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Note

Determination of the neuromuscular blocking drug gallamine in rat serum using high-performance liquid chromatography

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Gallamine (1, Fig. 1), a synthetic substitute for curare, has been in common clinical and experimental use as a non-depolarizing neuromuscular blocking agent since the early 1950s. Although its use has declined in recent years, due mainly to prolonged effects in renal failure, it remains a valuable experimental tool in pharmacology [1] and may prove to be a useful probe for examining the effects of disease(s) and other physiologic pertubations on the nicotinic cholinergic receptor system in vivo, since it is presumably not metabolized [2, 3], is bound negligibly to serum/plasma proteins [4] and its effective concentration range is above 1 μg/ml [5], making it easily quantifiable in small volumes of biological fluids during invasive studies in experimental animals.

Previous studies [5–10] with gallamine have utilized dye-binding fluorescence techniques and only recently [11] has a chromatographic method for its analysis been developed. This present report describes a simple method for quantitation of gallamine in small volumes of rat serum using a readily available internal standard. The assay has been utilized in preliminary pharmacokinetic and pharmacodynamic studies in rats and has general applicability for similar studies in humans.

EXPERIMENTAL

Drugs, chemicals and reagents

Gallamine, as the triethiodide salt, was from three different sources (May & Baker, Australia; ICN Pharmaceuticals, Plainview, NY, U.S.A.; and Sigma, St. Louis, MO, U.S.A.). *d*-Tubocurarine chloride, the internal standard (2, Fig. 1), was also purchased from Sigma.

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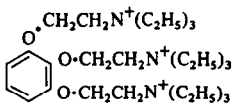
1



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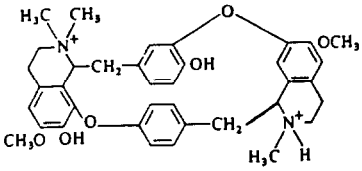

Fig. 1. Structures of gallamine (1) and internal standard *d*-tubocurarine (2).

HPLC-grade methanol, sodium tungstate dihydrate and sulfuric acid were from J.T. Baker (Phillipsburg, NJ, U.S.A.) and tetrabutylammonium (TBA) hydrogen sulfate was from Aldrich (Milwaukee, WI, U.S.A.).

Chromatography

The HPLC system consisted sequentially of a Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Waters WISP 710B autoinjector, a 5-cm C_{18} guard and a 25 cm×4.6 mm I.D., 10-μm C_{18} Waters μBondapak analytical column, a Waters 440 fixed-wavelength detector connected to a Waters extended wavelength module set at 229 nm and a Waters 740B data module. The column temperature was ambient and the detector was set at 0.02 a.u.f.s. The mobile phase consisted of 10% methanol in water containing 0.0075 *M* TBA, filtered and degassed prior to use. The flow-rate was 1.8 ml/min.

Sample preparation and processing

Rat serum (50 μl) was placed into plastic 1.5-ml Eppendorf tubes (Brinkman Instruments, Westbury, NY, U.S.A.) followed by addition of 10 μl of *d*-tubocurarine (0.1 mg/ml in water) as internal standard and 50 μl of protein precipitant consisting of an equal mixture of 10% (w/v) sodium tungstate and 0.335 *M* sulfuric acid. The mixture was vortexed for 15 s and centrifuged at 12 800 *g* for 2 min in an Eppendorf microcentrifuge (Brinkman Instruments). Of the clear supernatant, 30 μl were injected onto the column. For routine analyses, a calibration curve for gallamine between 1 and 20 μg/ml was constructed using drug-free rat serum. If necessary this curve could be extended to 100 μg/ml.

RESULTS

Using the described conditions, gallamine and internal standard (*d*-tubocurarine) had retention times of 6.35 and 10.0 min, respectively. There were no interfering peaks but a peak at 7.42 min was consistently seen even from blank serum. This peak was, however, baseline-resolved from both gallamine and *d*-

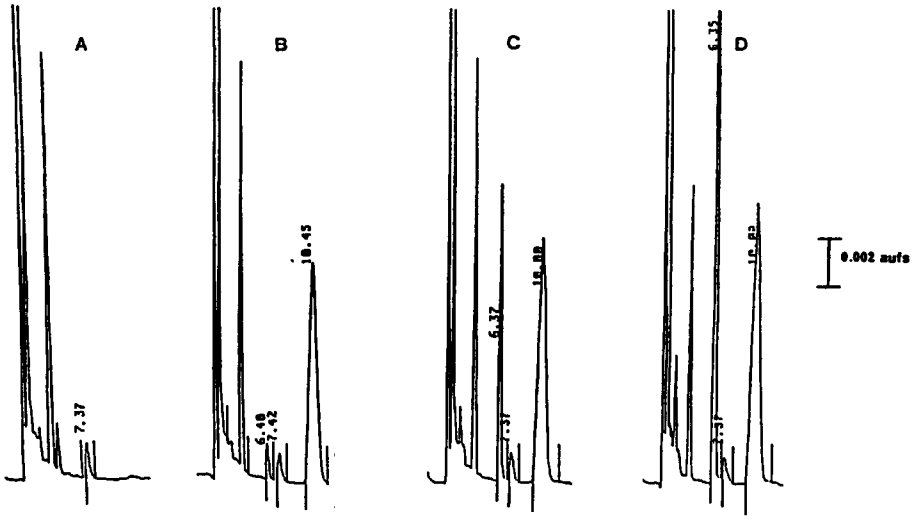

Fig. 2. Chromatograms of tungstate precipitated rat serum. (A) Blank serum; (B) serum spiked with 1 μg/ml gallamine and internal standard, *d*-tubocurarine; (C) serum spiked with 10 μg/ml gallamine and internal standard; (D) serum containing 31 μg/ml gallamine from a rat dosed with 2 mg/kg gallamine. Gallamine and *d*-tubocurarine peaks are at 6.4 and 10 min, respectively. Peak at 7.4 min is an unknown endogenous compound present in rat serum.

tubocurarine and did not present any problems. Fig. 2 shows several chromatograms of either blank rat serum (A), rat serum spiked with either 1 μg/ml (B) or 10 μg/ml (C) gallamine or rat serum when dosed with gallamine (D).

Gallamine-to-internal standard peak-area ratios were linear with gallamine concentrations between 1 and 100 μg/ml with correlation coefficients in excess of 0.99. The limit of detection for gallamine was 1 μg/ml using 50 μl of serum and the coefficient of variation of the assay was 5.3% ($n=5$) at 1 μg/ml and 2.5% at 20 μg/ml. Gallamine recovery and accuracy of the procedure was in excess of 97%. There were no interferences from other drugs used in anesthesiology (e.g., the barbiturates), other neuromuscular blockers (e.g., alcuronium or metocurine) or anti-cholinesterase drugs (e.g., neostigmine or edrophonium) used to reverse neuromuscular blockade clinically.

Fig. 3 shows a gallamine serum concentration–time profile from a rat administered gallamine 2 mg/kg as a bolus intravenous dose while Fig. 4 is a plot of the neuromuscular effect (muscle paralysis) as a function of gallamine serum concentration in the same rat. Both these plots illustrate the versatility of this newly developed assay for gallamine.

DISCUSSION

In preliminary work leading to the development of this assay for gallamine, the classical approach of using an octadecylsilane-bonded (C_{18}) column and either an acetonitrile or methanol–water mobile phase containing different sulfonic acid ion-pairing agents failed to retain gallamine on the column. Varying the concen-

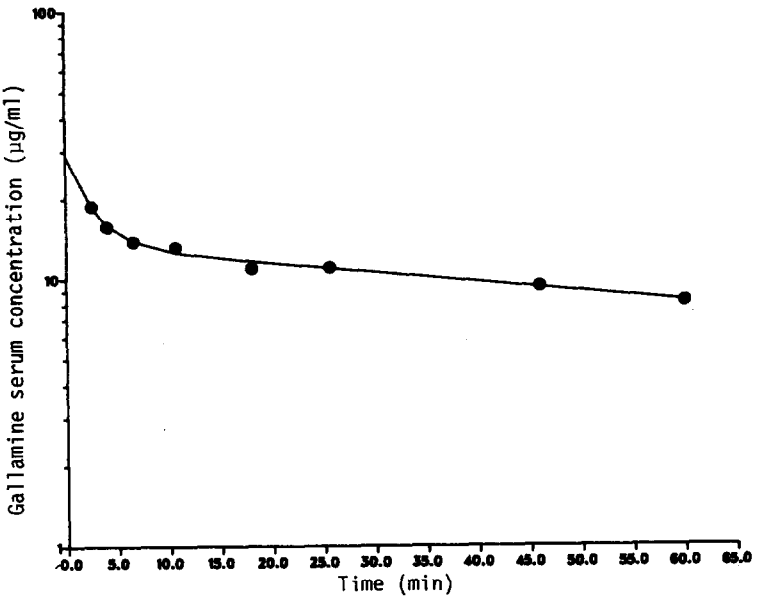

Fig. 3. Gallamine serum concentration-time profile in a rat dosed with 2 mg/kg gallamine intravenously. Serial blood samples (0.10 ml) were taken from a jugular vein cannula. Actual data points together with the curve predicted using a two-compartment pharmacokinetic model are shown.

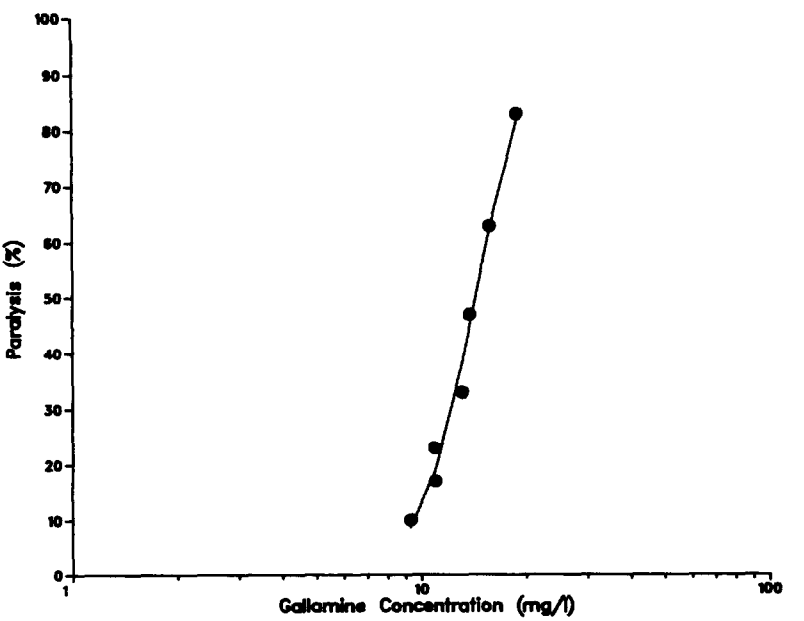

Fig. 4. Relationship between muscle paralysis and gallamine serum concentration during recovery from gallamine-induced paralysis in the same rat as in Fig. 3. Hind limb muscle activity was monitored following sciatic nerve stimulation. Actual data points and the theoretical relationship predicted by a non-linear form of the Hill equation are depicted.

tration and chain length of the ion-pairing agent or reducing the concentration of the organic modifier to 0% did not affect the results. Several C_{18}, C_1 and C_8 columns were also tried without success. Similar effects have been noted previously [11]; in fact gallamine could only be resolved from the solvent front if the flow-rate was reduced to 0.2 ml/min.

Acetonitrile or methanol–water mobile phases were also tried with several different amino (NH_2) columns and to the author's surprise gallamine was retained on the column and could be eluted by adding a sulfonic acid ion-pair reagent. However, other neuromuscular blockers or quaternary ammonium compounds which were potential internal standard candidates could not be retained appropriately under these conditions. In addition, on repeated use, the stability of the column was questionable. Strong cation-exchange columns using aqueous mobile phases containing different concentrations of various counter ions were also tried without success (gallamine was not retained while other compounds were retained). Finally, acetonitrile or methanol–water mobile phases containing different concentrations of TBA and a C_{18} column were tried and it was found that gallamine could be retained on the column with enhanced retention by lowering the concentration of the organic modifier or increasing the concentration of the quaternary ammonium ion-pair reagent. The best mobile phase was found to be 10% methanol in water with 0.0075 *M* TBA since it gave good resolution of gallamine and internal standard in a reasonable time.

The aberrant chromatographic behavior of gallamine appears to be related to the presence of three permanently charged quaternary ammonium groups on gallamine, each containing three bulky substituents (Fig. 1). Presumably steric hindrance around the positive centers is preventing ion pairs being formed with sulfonic acid reagents or ion-exchange mechanisms operating with cation exchangers. Gallamine's behavior on amino or C_{18} columns with mobile phases containing sulfonic acids or TBA, respectively, is difficult to rationalize but these ion-pairing agents are somehow changing the surface characteristics of the packing material. On the C_{18} column, gallamine is retained appropriately by the presence of a low concentration of TBA, and the internal standard, *d*-tubocurarine, is retained due to its structural complexity (Fig. 1) and a low concentration of organic modifier. Inorganic sulfate from TBA hydrogen sulphate is not acting as an ion-pair agent itself since other salts of TBA not containing sulfate gave similar results.

In summary, a sensitive, selective and reproducible method for the determination of gallamine in rat serum has been developed using a readily available internal standard. The procedure permits the pharmacokinetics and pharmacodynamics of gallamine to be determined using small volumes of biological fluids and thus is suitable for routine studies in infants and children where sample volumes are limited.

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