Modified Fluorometric Quantitation of Pancuronium Bromide and Metabolites in Human Maternal and Umbilical Serums

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Abstract □ The procedure for pancuronium bromide ion-pair extraction into chloroform using rose bengal and subsequent fluorometric measurement was modified by changing the extraction pH and eliminating phenol, ethanol, and acetone to give easier operation and enhanced fluorescence stability. Precision, accuracy, and sensitivity were evaluated over 0.14–0.82 μg/ml (CV = 14; relative error = 9%) and 0.05–0.17 μg/ml (CV = 19; relative error = 21%). Following a dose of 0.1 mg/kg for cesarean section in humans, the mean maternal arterial and umbilical venous serum concentrations of pancuronium bromide and metabolites were 0.52 and 0.12 μg/ml, respectively, at delivery (mean of 13 min after injection).

Keyphrases □ Muscle relaxants—pancuronium bromide, analysis, fluorometry, maternal arterial and umbilical venous serum □ Fluorometry—analysis, pancuronium bromide in maternal arterial and umbilical venous serum □ Pancuronium bromide—analysis, fluorometry, maternal arterial and umbilical venous serum, transplacental transfer □ Transplacental transfer—pancuronium bromide

Pancuronium bromide is used as a muscle relaxant during anesthesia for cesarean section (1). Therefore, the quantitation of maternal and fetal serum pancuronium bromide concentrations is important for evaluating transplacental drug transfer (2).

Pancuronium is a bisquaternary ammonium steroid that contains acetate ester groups at the 3- and 17-positions. Drug metabolism produces the 3-hydroxy, 17-hydroxy, and 3,17-dihydroxy derivatives through ester hydrolysis. A sensitive fluorometric quantitation procedure for pancuronium bromide and its metabolites in human plasma was described (3), and an initial modification to stabilize the fluorescence was reported (4). However, a higher intensity and more stable fluorescence signal was still desirable. In addition, a rigorous evaluation of the precision, accuracy, and sensitivity of the fluorometric plasma or serum pancuronium bromide determination has not appeared, although a minimum 0.02-μg/ml sensitivity, using 1 ml of plasma, was reported (3, 4).

Two reports on transplacental pancuronium bromide transfer have appeared (5, 6). In the earlier study (5), conclusions were based on the detection of pancuronium bromide and its metabolites in fetal urine using a limited sensitivity (0.6 μg/ml) spectrophotometric procedure. The second study (6) was based on maternal and umbilical cord blood samples taken during cesarean section delivery and assayed for pancuronium bromide using the fluorometric procedure; however, despite predosing with succinylcholine, no consideration was given to possible interferences in the pancuronium bromide assay due to succinylcholine or its metabolites.

In the present study, the fluorometric method was modified, and precision and accuracy were evaluated over the expected fetal and maternal concentration ranges. Serum, rather than plasma, was selected to facilitate the measurement of γ-globulin, to which pancuronium binds (7). The method was used to measure pancuronium bromide concentrations in maternal and umbilical cord blood samples obtained at cesarean section delivery.

EXPERIMENTAL

Chemicals and Reagents—Crystalline pancuronium bromide[1] was used as received. Rose bengal[2] was purified by washing six times with ethyl acetate[3] and three times with chloroform[4] and was desiccated (8). All other materials were reagent grade. All glassware was cleaned in 15% nitric acid. The centrifuge tubes were coated with dimethyldichlorosilane[5], rinsed with toluene[3], methanol[3], water, and acetone[3], and air dried. Pooled human serum[6] from normal subjects was used for the standard curves. The buffer was prepared by adding 0.2 M sodium bicarbonate to 0.2 M sodium carbonate until pH 10.5 was attained.

Previous Assay—To a 25-ml glass-stoppered centrifuge tube were added 7 ml of chloroform containing 2.5% phenol and 5% ethanol, 1 ml of serum, 1 ml of 0.1 M pH 7.8 phosphate buffer, and 0.5 ml of 0.2 mg of rose bengal/ml of 0.45 M dibasic potassium phosphate. The mixture was shaken 2 min on a vortex mixer followed by 30 min on a rotary mixer. The samples were centrifuged 10 min at 1500×g, and the aqueous layer was aspirated. An aliquot (0.5 ml) of the chloroform–phenol layer was added to 3.5 ml of acetone and vortexed. The relative fluorescence intensity was determined at 570 nm with excitation at 546 nm.

Modified Assay—To a 15-ml screw-capped (polytef-lined caps) centrifuge tube were added 1 ml of serum, 1 ml of 0.2 M sodium bicarbonate–carbonate buffer (pH 10.5), 0.5 ml of 0.5 mg of rose bengal/ml of 0.45 M dibasic potassium phosphate, and 7 ml of chloroform, with vortexing after each addition. After 30 min on a reciprocating horizontal shaker at 185 oscillations/min, the samples were centrifuged for 10 min at 1500×g; the aqueous layer was aspirated.

About 3 ml of the chloroform layer was transferred to a 1-cm silica cell. The relative fluorescence intensity was measured at 570 nm, with excitation at 546 nm, using a spectrofluorometer[7]. The instrument was adjusted daily to the same relative fluorescence intensity with 1 μg of quinine sulfate/ml of 0.1 N H_2SO_4 at 450 nm, with excitation at 350 nm. The blood samples were held for 2 hr at 0–4° and then centrifuged[8] for 10 min at 6000 rpm for serum preparation. All samples were assayed within 6 hr of blood sampling.

Standardization Procedure—To compensate for normal daily fluctuations in the spectrofluorometer xenon lamp output, a five-point linear least-squares fitted calibration curve was prepared each day using freshly prepared pancuronium bromide in serum over the range of 0–0.8 μg/ml. To establish precision, accuracy, and sensitivity, replicate pancuronium bromide samples in serum were prepared over the ranges of ~0.05–0.2 and 0.1–0.8 μg/ml. The resulting relative fluorescence intensity *versus* concentration data were fit by linear least squares; the coefficient of variation and the percentage deviation of each point (percent error) from the fitted line were calculated.

Human Study Protocol—Patients undergoing elective cesarean section delivery gave informed consent. The patients weighed 67 ± 10 (SD) kg and received 0.75 mg of hydroxyzine/kg im, 0.4 mg of atropine iv, 4 mg of thiamylal sodium/kg iv, 0.1 mg of pancuronium bromide/kg iv, and nitrous oxide–oxygen anesthesia. Blood samples included ma-

[1] Organon Inc., West Orange, N.J.
[2] Fisher, Pittsburgh, Pa.
[3] Certified ACS grade, Fisher, Pittsburgh, Pa.
[4] No. 4440, Mallinckrodt, St. Louis, Mo.
[5] Sigma, St. Louis, Mo.
[6] Immunopathology Laboratory, University Health Center, Pittsburgh, Pa.
[7] Aminco-Bowman model 4-8202.
[8] Sorvall RC2-B with SS-34 rotor.

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ternal predrug control, maternal arterial at delivery, and fetal venous from the clamped umbilical cord.

RESULTS AND DISCUSSION

A previous study in adults undergoing nonobstetric surgery showed that pancuronium concentrations ranged from 0.12 to 0.79 μg/ml during the 20-min interval after intravenous injection of ~0.1 mg/kg (9). Since pancuronium is a positively charged quaternary ammonium compound, its transfer rate across the placental membranes into the fetal blood would be slow; so fetal pancuronium concentrations would be much less than those in adults. For example, for the similar quaternary ammonium muscle relaxant metocurine, the human fetal plasma concentration was about one-tenth the maternal plasma concentration (10). Since the estimated fetal pancuronium bromide concentrations were near the lower limit for quantitation by the fluorometric method, its precision, accuracy, and sensitivity had to be evaluated.

In this laboratory, the relative fluorescence intensity varied by ±90% from the mean for four 1-μg/ml samples using the original (3) or modified (4) fluorometric assay. Since this variation was too great for the present study, the following changes were made: the extraction pH was increased from 7.8 to 10.5, phenol was eliminated from the extraction, and acetone and most of the ethanol were eliminated from the fluorescence measurement step.

The higher extraction pH increased the relative fluorescence from 0.27 ± 0.006 (*SD*) at pH 7.8 to 0.52 ± 0.03 (*SD*) at pH 10.5 for three samples of 0.5 μg of pancuronium bromide/ml of serum. The fluorescence decreased by 10% if the extraction was done at pH 12 instead of 10.5. This improvement could be expected if the rose bengal phenolic groups ionized to a greater extent at the higher pH. This would give a higher negative charge and improve complexation with the positively charged pancuronium ion. To obtain this desired pH change, a different buffer system (carbonate) was employed.

The phenol had been added in the extraction step to reduce emulsion formation during analysis (3, 8); however, there was still considerable emulsion formed. Unfortunately, the phenol also quenched the fluorescence (8). With the modifications, no advantage was observed from phenol addition; therefore, the phenol was omitted. Similarly, the ethanol used in the earlier procedures to stabilize the pancuronium–rose bengal complex fluorescence in acetone (3, 8) was omitted since the pancuronium–rose bengal–chloroform solutions without acetone showed only 3% change in fluorescence intensity over 30 min. The chloroform used in the present studies contained 0.75% ethanol as preservative; this amount was negligible compared with the 5% by volume in the chloroform–phenol solution used previously. Apparently, the combination of the higher pH, less alcohol, and no phenol minimized the emulsion formation by an unknown mechanism.

In these studies, the enhanced fluorescence obtained by mixing one volume of the pancuronium–rose bengal–chloroform solution with seven volumes of acetone was less than the loss in fluorescence due to dilution. For example, at 0.5 μg of pancuronium/ml, the relative fluorescence intensity was 0.52 ± 0.03 (*SD*) (n = 3) without acetone and only 0.19 ± 0.07 (*SD*) (n = 3) with acetone. Therefore, the acetone was omitted, which greatly helped to stabilize the fluorescence since the acetone fluorescence enhancement was transitory (4).

Precision and accuracy were evaluated by using serum to which known pancuronium bromide amounts were added. These samples were assayed as described. The standard curve was linear over the 0.14–0.82-μg/ml range, but some scatter was evident over the 0.05–0.17-μg/ml range. Representative results for the mean percent relative standard deviation (*CV*) and the mean relative error for each concentration range are shown in Table I. For the 0.05–0.14-μg/ml range, the validation was done on three separate groups (Table I). The averages of the *CV* for the high and low concentration ranges were 14 and 19, respectively; the averages of the mean relative error were 9% and 21% for the high and low ranges, respectively.

The mean fluorescence intensity at 0.05 μg/ml was three times the mean fluorescence intensity of the serum samples without added pancuronium bromide. Therefore, the minimum detectable level, defined as two times the blank fluorescence value, was less than 0.05 μg/ml. However, based on the *CV* values, the precision would be too poor to use at concentrations <0.05 μg/ml. Patient data with serum blanks that deviated more than 3 *SD* from the mean were omitted.

An evaluation to see if samples could be stored for later assay demonstrated that the fluorescence readings were significantly lower if the serum samples were not assayed promptly. In this evaluation, a solution of 0.5 μg of pancuronium bromide/ml of serum was prepared and divided into 15 1-ml aliquots. Three samples were assayed on the same day with a result of 0.49 μg/ml. The other samples were frozen. Three tubes were thawed and assayed after the days shown with the following results: 1 day, 0.42 μg/ml; 2 days, 0.41 μg/ml; 7 days, 0.28 μg/ml; and 13 days, 0.37 μg/ml. In addition, an umbilical cord sample assayed as described and again 24 hr later showed an apparent 30% decrease in pancuronium concentration.

Whether this change in fluorescence intensity was due to drug instability or to some other factor is not known. However, the results showed clearly that samples could not be stored and assayed later. Therefore, all samples were assayed within 4–6 hr after blood sampling. This finding raises an additional question concerning the data obtained in the previous placental transfer study of pancuronium using the fluorometric assay since the samples were frozen and assayed later (6). However, in that study, heparinized plasma rather than serum was used; pancuronium stability in heparinized plasma has not been reported.

The modified assay was utilized to determine the mean maternal arterial and umbilical vein concentrations in 15 patients undergoing elective cesarean section delivery. A preinduction maternal blood sample was used as a control. To test for possible interference in the pancuronium assay due to the other drugs used during cesarean section, the maximum maternal serum concentrations of the other drugs, except gaseous nitrous oxide, were estimated and added to samples of 0.5 μg of pancuronium bromide/ml of serum. The drugs, their concentrations, and the relative fluorescence following assay for pancuronium were: hydroxyzine hydrochloride, 50 μg/ml, 0.51 ± 0.04 (*SD*) (n = 3); atropine, 0.08 μg/ml, 0.49 ± 0.04 (*SD*) (n = 3); thiamylal sodium, 56 μg/ml, 0.44 ± 0.02 (*SD*) (n = 3); and control, 0.46 ± 0.05 (*SD*) (n = 9). It was concluded that no interference existed at the concentrations tested.

The mean maternal arterial and umbilical venous pancuronium concentrations were 0.52 ± 0.14 and 0.12 ± 0.04 μg/ml, respectively. All samples were obtained at delivery, which occurred at a mean time of 13 ± 4 min after the pancuronium injection. The resulting fetal to maternal ratio of the pancuronium concentrations at delivery was 0.23. This ratio was similar to the mean ratio of 0.24 reported earlier (6) for a smaller dose of pancuronium bromide but with a possible question concerning the absolute concentration values in the earlier study.

Table I—Representative Results for Precision and Accuracy Evaluation over Two Concentration Ranges [a]

Pancuronium Bromide Added, μg/ml	n	Calculated Experimental Mean (Range), μg/ml	*CV*	Relative Error, %
		Part 1: High Range		
0.14	4	0.14 (0.11–0.17)	24	17
0.38	3	0.40 (0.34–0.45)	15	12
0.82	4	0.81 (0.78–0.84)	3	3
		Part 2: Low Range		
0.05	3	0.04 (0.03–0.04)	15	18
0.06	6	0.06 (0.04–0.08)	22	22
0.07	4	0.11 (0.09–0.12)	11	47
0.12	5	0.10 (0.08–0.13)	22	20
0.14	5	0.14 (0.09–0.16)	20	13
0.15	3	0.13 (0.10–0.14)	16	16

[a] Unknown "test" and known "standard" samples of pancuronium in serum were prepared and assayed to give relative fluorescence values. All samples were randomized. The standard samples duplicated those used in preparing the daily reference curve and were used to plot a relative fluorescence intensity *versus* concentration reference curve. This curve was used to convert fluorescence intensity to concentration for the test samples. The results in this table are for the test samples.

REFERENCES

(1) J. B. Neeld, Jr., P. D. Seabrook, Jr., G. M. Chastain, and E. L. Frederickson, *Anesth. Analg.*, **53**, 7 (1974).

(2) L. B. Wingard, Jr., and D. R. Cook, *Clin. Pharmacokinet.*, **2**, 330 (1977).

(3) U. K. Kersten, D. K. F. Meijer, and S. Agoston, *Clin. Chim. Acta*, **44**, 59 (1973).

(4) M. J. Watson and K. McLeod, *ibid.*, **79**, 511 (1977).

(5) I. Speirs and A. W. Sim, *Br. J. Anaesth.*, **44**, 370 (1972).

(6) P. N. Booth, M. J. Watson, and K. McLeod, *Anesthesia*, **32**, 320 (1977).

(7) J. M. Thompson, *ibid.*, **31**, 219 (1976).
(8) E. N. Cohen, *J. Lab. Clin. Med.*, **61**, 338 (1963).
(9) S. Agoston, G. A. Vermeer, U. W. Kersten, and D. K. F. Meijer, *Acta Anaesth. Scand.*, **17**, 267 (1973).
(10) I. Kivalo and S. Saarikoski, *Br. J. Anaesth.*, **44**, 557 (1972).

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Isolation and Identification of 3,3′,5,5′-Tetrabis(*tert*-butyl)stilbenequinone from Polyethylene Closures Containing Titanium Dioxide and Butylated Hydroxytoluene

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Abstract □ A yellow compound was isolated from commercially available, discolored, polyethylene ophthalmic closures containing titanium dioxide and butylated hydroxytoluene (I). This compound was present at 7.46 ppm (w/w). It was identified by UV, IR, and mass spectra as 3,3′,5,5′-tetrabis(*tert*-butyl)stilbenequinone (II), a dimer of I. Further structural confirmation was obtained by NMR. Formation of II is catalyzed by titanium dioxide.

Keyphrases □ Tetrabis(*tert*-butyl)stilbenequinone—analysis, isolation from polyethylene closures, synthesis from butylated hydroxytoluene catalyzed by titanium dioxide □ Butylated hydroxytoluene—conversion to tetrabis(*tert*-butyl)stilbenequinone in polyethylene closures, catalyzed by titanium dioxide □ Polyethylene—drug packaging, discoloration, butylated hydroxytoluene conversion to tetrabis(*tert*-butyl)stilbenequinone catalyzed by titanium dioxide □ Titanium dioxide—in polyethylene closures, catalysis of butylated hydroxytoluene discoloration reaction

Polymers are widely used in industry as packaging materials for food and drugs. As they age, they undergo oxidation *via* a free radical mechanism initiated by heat, light, or radiation. One result is scission or branching in the polymer chain, leading to eventual degradation in the mechanical properties of the polymer.

Antioxidants are added to the polymer in small amounts to prevent degradation. These substances can inhibit free radical formation and may become degraded instead of the polymer. Some antioxidants can convert to colored substances.

The oxidation products of antioxidants have been linked to the discoloration of polyethylene (1). An oxidation product of I caused a marked yellow discoloration of clear polyethylene (2). This colored compound was 3,3′,5,5′-tetrabis(*tert*-butyl)stilbenequinone (II). It has been isolated from cooking oil to which I was added (3). The corresponding yellowing of pigmented polyethylene caused by I has not been reported. The pigment, titanium dioxide, is used extensively as an opacifier for food and drug packaging.

This work was undertaken to isolate and identify the yellow discoloration product in titanium dioxide-pigmented polyethylene in which I is the antioxidant and to evaluate the titanium dioxide role in the discoloration reaction.

EXPERIMENTAL

Closures—Commercially available, opaque, ophthalmic, 15-mm closures, consisting of polyethylene with 1.5% titanium dioxide as the pigment and I as the antioxidant, were used. Each closure weighed ~0.2922 g with a specific gravity of 0.931.

Extraction—Extraction was performed using a soxhlet extractor under defined reproducible conditions.

Separation and Purification of II—*Column Chromatography*—The silica gel column was purged with hexane, toluene, and chloroform. The yellow band was removed using hexane and concentrated by blowing nitrogen on it at room temperature.

TLC—Hexane–toluene (80:20, v/v) was used as the developing medium on silica gel 60 F 254, 25-mm glass plates. On separation, the yellow band was scraped off and reeluted for further purification.

Spectrophotometry—A spectrophotometer was used to determine spectra in the visible and UV regions. IR spectrophotometric analysis was performed with a beam condenser in conjunction with an ultramicro liquid cell.

Mass Spectrometry—A mass spectrometer with a single-focusing magnetic mass analyzer was used. Temperature programming was such that spectra were obtained rapidly at increasing temperatures. All samples were analyzed at 70 ev.

NMR—An NMR spectrometer analyzed the sample in deuterated chloroform with tetramethylsilane as the internal marker. The sample was run at ambient temperature at 100.1 MHz in the Fourier transfer mode using a computer. Chemical shift was expressed as δ (delta) in parts per million.

GLC—GLC analysis was at 200° using a 3.08-m column (0.64 cm in diameter) with 10% SE-30 on 90–100-mesh packing. Nitrogen was the carrier gas with a flow rate of 60 ml/min.

Quantification—The amount of I injected *versus* response was generated using the gas chromatograph. Twelve solutions of I in acetone ranging from 1.0×10^{-5} to 12.0×10^{-5} g were prepared by linear stepwise dilution to quantify I in the 250–500-ppm region. Chloroform was unsuitable because both spectral and analytical grades produced a response at the same retention time as I around or below 9.0×10^{-8} g. With identical extraction conditions, the amount of I extracted per hour from nondiscolored caps was determined.

RESULTS AND DISCUSSION

At room temperature, the yellow material was slightly soluble in chloroform. A total of 913 closures, weighing 266.7786 g, was placed in this solvent. After the extraction procedure, they weighed 265.0773 g, a difference of 1.7013 g. A 10-μl injection of the concentrated crude extract into the gas chromatograph revealed 29 distinct substances.

Spectrophotometric examination of an aliquot of the extract showed major absorption bands at 418 and 442 nm and two minor absorption bands at 396 and 218–233 nm.

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