFH at pH > 10.5. The binding of SFH to morphine appeared to be covalent in nature. SFH prolonged the $t_{\frac{1}{2}}$ of morphine by altering the Vd. In contrast, FDA prolonged the $t_{\frac{1}{2}}$ of morphine by altering the Cl.

2. Materials and Methods

a. Animals

Young, adult, male Sprague-Dawley rats weighing 250 to 350 g (Sasco, Omaha, NE) were used for the study.

b. FDA

Fluosol DA 20% with the following final composition was kindly supplied by Alpha Therapeutics, Los Angeles, CA, for these studies: perfluorodecalin (14.0 w/v%), perfluorotripropylamine (6.0 w/v%), pluronic F-68 (2.7 w/v%), yolk phospholipids (0.4 w/v%), potassium oleate (0.032 w/v%), glycerol (0.8 w/v%), NaCl (0.6 w/v%, KCl (0.034 w/v%), $MgCl_2$ (0.02 w/v%), $CaCl_2$ (0.028 w/v%), $NaHCO_3$ (0.21 w/v%), glucose (0.18 w/v%), and hydroxyethylstarch (3.0 w/v%).

c. SFH

A 72 mg/ml solution of human stroma-free hemoglobin in 0.9% NaCl was generously supplied by the Letterman Army Institute of Research, The Presidio of San Francisco, CA.

d. Morphine

Morphine sulfate injectable, 10 mg/ml, was obtained from Wyeth, Philadelphia, PA. Morphine sulfate reference standard was obtained from the U.S. Pharmacopoeia, Rockville, MD. Tritiated morphine was obtained through New England Nuclear, Boston, MA.

e. Morphine Assay

Morphine concentrations were determined by reverse-phase HPLC with fluoresence detection. To 100 µl of a sample to be assayed, 50 µl of codeine phosphate (U.S. Pharmacopoeia, Rockville, MD), 1.5 µg/ml in water as internal standard, and 50 µl of 0.20 M NaOH were added and mixed. The sample was then extracted by adding 1.5 ml of an ethyl acetate/isoamyl alcohol mixture (10:1, v/v) and vortexing at 600 rpm for 60 sec. Following centrifugation at 1000 x g for 10 min, the organic layer was transferred to a clean test tube. The solvent was evaporated to dryness in a 50°C water bath under a stream of nitrogen gas. The residue was dissolved in 50 µl of mobile phase (45:55 acetonitrile:water, v/v; 0.50 g sodium lauryl sulfate/L; and 6.0 ml glacial acetic acid/L) and 20 µl were injected onto the chromatograph column. The apparatus consisted of Waters 6000 A pump (flow rate = 2.0 ml/min), a 30 cm x 4 mm reverse-phase C_{18} column (Waters, Milford, MA) and a Schoeffel FS 970 fluorometer (excitation = 215 nm, emission = 340 nm). The retention times of morphine and codeine were 4.8 and 6.0 min, respectively. Morphine concentration was determined by comparing the peak height ratio of morphine to codeine with a plasma standard curve. The lower limit of sensitivity of this assay was 0.1 µg/ml.

f. PBS

A PBS solution was prepared by adding a sufficient amount of a 0.1 M NaOH solution to 50 ml of a 0.1 M NaH_2PO_4 solution to produce a measured pH of 7.4. Both starting solutions were prepared in a 0.1 M NaCl solution.

g. pH Titration

To 100 µl of plasma, FDA, or SFH samples containing 3.0 µg/ml morphine, 50 µl of each of eight two-fold serial dilutions of 1.0 M NaOH in PBS were added. Subsequently, 50 µl of internal standard were added to each. The samples were extracted and assayed for morphine. The pH of extraction ranged from 12.5 to 7.4. The experiment was done in triplicate.

h. Titration of Fluids

Two-fold serial dilutions of plasma, FDA, and SFH in PBS, each containing 3.0 µg/ml of morphine, were prepared. To 100 µl of each, 50 µl of codeine phosphate internal standard were added plus 50 µl of 2.0 M NaOH or PBS. Samples were extracted and assayed for morphine. The experiment was performed in triplicate.

i. Extraction Efficiency

The extraction efficiency of morphine from plasma, FDA, and SFH at pH 7.4 and 12.5 was determined by extracting 0.5 ml samples of each fluid containing 3.0 µg/ml ^{3}H-morphine (specific activity = 10.5 mCi/mmol). To each sample, 0.25 ml of codeine internal standard and 0.25 ml of either PBS or 2.0 M NaOH were added. The samples were then extracted with 7.5 ml of ethyl acetate/isoamyl alcohol (10:1, v:v) and centrifuged at 1000 g x 10 min. The organic layer was drawn off, evaporated to dryness, and reconstituted with 4.0 ml of aqueous counting fluid (ACS, Amersham Corporation, Arlington Heights, IL). The samples were counted on a Packard Tri-Carb Model 3255 liquid scintillation spectrometer.

j. Tight Binding at Extraction

SFH containing 3.0 µg/ml morphine was prepared. To 100 µl samples, 50 µl of internal standard and 50 µl of 2.0 M NaOH were added. Either 100 mg NaCl, 50 µl 5 M urea, or enough 2 M HCl to bring the pH down to 7.4 was added to these preparations. The samples were then extracted and assayed for morphine. Each test was run in triplicate.

k. Apparent Covalent Binding

To 1.0 ml samples of SFH containing 3.0 µg/ml ^{3}H-morphine (specific activity = 10.3 mCi/mmol) were added 0.50 ml of internal standard and either 0.50 ml of 2.0 M NaOH (final pH = 12.5) or 0.50 ml of PBS (final pH = 7.4). Samples were extracted with 15 ml ethyl acetate/isoamyl alcohol (10:1, v/v), and centrifuged at 1000 x g for 10 min. The organic layer was drawn off and 3.0 ml of 0.8 M TCA were added to the aqueous layer to precipitate the hemoglobin. The sample was vortexed at 600 rpm for 60 sec and then centrifuged at 1000 x g for 10 min. The supernatant was discarded and two identical washings were performed with 0.4 M TCA. The supernatant was discarded and seven similar washings were performed with 3.0 ml methanol:water, 80:20, v/v. The final precipitate was digested with 1.0 ml 10 N NaOH in a boiling water bath. An aliquot (50 µl) was added to 10 ml of scintillation fluid composed of 14 g 2, 5-bis [5'-tert-butylbenzoxazolyl-(2')]-thiophene (Sigma, St. Louis, MO) and 280 g naphthalene dissolved in 2000 ml toluene and 1400 ml ethylene glycol monomethyl ether, and counted on a Packard Tri-Carb 460 CD liquid scintillation spectrometer.

l. Equilibrium Dialysis

Binding of ^{3}H-morphine to plasma, FDA, SFH, and plasma from rats immediately after transfusion with either FDA or SFH at pH 7.4 was determined by dialyzing 1.0 ml samples of these fluids containing 3.0 µg/ml morphine against 1.0 ml of PBS across a membrane with a cutoff of 3,500 daltons (Spectraphor, Spectrum Medical Industries, Inc., Los Angeles, CA). The dialysis cells were incubated at 37°C for 18 hr and the contents immediately assayed. The morphine specific activity was 3.5 mCi/mmol. Aliquots of buffer, plasma, FDA, SFH, and plasma from FDA and SFH transfused rats were analyzed for radioactivity in 4 ml of scintillation fluid as a control.

m. Transfusions

Rats were anesthetized with methoxyflurane. A plastic cannula (PE-50, Clay Adams, Persippany, NJ) for blood sampling was inserted cephalad into the left external jugular vein and anchored with a ligating suture. FDA transfused animals were cannulated in a lateral tail vein with a 25 gauge scalp vein needle for administration of the emulsion. SFH was infused through a cannula inserted caudad into the left jugular vein and anchored with a ligating suture. The cannulae were kept patent with a heparin solution of 400 units/ml. An isovolumetric exchange transfusion was performed by infusing 25 ml of FDA or SFH while simultaneously withdrawing blood at a rate of 1.0 ml/min with a syringe pump (model 341A, Sage Instruments, Cambridge, MA). This procedure accomplished a 71 ± 4% (mean ± SD) exchange for FDA and a 73 ± 5% exchange for SFH. After infusion, the delivery cannulae were removed. The remaining cephalad jugular cannulae were used for serial blood sampling. Control animals were treated identically except that no transfusion was performed. Light anesthesia was maintained for the duration of the transfusion procedure.

n. Pharmacokinetic Studies

Immediately after transfusion with SFH or FDA, 0.5 mg of morphine sulfate was administered to each animal by bolus IV injection. Blood, 0.5 ml, for plasma samples was collected prior to dosing and at 15, 30 45, 60, 120, 180, 240, and 360 min after dosing. Samples were stored at -70°C until assayed.

o. Hemoglobin Assay

Plasma samples were assayed for hemoglobin as previously described (23). Briefly, to 100 µl of sample, 1.0 ml of a 1.0 g/L solution of leucomalachite green [p,p'-benzylidenebis-(N,N-dimethylaniline), Eastman Organic Chemicals, Rochester, NY] in 3.3 M acetic acid and 1.0 ml of 0.10% H_2O_2 were added. Ten minutes were allowed for color development and optical absorbance at 617 nm was determined on a Hitachi/Perkin-Elmer 139 UV-VIS Spectrophotometer. Standards of 45, 90, 240, and 360 µg/ml were used. Samples from the SFH transfused animals were diluted with water to bring the hemoglobin concentration within the range of the standards.

p. Statistical Analyses

Pharmacokinetic parameters were calculated for each animal, adjusted for the weight of each animal where appropriate, and compared across treatment groups. The AUC was computed using the trapezoidal rule and converted to Cl using the relation:

$$Cl = Dose/AUC$$

Either a one-compartment or a two-compartment model was fit for each animal. The elimination $t\frac{1}{2}$ was computed using the relation:

$$t\frac{1}{2} = LN(2)/k$$

and the Vd was computed using the relation:

$$Vd = Cl/k$$

where k = slope of the elimination compartment. Summary statistics, including the median, range, mean, and standard deviation were computed for each group of animals. The SFH and FDA groups were compared to the control group using the Wilcoxon Rank Sum Test for each of the pharmacokinetic parameters. All calculations were performed using the BMDP Statistical software.

3. Results

IN VITRO INTERACTION OF MORPHINE WITH RESUSCITATION FLUIDS

Using the original assay, the detection of morphine in plasma was hampered by the presence of an interfering peak in 8 of 20 samples tested (Fig. 4). For these assays, the samples were extracted after the addition of 0.20 M NaOH, which produced an extraction pH of 11.1. Similar interfering peaks were never observed when morphine was assayed in FDA and SFH containing samples. When extraction of plasma samples was done at pH greater than or less than 11.1, the interfering peak was not observed. No further characterization of this interfering material was done.

In an attempt to eliminate the interfering peak, the concentration of the NaOH solution added prior to extraction was increased to 2.0 M. This raised the extraction pH to 12.5. The resulting chromatograms showed morphine in samples from all control animals and FDA transfused animals. However, no morphine was detected in samples obtained as early as 15 minutes after injection into animals transfused with SFH (Fig. 5). Samples of SFH containing 3.0 µg/ml of morphine, which was readily detected in a plasma standard, also failed to show morphine. This suggested that an assay phenomenon, rather than erratic pharmacokinetics, was responsible for the lack of detectable morphine in the SFH samples. Experiments were performed to further characterize this phenomenon.

a. pH Titration

The ability to detect morphine in plasma, FDA, and SFH was pH dependent (Fig. 6). The recovery of morphine was maximal at or near the pKa of morphine (pH 8.9) and dropped off at pH > 10.5. Although morphine was still detectable in plasma and FDA extracted at pH 12.5, morphine was not detectable in SFH extracted at pH $\geq$ 11.7. The recovery of codeine, the internal standard for the assay, was unaffected by alteration of the extraction pH.

b. Titration of Fluids

Serial two-fold dilutions of plasma, FDA, and SFH, when assayed at pH 7.4, showed consistent recovery of morphine (Fig. 7A). Recovery of morphine at pH 12.5 was inversely related to the amount of plasma, FDA, or SFH present (Fig. 7B). Most striking, in the case of the SFH samples, no morphine was recovered at hemoglobin concentrations $\geq$ 2.3 mg/ml ($\log_2$ dilution^{-1} = 5), and recovery was still poor at a hemoglobin concentration of 18 µg/ml ($\log_2$ dilution^{-1} = 12). There was also a consistent trend for the recovery in plasma to exceed that in FDA. Again, the recovery of codeine was unaffected by either changes in the concentrations of plasma, FDA, and SFH or extraction pH.

c. Extraction Efficiency

The efficiency of extracting morphine at pH 7.4 from plasma, FDA, and SFH was 88 ± 7.5% (mean ± S.D.), 83 ± 5.1%, and 84 ± 5.9%, respectively. However, when the pH of extraction was increased to 12.5, the efficiency fell off markedly to 47 ± 1%, 28 ± 3.5% and 0.11 ± 0%, respectively.

d. Binding of Morphine to SFH

Morphine was not detected in samples of SFH containing morphine extracted at pH 12.5 following saturation with NaCl, addition of urea, or titration with HCl back to pH 7.4. Apparent covalent binding was determined by quantitating the radioactivity associated with the protein precipitate following repeated TCA and methanol/water extractions. This binding was also pH dependent. Following extraction at pH 7.4, only 1.9% of the morphine was bound to the hemoglobin protein. In contrast, after extraction at pH 12.5, 65% of the morphine was bound to the hemoglobin protein.

e. Modification of Assay

On the basis of these findings, the original assay was modified by substituting PBS for the sodium hydroxide solution. This yielded an extraction pH of 7.4.

f. Equilibrium Dialysis

When dialyzed against PBS at 7.4, 24 ± 1.1%, 10 ± 1.6%, and 0.68 ± 0% of ^{3}H-morphine was bound to plasma, FDA, and SFH, respectively. When plasma from rats previously transfused with FDA or SFH was dialyzed against PBS at pH 7.4, 17 ± 1.7% and 10 ± 2.8% of ^{3}H-morphine, respectively, was bound.

IN VIVO INTERACTION OF MORPHINE WITH RESUSCITATION FLUIDS

a. Pharmacokinetics

The mean time-concentration curves for morphine demonstrated differences among the three groups of animals (Fig. 8). All groups showed a distribution (alpha) phase during the first hour, and an elimination (beta) phase over the following five hours. However, the plasma concentration of morphine in the FDA transfused groups consistently exceeded that of the control group for the duration of the 6 hr time course. In addition, the plasma concentration of morphine in the SFH transfused group was lower than in the control group for the first three hours, after which it slightly exceeded that of the controls for one hour before both groups dropped to undetectable levels at five hours.

Pharmacokinetic parameters were calculated for each groups of animals (Table 2). The t of morphine was significantly prolonged from 1.02 ± 0.50 hr (mean ± SD) to 2.46 ± 1.68 hr ($p = 0.03$) after transfusion with SFH and to 2.05 ± 0.95 hr ($p = 0.006$) after transfusion with FDA. The Vd was increased from 1.35 ± 0.81 $L \cdot Kg^{-1}$ to 2.99 ± 1.45 $L \cdot Kg^{-1}$ ($p = 0.004$) after transfusion with SFH; no difference was observed after transfusion with FDA ($p = 0.86$). The AUC was increased from 2.37 ± 1.78 $mg \cdot hr \cdot L^{-1}$ to 6.02 ± 6.61 $mg \cdot hr \cdot L^{-1}$ ($p = 0.02$) and Cl was decreased from 1.02 ± 0.53 $L \cdot hr^{-1} \cdot Kg^{-1}$ to 0.55 ± 0.36 $L \cdot hr^{-1} \cdot Kg^{-1}$ ($p = 0.01$) after transfusion with FDA; no significant differences were observed after transfusion with SFH ($p = 0.48$ and $p = 0.81$, respectively). These data show that SFH prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Vd. In contrast, FDA prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Cl.

b. Hemoglobin Assay

All of the 0 hr plasma samples for the pharmacokinetic study contained detectable amounts of hemoglobin (Table 3).

4. Discussion

Various methods have been developed for the quantitation of morphine in biological fluids, including radioimmunoassay (24,25), gas-liquid chromatography (26,27), and high performance liquid chromatography (28,29). The sensitivity of an assay can be improved by extraction of the morphine from the same with an organic solvent and concentration of the extract by evaporation. The pH of a sample becomes an important consideration when extracting an amphoteric drug such as morphine. Since morphine is a weak base, it exists predominantly in the nonionized form at a pH at or greater than its pKa of 8.9. The extraction pH of reported assay techniques ranges from 8.7 to 10.0 (30-32). In the first two assay techniques we used, namely pH 11.1 and 12.5, most of the morphine in the sample would be shifted into the nonionized form. As such, it should have been easily extracted into the organic phase. However, an unexpected interaction between morphine and SFH was observed. At pH 12.5, no morphine was detected in the extract.

Further study revealed that plasma and FDA also interact with morphine, although to a lesser extent than SFH. The interaction has several unusual characteristics. First, the interaction is pH dependent. In the case of SFH, the interaction parallels the alkaline denaturation of hemoglobin. Human hemoglobin dissociates into dimers above pH 10.0 (33), the point at which the observed binding effect begins to become pronounced. At pH 11.6, denaturation of the protein chains begins (34). This corresponds to the point at which morphine was no longer detected. Denaturation is brought about by the alkaline ionization of buried weakly acidic side chains, chiefly tyrosine and cysteine, which stablize the unfolded form of the molecule (35). These are possible sites for binding of morphine. Second, the interaction is dependent upon the amount of plasma, FDA, or SFH present. At pH 7.4, the recovery of morphine is consistent regardless of the amount of plasma, FDA, or SFH present. In contrast, at pH 12.5, the amount of morphine recovered was inversely proportional to the amount of plasma, FDA, or SFH present. Third, the interaction we observed is specific for morphine. Codeine, used as the internal standard, did not interact with plasma, FDA, or SFH, although it differs from morphine at only the 3 position (Fig. 9). In the case of SFH, this interaction would not have been predicted solely on the basis of hemoglobin denaturation by alkaline pH. If this were the case, then codeine also should have been bound tightly to the hemoglobin. These considerations suggest that the 3-hdyroxyl group of morphine is the site for binding to the hemoglobin molecule. Fourth, in the case of SFH, the interaction is tight. The covalent binding procedure described is a modification of the technique of Jollow and colleagues (36) and is presumptive evidence of covalent binding. Hydrogen bonding or Van der Vaals forces are too weak to withstand the conditions of this assay. Although we do not have direct chemical proof of a covalent bond between morphine and the hemoglobin molecule at pH 12.5, our data suggest that this binding is very tight. Fifth, although we have no data, the decrease in extraction efficiency with increasing pH may in part be due to ionization of the 3-hydroxy group of morphine.

The assay that was finally used was chosen because at pH 7.4 the recovery of morphine from plasma, FDA, or SFH was consistent and was unaffected by the amount of plasma, FDA, or SFH present. This was an important consideration because the amounts of plasma and FDA or SFH in samples from transfused animals are neither constant nor readily determined.

These findings, also, have implications regarding human plasma samples analyzed for morphine. Human plasma normally contains 5 to 50 µg/ml free hemoglobin and as much as 250 to 300 µg/ml in hemolytic anemias (37). This is sufficient hemoglobin to cause the binding phenomenon that we observed. If an extraction procedure is used to process the sample, a pH of no greater than 10.0 should be used.

Morphine is a rapidly metabolized drug (38). It has been reported to have a $t\frac{1}{2}$ of approximately two hours in humans and rats (38,39). The $t\frac{1}{2}$ of morphine in our control group was determined to be approximately one hour. The $t\frac{1}{2}$ of morphine in animals transfused with SFH and FDA was significantly increased. In the case of animals transfused with SFH, the prolongation of the $t\frac{1}{2}$ of the drug with a concomitant two-fold increase in the Vd would suggest that decreased binding of morphine to SFH, compared to plasma, is an important factor in the observed pharmacokinetics. In the case of animals transfused with FDA, the prolonged $t\frac{1}{2}$ was accompanied by a decrease in Cl, which would implicate an inhibition of morphine metabolism and/or a decrease in hepatic blood flow as the cause of

the prolonged $t_{\frac{1}{2}}$. We cannot distinguish between these possibilities with our data. Since the primary metabolic pathway for clearance of morphine is glucuronidation in the liver (40), future study of the effect of perfusion with FDA on this metabolic pathway as well as on hepatic blood flow would be of interest. Demonstration of altered pharmacokinetics of morphine in the rat model suggest similar phenomena may occur in man. Careful monitoring of the effect of morphine and, possibly, monitoring of morphine serum concentrations in humans transfused with either resuscitation fluid are indicated.

5. Conclusions

   a. Morphine interacts with plasma, FDA, and SFH at pH > 10.5.

   b. The binding of SFH to morphine is covalent.

   c. SFH prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Vd.

   d. FDA prolongs the $t_{\frac{1}{2}}$ of morphine by altering the Cl.

6. Recommendations

   a. Morphine assays be done at pH < 10.5.

   b. The duration of altered pharmacokinetics of morphine after transfusion with FDA or SFH should be determined.

   c. The mechanism of altered morphine clearance after transfusion with FDA should be determined.

   d. The biologic effect of altered morphine pharmacokinetics after transfusion with FDA and SFH should be determined.

C. EFFECT OF EXCHANGE TRANSFUSION WITH FLUOSOL DA OR STROMA-FREE HEMOGLOBIN ON PHARMACOKINETICS OF ANTIPYRINE, DIAZEPAM, PENICILLIN, AND SULFAMETHAZINE

1. Introduction

Perfluorocarbon emulsions and hemoglobin solutions have been the most extensively studied oxygen-carrying resuscitation fluids (5,11,14). One perfluorocarbon emulsion, FDA, has shown enough promise that clinical trials have been initiated (14,15). The major uses of these fluids will be to treat refractory anemias, as well as organ perfusion and preservation (16). Another important use of a blood substitute will be to resuscitate trauma victims while waiting for blood or blood products to reach them. Ease of storage as well as elimination of blood typing procedures make these substitutes ideal for such purposes. However, patients who receive blood substitutes under these circumstances usually also will receive therapy with other drugs. When coadministered with FDA or SFH, the presence of these substitutes may alter drug disposition, and depending on the particular interaction, may render normal dosages subtherapeutic, toxic, or lethal.

Because of these considerations, this study was performed to determine the pharmacokinetics of antipyrine, diazepam, penicillin, and sulfamethazine in rats transfused with either FDA or SFH. These drugs represent a variety of biologic

actions, binding properties, and metabolic and excretory pathways. Therefore, alteration in their pharmacokinetics would suggest that the resuscitation fluids alter drug disposition.

The experiments performed showed no significant differences in the pharmacokinetic parameters of the drugs except in the case of sulfamethazine. When compared to appropriate controls, sulfamethazine treated animals transfused with FDA showed an increased $t\frac{1}{2}$ and Vd. In addition, animals transfused with SFH showed decreased AUC and increased Cl and Vd for sulfamethazine.

2. Materials and Methods

a. Animals

Young, adult, male Sprague-Dawley rats weighing 325 ± 33 g (mean ± S.D.) were used for the study.

b. SFH

A 72 mg/ml solution of stroma-free hemoglobin in 0.9% NaCl was generously supplied by the Letterman Army Institute of Research, The Presidio of San Francisco, CA.

c. FDA

Fluosol DA 20% with the following final composition was kindly supplied by Alpha Therapeutics, Los Angeles, CA, for these studies: perfluorodecalin (14.0 w/v%), perfluorotripropylamine (6.0 w/v%), pluronic F-68 (2.7 w/v%), yolk phospholipids (0.4 w/v%), potassium oleate (0.032 w/v%), glycerol (0.8 w/v%), NaCl (0.6 w/v%), KCl (0.034 w/v%), $MgCl_2$ (0.02 w/v%), $CaCl_2$ (0.028 w/v%), $NaHCO_3$ (0.32 w/v%), glucose (0.18 w/v%), and hydroxyethylstarch (3.0 w/v%).

d. Transfusions

Rats were anesthetized with methoxyflurane. A plastic cannula (PE-50, Clay Adams, Persippany, NJ) for blood sampling was inserted cephalad into the left external jugular vein and anchored with a ligating suture. FDA transfused animals were cannulated in a lateral tail vein with a 25 gauge scalp vein needle for administration of the emulsion. SFH was infused through a cannula inserted caudad into the left jugular vein and anchored with a ligating suture. The cannulae were kept patent with a heparin solution of 400 units/ml. An isovolumetric exchange transfusion was performed by infusing 25 ml of FDA or SFH while simultaneously withdrawing blood at a rate of 1.0 ml/min with a syringe pump (Model 341A, Sage Instruments, Cambridge, MA). After infusion, the delivery cannulae were removed. The remaining cephalad jugular cannulae were used for serial blood sampling. Control animals were treated identically except that no transfusion was performed. Light anesthesia was maintained for the duration of the transfusion procedure. Percent blood volume replacement is listed in Table 4.

e. Pharmacokinetic Studies

Each drug was administered by bolus intravenous injection immediately after transfusion with FDA or SFH. Plasma samples were collected by withdrawing 0.5 ml of blood at varying intervals after injection. Samples for antipyrine (Sigma,

St. Louis, MO; dose = 5 mg in FDA transfused animals and 10 mg in SFH transfused animals) were taken at 0, 15, 60, 120, 180, 240, 300, and 360 min. Total urine for 24 h was also collected by placing animals in metabolic cages (Plas-Labs, Lansing, MI). Samples for diazepam (Roche, Nutley, NJ; dose = 2 mg) were taken at 0, 15, 30, 45, 60, 120, 180, 240, 300, and 360 min. Samples for penicillin (E.R. Squibb & Sons, Princeton, NJ; dose = 1750 U/animal) were taken at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 min. Samples for sulfamethazine (Sigma, St. Louis, MO; dose = 1.5 mg) were taken at 0, 15, 30, 45, 60, 120, 180, and 360 min and 24, 48, and 72 hr. Light anesthesia was maintained for the first 6 h. Samples were stored at -70°C until assayed.

DRUG ASSAYS

a. Antipyrine and Metabolites

Plasma concentration of AP and urine concentrations of AP and its metabolites, $30HCH_3AP$, 40HAP, and NdemAP, were determined by HPLC. A mobile phase consisting of 86% 0.02 M acetate buffer (pH 5.4) and 14% isopropanol/acetonitrile (55:45 v:v) was used with a Waters μ Bondapak C_{18} column. Flow rates were 2.0 ml/min for $30HCH_3AP$ extractions and 1.5 ml/min for NdemAP and 40HAP extractions. Ultraviolet absorbance at 244 nm was detected at a sensitivity of 0.1 absorbance units full scale.

A standard curve was prepared in the following manner in order to prevent subjecting the metabolites to potentially degrading conditions: 240 μl of a 1.0 mg/ml $30HCH_3AP$ solution and 30 μl of a 1.0 mg/ml AP solution, both in methanol, were dried under nitrogen at 37°C. The residue was reconstituted in 240 μl of a 1.0 mg/ml solution of both NdemAP and 40HAP. To this was added, 1200 μl of blank urine and four serial 1:1 dilutions with blank urine gave the solutions for the standard curve. This gave concentrations of 20.8, 10.4, 5.2, 2.6, and 1.3 μg/ml of AP and 166, 83, 41.5, 20.8, and 10.4 μg/ml for each of the three metabolites.

A solution of acetophenetidin, 100 μg/ml in water, was used as an internal standard. Fifty microliters of this solution was added to 500 μl of the standards and the experimental urines. To hydrolyze glucuronides, 500 μl samples of urine were incubated for 30 min at 37°C after addition of 1000 μl of 0.1 M pH 4.5 acetate buffer, 50 μl β-glucuronidase (5000 Fishman units bovine enzyme, Sigma, St. Louis, MO), and 100 μl $Na_2S_2O_5$, 80 mg/ml, in acetate buffer. The metabolites 40HAP and NdemAP were extracted in the following manner: to 500 μl urine, 2.5 ml of a 70:30 pentane:methylene chloride solution (v/v) were added and the mixture shaken for 10 min. Following centrifugation at 700 g for 5 min, the organic layer was drawn off and the aqueous layer re-extracted by the same procedure. The combined organic layers were dried under nitrogen at 37°C and the residue reconstituted with 200 μl mobile phase. One hundred microliters were injected onto the HPLC apparatus. Antipyrine and $30HCH_3AP$ were extracted in the following way: to 500 μl urine, 100 mg Na_2SO_4 and 50 μl 5N NaOH were added and mixed well. The sample was subsequently extracted with 5 ml methylene chloride by shaking for 10 min and centrifugation for 5 min at 700 g. The aqueous layer was removed, the organic layer was decanted and dried under nitrogen at 37°C. The residue was reconstituted with 200 μl of mobile phase and 100 μl injected onto the HPLC. Plasma concentrations of AP were determined identically, except that plasma was used for preparation of the standard curve.

b. Diazepam

Plasma concentrations of DZ were determined by HPLC. A mobile phase consisting of 36:64 acetonitrile:water (v:v) with 1 g/L sodium dodecyl sulfate and 6.0 ml/L acetic acid at a flow of 2.0 ml/min was used with a Waters μ Bondapak C_{18} column. A Waters 440 absorbance detector set at 0.02 absorbance units full scale at 254 nm was used. To 200 μl samples of plasma, 100 μl of oxazepam internal standard (20 μg/ml in methanol) were added. To this, 500 μl of a saturated Na_3PO_4 solution were added and the sample was extracted with 2 ml ethyl acetate by vortexing for 60 seconds. Following centrifugation at 1000 g for 10 min, the organic layer was drawn off and dried under nitrogen at 50°C. The sample was reconstituted with 150 μl mobile phase and 20 μl were injected onto the HPLC. DZ concentrations were determined by comparing the peak height ratio of DZ and oxazepam in samples to a curve of plasma samples to which was added 20, 10, 5, 2.5, 1.0, 0.50, and 0.25 μg/ml of DZ plus the internal standard. The lower limit of detection of this assay was 0.1 μg/ml.

c. Penicillin Assay

The concentrations of PC were determined using a disk diffusion method. Blank, 6.4 mm antibiotic susceptibility disks were saturated with either the test samples or reference standards diluted in pooled, normal rat plasma. The disks were placed on the surface of antibiotic medium A, pre-seeded with Staphylococcus aureus (ATCC 6538-P) in 150 mm petri dishes. All test and references samples were incubated at 37°C for 18 hours. Zones of inhibition around the disks were measured with a vernier caliper. Antibiotic concentrations were determined by comparing the mean zone of inhibition of each sample tested in triplicate with a curve constructed from the mean zones of inhibition around the reference standards. The lower limits of sensitivity of this assay was 0.05 U/ml.

d. Sulfamethazine Assay

SM in plasma was assayed by UV-absorbance coupled with HPLC. A mobile phase of 25% methanol in water at 2.0 ml/min was used with a Waters μ Bondapak C_{18} column. The ultraviolet absorbance of the eluate was detected with a Waters 440 absorbance detector at 254 nm, 0.1 absorbance units full scale. Samples were prepared by adding 100 μl of 15% trichloroacetic acid to 100 μl of sample plus 50 μl of sulfisoxazole (20 μg/ml in water), as an internal standard. Samples were mixed and centrifuged at 1000 g for 5 min, and 20 μl of the supernatant injected onto the HPLC. A standard curve as prepared by adding sulfamethazine to plasma to give concentrations of 16, 8.0, 4.0, 2.0, 1.0, 0.50, 0.25, and 0.125 μg/ml and assayed as above. The lower limit of detection of this assay was 0.02 μg/ml.

e. Pharmacokinetic Analysis

The plasma concentration-time data were fitted to an exponential equation of the type: $Cp = \sum_{i=1}^{n} A_i e^{-\beta_i t}$ (41) where, Cp = plasma drug concentration, t = time, A_i = coefficient, β_i = exponent. Data were fitted for values of i = 1 (one compartment model) and i = 2 (two compartment model). The model which best described each set of concentration-time data was determined by a F-test as described by Boxenbaum and colleagues (41) and the Akaike's Information Criteria reported by Yamoaka and colleagues (42).

Area under the plasma concentration-time curve for the sampling period, $(AUC)_0^\tau$, was calculated by the trapezoidal method. Area under the curve from the last sampling time to infinity, $(ACU)_\tau^\infty$, was calculated using the equation: $(AUC)_\tau^\infty = Cp\ (t)/\beta_i$ (42) where Cp (t) = last plasma concentration value B_i = terminal rate constant. The total area under the plasma concentration-time curve $(AUC)_0^\infty$ was obtained from the sum of $(AUC)_0^\tau + (AUC)_\tau^\infty$. Plasma clearance, Cl, was calculated by the equation: $Cl = Dose/(AUC)_\tau^\infty$ (43). Volume of distribution, $Vd(\beta)$ was obtained by dividing Cl/β_i. Terminal half-life was determined by dividing 0.693 by β_i.

f. Statistical Analysis

The non-parametric, Mann-Whitney test (43) was used to test for significant differences between the pharmacokinetic parameters obtained in control and perfused rats.

3. Results

Mean concentrations of AP 15 min after dosing was higher in the FDA transfused group than the respective controls. Both elimination curves were similar and drug concentrations above 5.0 µg/ml were detectable in both groups at the end of the six-hour sampling period (Table 5). All four urine metabolites were recovered in similar amounts from FDA transfused animals and control animals (Table 6). The plasma AP concentrations in the SFH transfused group and the respective control group declined steadily after an initial peak at 15 min. AP was detectable at the end of the six-hour sampling period. All four urine metabolites were recovered in similar amounts from SFH transfused and control animals. There were no significant differences between the calculated pharmacokinetic parameters for FDA transfused animals and the respective control animals or SFH transfused animals and respective control animals (Table 7).

The mean plasma concentrations of DZ in FDA and SFH transfused animals were lower than in the respective control animals (Table 8). DZ was undetectable in three of the four groups by 6 h. Calculation and analysis of the pharmacokinetic parameters for both groups, however, showed no statistically significant differences (Table 9).

The mean plasma concentrations of PC dropped to low or undetectable levels within 3 h in all groups (Table 10). PC concentrations in FDA and SFH transfused animals were similar to their respective controls. Calculation and analysis of the pharmacokinetic parameters for both groups showed no statistically significant differences (Table 11).

The mean plasma concentrations of SM declined slowly over the 72 h observation period (Table 12). The mean plasma concentrations in the control animals consistently exceeded those in the transfused animals in both groups. Pharmacokinetic parameters were significantly different for both FDA transfused animals and SFH transfused animals and the respective controls (Table 13). In the case of animals transfused with FDA, the $t_{\frac{1}{2}}$ and Vd were increased. Animals transfused with SFH demonstrated a decrease in AUC and an increase in Cl and Vd.

4. Discussion

The drugs used in this study were chosen for their diversity of pharmacodynamics, biologic action, and metabolic pathways. Changes in their pharmacokinetic parameters would imply that transfusion with FDA or SFH altered the disposition of the drug under study.

Antipyrine is a drug whose metabolic pathways have been well established (44). Because it distributes in body water (44), its distribution would depend less on plasma binding than the other drugs in this study. The lack of significant changes in the pharmacokinetic parameters and metabolites excreted in the urine would indicate that neither FDA nor SFH significantly effects distribution of AP in body water and, furthermore, neither substitute significantly alters liver metabolism of AP and renal excretion of AP and its metabolites.

DZ is a highly protein-bound drug that is very lipid soluble (45). Transfusion with FDA or SFH would presumably diminish levels of serum albumin and decrease the numbers of remaining binding sites. This effect would have its greatest impact on a highly bound drug such as DZ. Thus, $t_{\frac{1}{2}}$ may be prolonged and the Vd increased. Lack of significant changes in the pharmacokinetic parameters in either group, however, indicates that binding effects were not significantly altered.

PC is rapidly cleared by renal tubular secretion (46). Thus, a defect in active transport caused by transfusion with either FDA or SFH might be demonstrated by altered pharmacokinetics. Since no significant changes were observed, it can be inferred that transfusion with either substitute does not alter tubular secretion of PC.

SM is acetylated in the liver and is excreted in the urine as both the parent compound and the acetylated derivative (47). It is with this drug that significant changes in pharmacokinetics were observed for both substitutes. With FDA, the prolonged $t_{\frac{1}{2}}$ and increased Vd would be caused by decrease plasma binding. However, SM is less plasma bound than DZ (48) which was not influenced by transfusion with FDA. Alternatively, FDA may interfere in some way with the process of acetylation. In contrast, SFH decreased the AUC and increased the Vd and Cl of SM. How SFH may promote improved elimination of SM is not clear. SFH may have the effect of enhancing acetylation of SM. However, we do not have direct data to confirm this. Studies of the acetylated SM in plasma and urine would be of interest.

5. Conclusions

a. Neither FDA nor SFH alter the pharmacokinetics of antipyrine, diazepam, and penicillin.

b. Both FDA and SFH alter the pharmacokinetics of sulfamethazine.

6. Recommendations

Studies of acetylated sulfamethasone in plasma and urine or rats after transfusion of FDA or SFH should be done.

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VIII. Glossary

AP:	Antipyrine
ATCC:	American Type Culture Collection
AUC:	Area under the curve
CFU:	Colony forming units
Cl:	Total body clearance
DZ:	Diazepam
FDA:	Fluosol-DA 20%
HPCL:	High pressure liquid chromatography
4OHAP:	4-hydroxyantipyrine
IV:	Intravenous
$3OHCH_3AP$:	3-methoxyantipyrine
mCi:	milliCurie
mmol:	millimole
NdemAP:	N-demethylantipyrine
PC:	Penicillin
PBS:	Phosphate buffered saline
SFH:	Stroma-free hemoglobin
SM:	Sulfamethazine
$t_{\frac{1}{2}}$:	Half-Life
TCA:	Trichloroacetic acid
Vd:	Volume of distribution

TABLE 1

Results of culture of blood, pleural fluid, and peritoneal fluid at time of demise of penicillin treated animals.

Transfusion Group	Total No.	No. Died	No. Cultured	No. with a Culture Positive for Pneumococcus
FDA + penicillin	13	10	7	6
Sham + penicillin	15	3	3	2

TABLE 2

Pharmacokinetic parameters for morphine in control rats and rats after exchange transfusion with SFH or FDA.

Group	No. of animals	$t_{\frac{1}{2}}$ (hr) median (range)	$t_{\frac{1}{2}}$ (hr) mean (± SD)	Vd ($L \cdot Kg^{-1}$) median (range)	Vd ($L \cdot Kg^{-1}$) mean (± SD)	AUC ($mg \cdot hr \cdot L^{-1}$) median (range)	AUC ($mg \cdot hr \cdot L^{-1}$) mean (± SD)	Cl ($L \cdot hr^{-1} \cdot Kg^{-1}$ median (range)	Cl ($L \cdot hr^{-1} \cdot Kg^{-1}$ mean (± SD)
Control	14	1.06 (0.24-2.10)	1.02 (±0.50)	1.25 (0.35-2.61)	1.35 (+0.81)	1.67 (0.75- 7.17)	2.37 (±1.78)	1.04 (0.21-2.08)	1.02 (±0.53)
SFH	10	1.79 (0.85-9.90) p = 0.03*	2.46 (±2.68)	2.77 (0.90-5.62) p = 0.004	2.99 (±1.45)	1.59 (0.92- 3.97) p = 0.48	1.75 (±0.92)	1.02 (0.39-1.82) p = 0.81	1.09 (±0.46)
FDA	10	2.09 (0.32-3.47) p = 0.006	2.05 (±0.95)	1.36 (0.23-4.00) p = 0.86	1.46 (±1.17)	3.36 (1.64-22.71) p = 0.02	6.02 (±6.61)	0.49 (0.07-1.05) p = 0.01	0.55 (±0.36)

*The pharmacokinetic parameters of the SFH and FDA groups were compared to the control group using the Wilcoxon Rank Sum Test.

TABLE 3

Hemoglobin concentrations in 0 hr plasma samples from control rats and SFH or FDA transfused rats.

Transfusion Group	Hemoglobin Concentration (μg/ml ± SD)
Control	40 ± 32
SFH	36,000 ± 15,000
FDA	37 ± 16

TABLE 4

Amount of blood replacement with FDA or SFH.

Drug Group	Transfusate					
	FDA			SFH		
	Before Transfusion	After Transfusion	Percent Exchange	Before Transfusion	After Transfusion	Percent Exchange
Antipyrine	45 ± 3*	17 ± 3	62 ± 7	40 ± 1	12 ± 1	72 ± 7
Diazepam	44 ± 6	16 ± 2	63 ± 6	44 ± 2	13 ± 2	71 ± 4
Penicillin	43 ± 3	18 ± 3	60 ± 10	41 ± 2	10 ± 2	75 ± 3
Sulfamethazine	41 ± 4	18 ± 3	57 ± 6	42 ± 2	12 ± 1	71 ± 1

*Mean ± S.D.

Table 5

Plasma antipyrine concentrations (μg/ml, mean ± S.D.) in rats after transfusion with FDA (dose = 5 mg) or SFH (dose = 10 mg) and their respective controls.

Transfusate	No. of Animals	Time (min)							
		0	15	60	120	180	240	300	360
Sham	4	0	20.3 ± 7.5	14.5 ±6.4	12.9 ± 3.6	7.6 ±3.3	6.8 ±3.3	7.0 ±2.9	5.2 ±3.4
FDA	5	0	27.6 ±10.4	12.7 ±3.1	9.9 ± 3.5	8.4 ±3.0	6.0 ±1.9	6.4 ±2.4	6.0 ±2.1
Sham	4	0	35.7 ± 3.0	30.0 ±9.2	21.5 ±11.7	14.3 ±4.4	9.1 ±3.5	8.3 ±3.8	6.6 ±3.3
SFH	5	0	33.9 ±11.8	29.7 ±5.8	15.4 ± 6.3	13.6 ±3.0	11.2 ±3.6	6.8 ±0.8	8.0 ±2.6

Table 6

Total amount (μg, mean ± S.D.) of antipyrine and antipyrine metabolites excreted in urine of rats transfused with FDA (dose = 5 mg) or SFH (dose = 10 mg) and their respective controls during 24 h after dosing.

Transfusate	Animals	$30HCH_3AP$	NdemAP	4OHAP	AP
Sham	4	387 ± 65	211 ± 42	251 ± 72	40 ± 11
FDA	5	334 ± 109	290 ± 109	230 ± 74	41 ± 20
Sham	4	1500 ± 230	920 ± 200	490 ± 190	150 ± 54
SFH	5	1500 ± 230	890 ± 390	450 ± 310	200 ± 54

Table 7

Pharmacokinetic parameters (mean ± S.D.) of antipyrine in rats after transfusion with FDA (dose = 5 mg) or SFH (dose = 10 mg) and their respective controls.

Transfusate	No. of Animals	$t_{\frac{1}{2}}$ h	Cl ($ml \cdot h^{-1}$)	Vd (ml)	ACU ($\mu g \cdot h \cdot ml^{-1}$)
Sham	4	8.39* ±9.66	95.65 ±49.89	880 ± 754	61.4 ± 24.0
FDA	5	12.3 ±15.4	79.7 ±24.4	1759 ±2679	67.1 ± 17.9
Sham	4	2.76 ±0.98	96.9 ±34.6	418 ± 276	114.2 ± 41.3
SFH	5	3.34 ±2.64	102.1 ±25.5	465 ± 310	103.7 ± 28.9

*$p > 0.05$ for all transfused groups compared to their respective control group.

Table 8

Plasma diazepam concentrations (μg/ml, mean ± S.D.) in rats after transfusion with FDA or SFH (dose = 2 mg) and their respective controls.

Transfusate	No. of Animals	Time (min)									
		0	15	30	45	60	120	180	240	300	360
Sham	4	0	3.3 ±2.7	2.6 ±2.2	1.7 ±2.0	0.7 ±0.4	0.2 ±0.3	0.2 ±0.4	0.1 ±0.2	0.1 ±0.2	0.1 ±0.2
FDA	4	0	1.1 ±0.5	0.8 ±0.4	0.4 ±0.2	0.4 ±0.1	0.2 ±0.3	0.1 ±0.3	0*	0	0
Sham	5	0	7.5 ±4.0	3.2 ±1.5	2.0 ±1.3	0.9 ±0.5	0.7 ±0.7	0.5 ±0.9	0.2 ±0.2	0.2 ±0.3	0
SFH	5	0	2.5 ±1.6	1.3 ±0.7	0.7 ±0.1	0.4 ±0.1	0.4 ±0.2	0.3 ±0.1	0.2 ±0.2	0	0

*Less than 0.1 μg/ml.

Table 9

Pharmacokinetic parameters (mean ± S.D.) of diazepam in rats after transfusion with FDA or SFH (dose = 2 mg) and their respective controls.

Transfusate	Animals	$t_{\frac{1}{2}}$ (h)	Cl ($ml \cdot h^{-1}$)	Vd (ml)	AUC ($\mu g \cdot h \cdot ml^{-1}$)
Sham	4	0.29 ± 0.09*	1108 ± 1221	396 ± 361	4.16 ± 3.72
FDA	4	1.2 ± 1.1	2150 ± 1560	2970 ± 2160	1.26 ± 0.668
Sham	5	0.72 ± 1.06	429 ± 314	251 ± 186	7.40 ± 5.05
SFH	5	5.57 ± 9.02	829 ± 398	3950 ± 4480	2.97 ± 1.57

*$P > 0.05$ for all transfused groups compared to their respective control groups.

Table 10

Plasma penicillin concentrations (U/ml, mean ± S.D.) in rats after transfusion with FDA or SFH (dose = 1750 U) and their respective controls.

Transfusate	No. of Animals	Time (min)									
		0	5	10	15	20	25	30	60	120	180
Sham	5	0	4.1 ±1.2	3.5 ±0.9	2.6 ± 1.2	1.6 ±0.6	1.3 ±0.4	0.8 ±0.4	0.2 ±0.1	0*	0
FDA	5	0	4.9 ±1.9	3.7 ±1.6	2.9 ±1.8	2.5 ±1.9	2.1 ±1.8	1.6 ±1.1	0.7 ±0.4	0.1 ±0.2	0
Sham	5	0	14.1 ±9.5	6.2 ±1.7	3.4 ±1.1	2.2 ±0.7	1.8 ±0.5	1.2 ±0.5	0.5 ±0.4	0	0
FDA	4	0	18.8 ±6.5	4.9 ±1.6	2.6 ±0.8	1.9 ±0.9	1.5 ±1.1	1.2 ±1.0	0.7 ±0.8	0.4 ±0.8	0.3 ±0.7

*Less than 0.05 U/ml.

Table 11

Pharmacokinetic parameters (mean ± S.D.) of penicillin in rats after transfusion with FDA or SFH (dose = 1750 U) and their respective controls

Transfusate	No. of Animals	$t_{\frac{1}{2}}$ (min)	Cl ($ml \cdot min^{-1}$)	Vd (ml)	AUC ($\mu g \cdot ml \cdot min^{-1}$)
Sham	5	11.5 ± 3.47*	18.3 ± 3.8	309 ± 140	99.2 ± 19.5
FDA	5	19.5 ± 9.11	12.5 ± 5.3	324 ± 115	170 ± 97
Sham	5	9.45 ± 5.56	8.44 ± 4.09	98.4 ± 57.5	310 ± 291
SFH	4	36.9 ± 51.7	3.88 ± 1.95	122 ± 112	565 ± 335

*$p > 0.05$ for all transfused groups compared to their respective control group.

TABLE 12

Plasma sulfamethazine concentrations (μg/ml, mean ± S.D.) in rats after transfusion with FDA or SFH (dose = 1.5 mg) and their respective controls.

Transfusate	No. of Animals	Time (h)									
		0	0.25	0.5	0.75	1	2	3	6	24	48	72
Sham	5	0	18.2 ±3.5	18.2 ±1.6	17.2 ±2.3	16.2 ±2.0	13.2 ±2.4	10.1 ±1.8	6.4 ±2.1	1.0 ±2.2	0*	0
FDA	5	0	8.7 ±0.8	9.9 ±2.1	9.0 ±1.7	8.4 ±1.2	7.4 ±1.6	7.8 ±0.5	6.4 ±2.4	0.3 ±0.1	0.01 ±0.03	0
Sham	5	0	24.8 ±4.9	18.8 ±3.1	16.0 ±1.6	14.4 ±1.1	12.4 ±1.7	9.7 ±2.1	5.8 ±2.1	0.07 ±0.05	0	0
SFH	5	0	11.2 ±2.6	8.7 ±1.3	8.6 ±2.0	7.9 ±1.9	7.4 ±1.2	6.2 ±1.2	3.8 ±2.4	0.3 ±0.5	0	0

*Less than 0.02 μg/ml.

Table 13

Pharmacokinetic parameters (mean ± S.D.) of sulfamethazine in rats after transfusion with FDA or SFH (dose = 1.5 mg) and their respective controls

Transfusate	No. of Animals	$t_{\frac{1}{2}}$ (h)	Cl ($ml \cdot h^{-1}$)	Vd (ml)	AUC ($\mu g \cdot h \cdot ml^{-1}$)
Sham	5	3.15 ± 0.56*	14.0 ± 5.1	60.7 ± 17.5*	127 ± 69
FDA	5	7.65 ± 2.40*	14.8 ± 4.1	152 ± 16*	108 ± 30
Sham	5	3.56 ± 1.0	12.2 ± 3.4*	60.1 ± 11.0*	129 ± 28*
SFH	5	5.02 ± 3.1	20.2 ± 6.0*	132 ± 49*	80.5 ± 27.0*

*$p < 0.05$ for transfused animals compared to their respective control group.

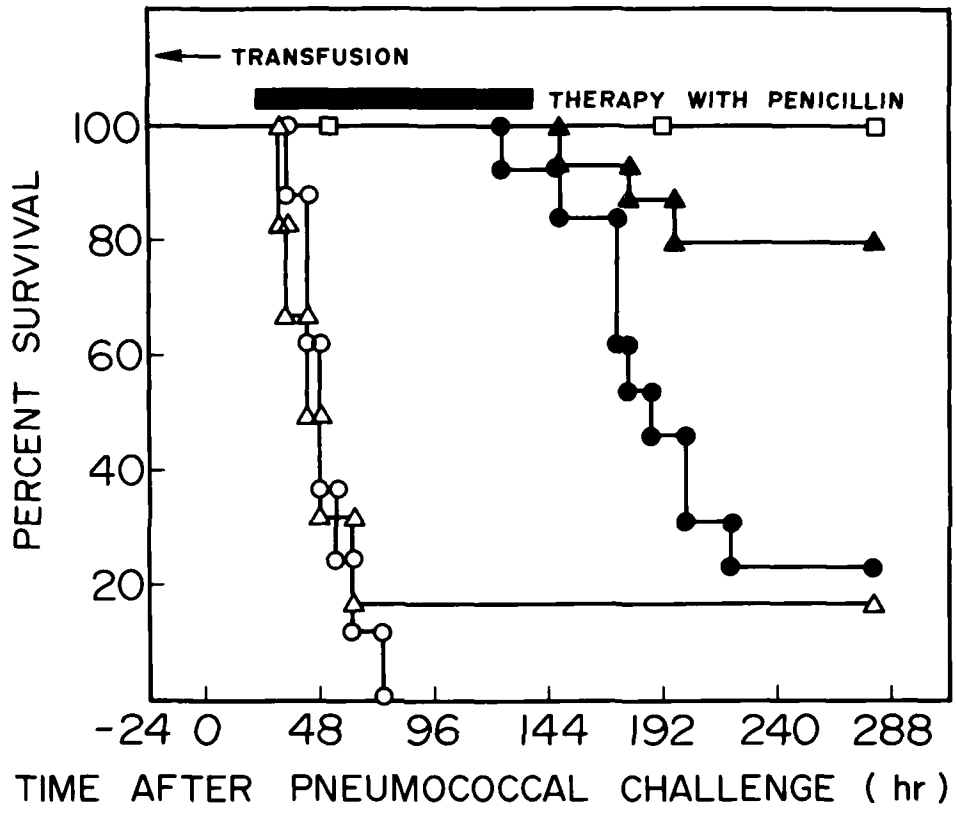


Figure 1. Survival of rats after transfusion with FDA (□—□); transfusion with FDA, challenge with pneumococcus, and saline treatment (○—○); transfusion with FDA, challenge with pneumococcus, and penicillin therapy (●—●); sham transfusion, challenge with pneumococcus, and saline treatment (△—△); and sham transfusion, challenge with pneumococcus, and penicillin therapy (▲—▲).

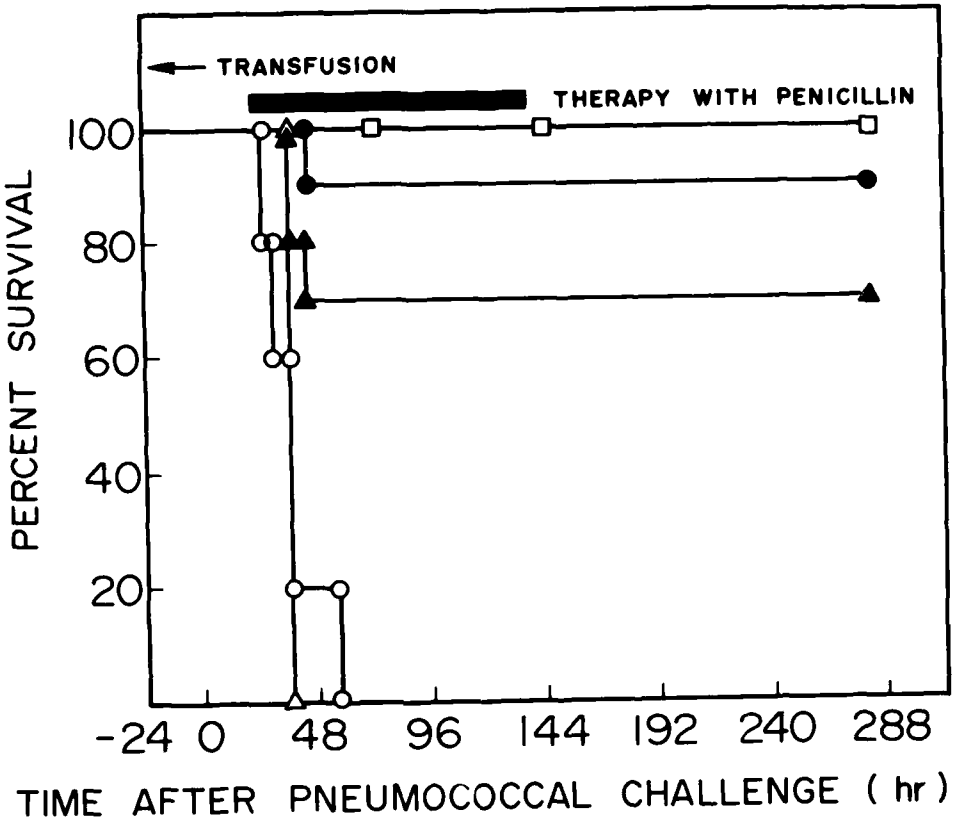


Figure 2. Survival of rats after transfusion with SFH (□—□); transfusion with SFH, challenge with pneumococcus, and saline treatment (○——○); transfusion with SFH, challenge with pneumococcus, and penicillin therapy (●——●); sham transfusion, challenge with pneumococcus, and saline treatment (△—△); and sham transfusion, challenge with pneumococcus, and penicillin therapy (▲—▲).

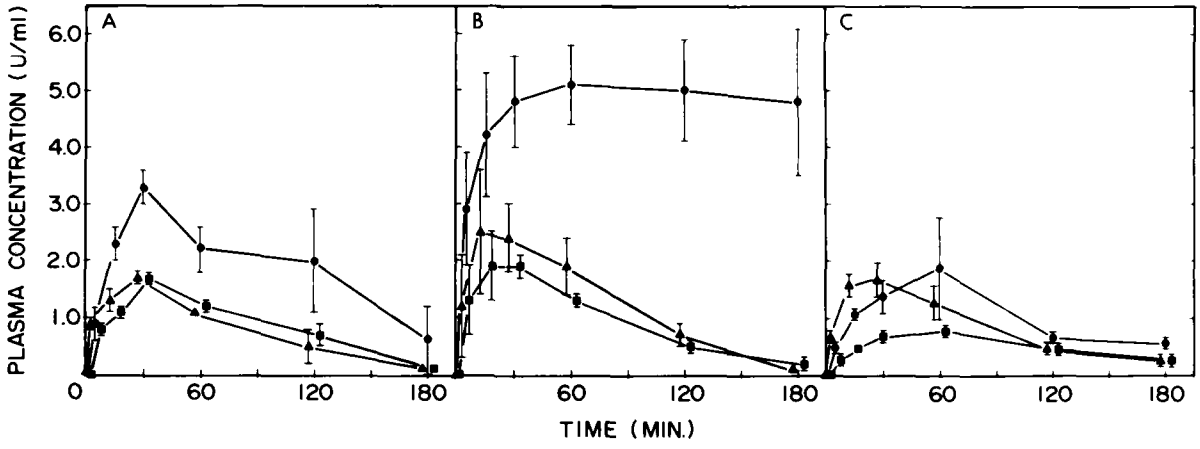


Figure 3. Penicillin concentrations (mean ± standard error of the mean) after IP injection of 1750 U immediately (n = 4, ●—●), 48 h (n = 3, ▲—▲) and 168 h (n = 3, ■—■) after sham transfusion (A), transfusion with FDA (B), and transfusion with SFH (C).

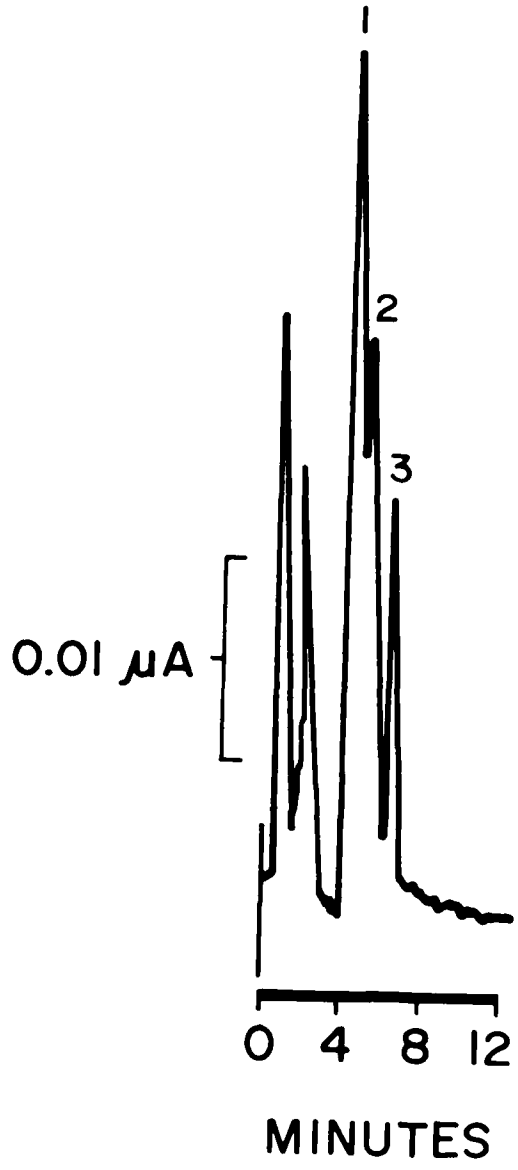


Figure 4. HPLC chromatogram of morphine (peak 2) in plasma from a control animal; peak 1 is an unidentified interfering substance; peak 3 is the codeine internal standard; pH of extraction, 11.1.

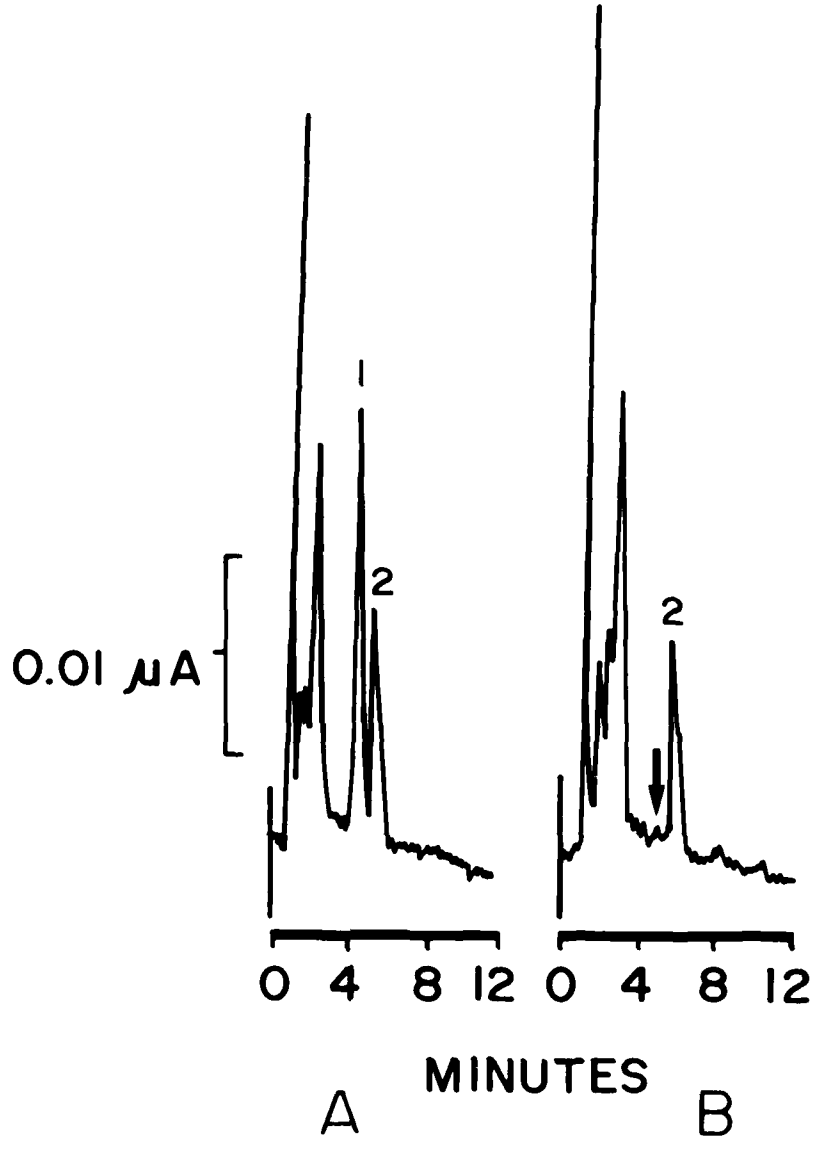


Figure 5. (A) HPLC chromatogram of pH 12.5 extracted plasma sample from a control rat 15 min after IV injection of morphine (0.5 mg) showing morphine (peak 1) and codeine (peak 2). (B) HPCL chromatogram of pH 12.5 extracted plasma sample from an SFH transfused rat 15 min after IV injection of morphine (0.5 mg) showing codeine (peak 2) and absence of morphine (arrow).

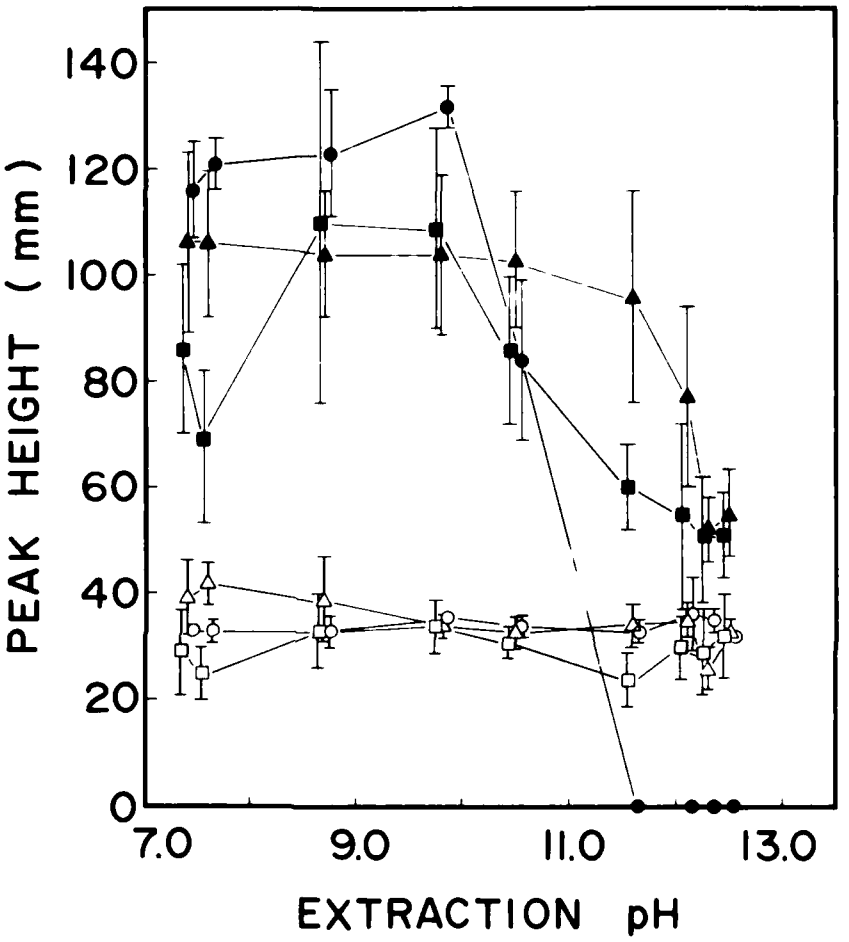


Figure 6. Heights (mean ± SD; n = 3) of morphine (closed symbols) and codeine (open symbols) peaks on HPLC chromatogram after addition of morphine (3.0 µg/ml) and codeine (1.5 µg/ml) to plasma (triangles), FDA (squares), and SFH (circles) and extracted at various pHs. Where SD ≤ ± 1.0 mm, no limits are shown.

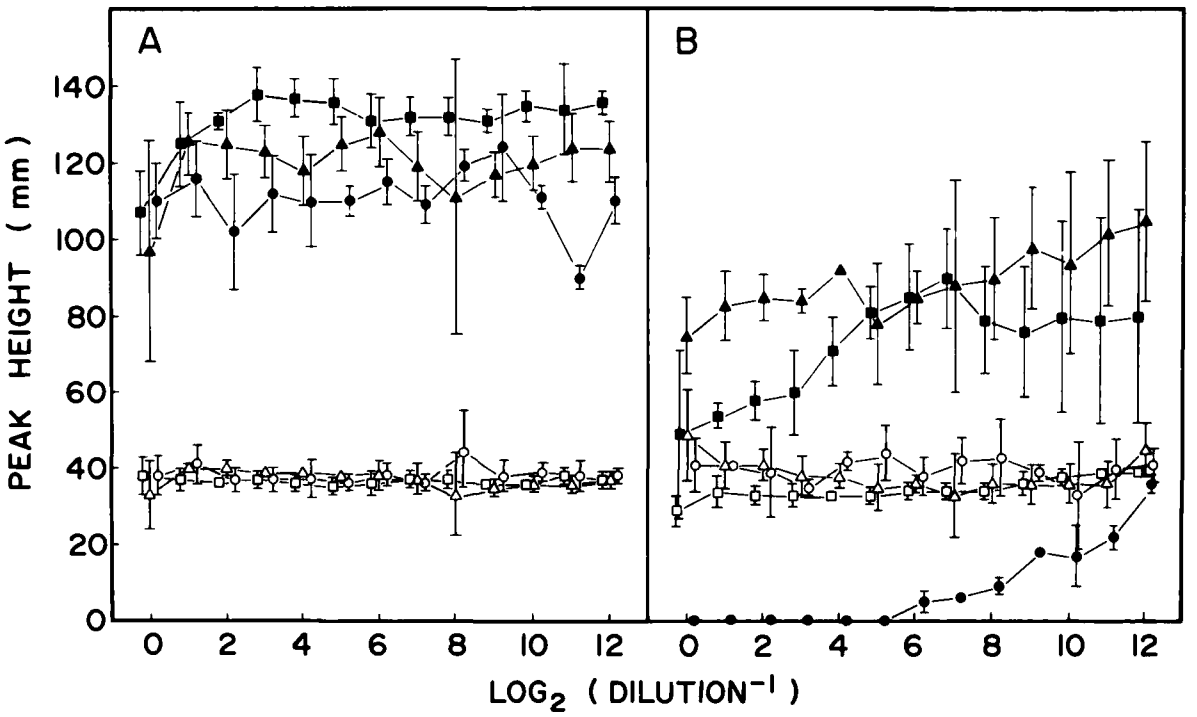


Figure 7. Heights (mean ± SD; n = 3) of morphine (closed symbols) and codeine (open symbols) peaks on HPLC chromatograms after addition of morphine (3.0 µg/ml) and codeine (1.5 µg/ml) to serial two-fold dilutions with PBS of plasma (triangles), FDA (squares), and SFH (circles). (A) Extraction at pH 7.4. (B) Extraction at pH 12.5. Where SD $\leq \pm$ 1.0 mm, no limits are shown.

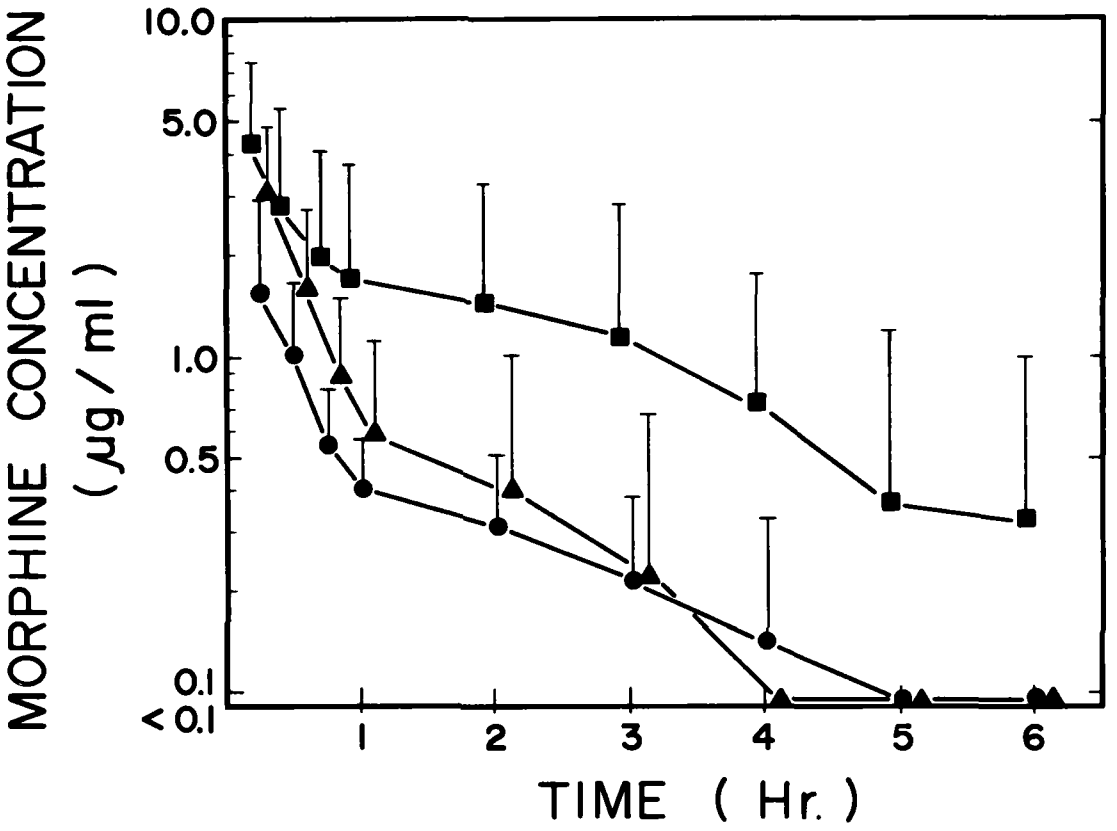
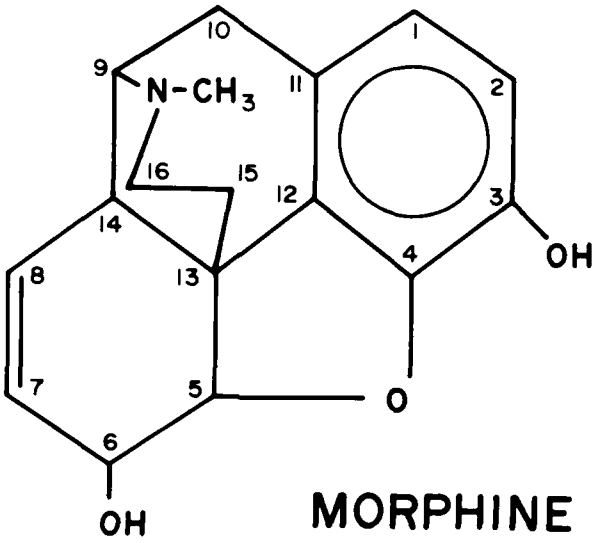
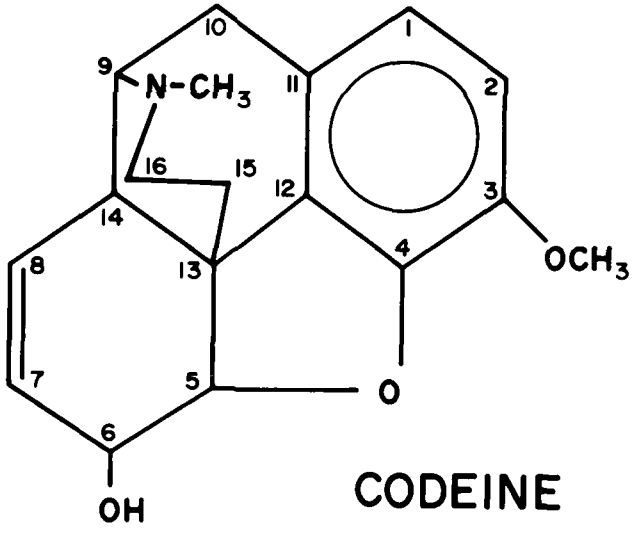


Figure 8. Time vs plasma concentration (mean ± SD) of morphine in control rats (triangles) and animals transfused with FDA (squares) or SFH (circles). The animals were injected IV with 0.5 mg morphine immediately following transfusion.



MORPHINE



CODEINE

Figure 9. Structures of morphine and codeine.

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