

**NEW TECHNIQUE USING [¹²⁵I]LABELED ROSE BENGAL FOR THE
QUANTIFICATION IN BLOOD SAMPLES OF PIPECURONIUM BROMIDE,
A MUSCLE RELAXANT DRUG**

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SUMMARY

A new technique involving the use of [¹²⁵I]labeled rose bengal for the quantification of pipecuronium bromide (a muscle relaxant drug) is presented. This technique, which is based on the ability of rose bengal to react with pipecuronium and then form a complex which can be extracted into an organic solvent, involves two steps: the purification and labeling of rose bengal with ¹²⁵I, and the quantification of pipecuronium. The specific activity of the compound (106 μCi/mg) allows for the quantification of pipecuronium in biological samples at concentrations as low as 5 ng/ml.

Key words: Iodine-125, Rose bengal, Pipecuronium, Pharmacokinetics

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INTRODUCTION

The original rose bengal ion-complexing method for the quantification of bis-quaternary ammonium compounds such as d-tubocurarine or pipecuronium was first described by Cohen (1). The drug interacts with the dye rose bengal to form an ion-pair complex which is readily extractable into an organic solvent and whose fluorescence is measurable. As the complex formed is not stable and the method was not sufficiently sensitive when drug concentrations were low or with

small volumes of biological fluids, Szabo (2) recommended using the catalytic effect of iodine on cerium (IV)-sulphate-arsenic (III) acid redox solution. With this method, the lower concentration limit of detection is 0.06 $\mu\text{g/ml}$ of pipercuronium, which is still not sufficiently sensitive for long-term pharmacokinetic studies.

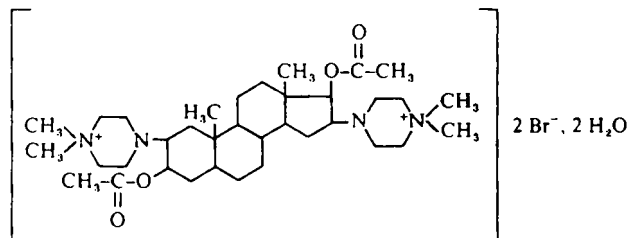


Figure 1. Chemical structure of pipercuronium bromide.

Since pipercuronium is a potent muscle relaxant, the doses administered and consequently the plasma concentrations, are low. For this reason, a very sensitive method of determination is needed for pharmacokinetic studies of this drug. We have therefore developed an assay based on the use of [^{125}I]labeled rose bengal, which allows the quantification of the complex formed by the pipercuronium and labeled rose bengal by measuring the radioactivity with a γ counter.

MATERIALS AND METHODS

This method involves two main steps : first the purification and labeling of rose bengal and then the quantification of pipercuronium.

a) Purification of commercial rose bengal

Due to the presence of impurities observed in the commercially available material, the rose bengal was purified by means of column chromatography. Silica (kieselgel 60, 70-230 Mesh ASMT, Merck, Darmstadt, Switzerland) (150 g) was stirred with 500 ml of eluting solution composed of ethyl acetate:methanol:ammonia (4:1:0.75) (vol/vol). The thick slurry was transferred

to the column (30 X 500 mm) and allowed to settle for 4 h. The top surface of the column was protected from any disturbance by covering it with about 2 mm of micro glass beads.

The crude rose bengal (1.5 g) was dissolved in 2 ml of the eluting solution and carefully loaded on the column. The solution was allowed to seep into the surface of the bed material and elution with the same solvent was started. The column was operated at a flow rate of 2.5 ml/min. The eluted fractions were assayed by thin layer chromatography using the same solvent and by spectrophotometric analysis with a variscan 635 LC spectrophotometer (Varian). The fractions having an adsorption maxima at 5500 nm were combined, the solvent was evaporated under vacuum, the residue dissolved in 0.01 N sodium hydroxide, precipitated with 0.1 N hydrochloric acid and filtered. The purified rose bengal was dried in an desiccator containing phosphorous pentoxide. An aliquot was then analyzed by thin layer chromatography and by spectrophotometric analysis as described above.

b) Labeling of rose bengal

10 mg of purified rose bengal were labeled according to the method described by Hupf (3), using 2 mCi of reductant-free [¹²⁵I]sodium iodide (100 mCi/ml). The solution was stirred for 45 min at room temperature. Labeled rose bengal was purified by means of a C₁₈ solid extraction cartridge. 1 M Hydrochloric acid (2 ml) was added to the solution and the remaining free iodine was then removed using Sep-Pak C₁₈ cartridges primed with 2 ml of 1 M hydrochloric acid. The cartridge was washed with 10 ml 1 M hydrochloric acid and labeled rose bengal eluted with 10 ml of a mixture of ethanol and 1 M sodium hydroxide (8:2 vol/vol). This solution was stored at room temperature and was stable for several weeks. The purity of the labeled rose bengal was tested by thin layer chromatography and absorption spectrophotometry as described above, and the absence of free iodine was confirmed by autoradiography.

The yield of the labeling reaction and the specific activity of labeled rose bengal were determined using absorption spectrophotometry and γ counting.

For daily determinations of pipecuronium, 20 μl of this stock solution were diluted with 10 ml of 0.45 M potassium dihydrogenphosphate.

c) Preparation of plasma samples

Volumes of 1 ml of human whole blood were collected, placed in heparinized tubes and immediately centrifuged at 1000 g for 30 s. 500 μl of plasma were transferred to an Eppendorf tube which was quickly frozen to -70°C in a mixture of acetone and solid carbon dioxide and stored at this temperature until the pipecuronium content was quantified, usually no more than 1 week later.

Deproteinization of plasma samples was achieved by ultrafiltration at 1500 g for 30 min using micropartition system MPS-1 (Amicon, Wallisellen, Switzerland) which allows rapid separation of unbound analytes from proteins in biological samples.

d) Determination of the pipecuronium content

The ultrafiltrate was diluted to 1 ml with phosphate buffer at pH 7.4. Each sample was mixed with 200 μl of the labeled rose bengal solution and then placed in a 5 ml stoppered extraction tube and mixed for 5 s on a vortex mixer to form the complex which was then extracted with 1 ml of chloroform containing 2.6 % phenol. After pipetting off of the aqueous phase, an aliquot of the organic phase was transferred to another tube and its radioactivity was then measured with a γ counter (Kontron Gamma Matic).

Calibration curve

Standard solutions used to establish the calibration curve were prepared by diluting pipecuronium in human plasma which was then ultrafiltered and quantified as described above. A five-point calibration curve was generated by plotting the dpm measured with the γ counter as a function of the known concentrations of the standards. The linearity of the calibration curve was verified by linear least-squares regression analysis.

Precision and accuracy

Precision was evaluated by serial assaying of 10 identical control plasma

samples spiked with known amounts of pipecuronium. The means, standard deviations and coefficients of variation (c.v.) were calculated for each concentration. Additionally, recovery was calculated by determining the ratio of equivalent amounts of unextracted standards in a phosphate buffer solution at pH 7.4 and extracted standard solutions.

RESULTS

Figure 2 shows that the crude rose bengal starting material contains at least 6 compounds which are probably dehalogenated degradation products. After purification by means of column chromatography, only one compound, with an *rf* of 0.5, was detected on the TLC. The absorption maximum of this compound was 5500 nm. The purification of 1.5 g of crude rose bengal gave a yield of 30 %.

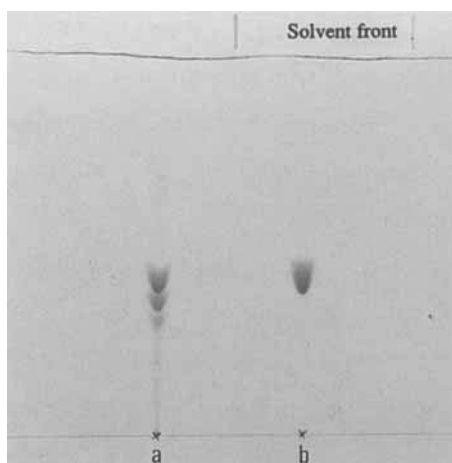


Figure 2. Thin layer chromatography of rose bengal (a) before and (b) after purification by means of column chromatography.

Figure 3 shows no degradation of the rose bengal during the labeling procedure. The absorption maximum of the compound was 5500 nm. The presence of free iodine in the solution necessitated the purification of the labeled rose bengal. After purification, the absence of free iodine in the solution was confirmed by autoradiography. The specific activity of the rose bengal was 106 $\mu\text{Ci}/\text{mg}$. The chemical yield of the reaction, which lasted 45 minutes, was 95 %.

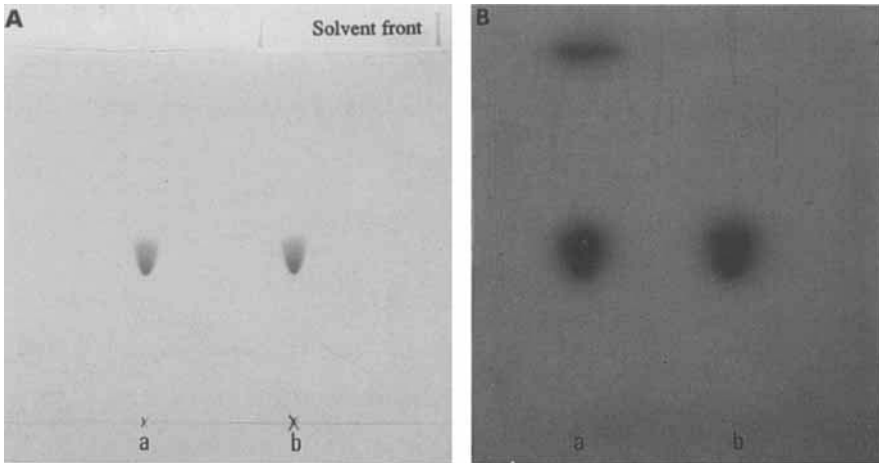


Figure 3. (A) Thin layer chromatography and (B) autoradiography of rose bengal (a) before and (b) after labeling with 125 iodine.

Figure 4 shows a calibration curve obtained from pipecuronium extracted from human plasma. The coefficient of correlation was 0.9998. The detection limit, fixed as a value two times greater than the measured background level of the counter used in this technique, was 5 ng/ml. The calibration curve was found to be linear within the range 5-5000 ng.

Reproducibility assays were performed on control samples of human plasma to which either 10 or 100 ng of pipecuronium had been added. Results are showed in table I. For all samples, the coefficients of variation were less than 16 %. The recovery of analytes from plasma, estimated by comparing results obtained with unextracted standards measured in phosphate buffer at pH 7.4 with those obtained with standards extracted from control human plasma, was at least 95 % for all samples.

The new technique with labeled rose bengal was then used for the determination of pipecuronium in human plasma samples obtained after i.v. administration of a bolus dose of 120 μ g/kg. This study, which will be soon submitted for publication, involved 18 humans but the results for only one subject will be presented here, and are shown in figure 5.

This technique has also been used for the determination of pipecuronium in plasma samples during and after bilateral renal exclusion and orthotopic liver transplantation in the pig (4).

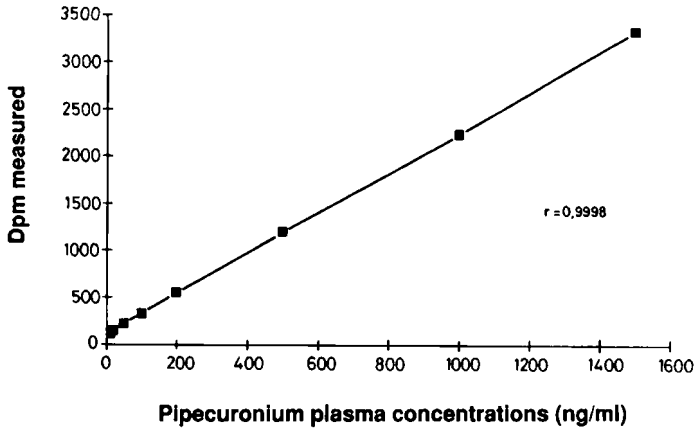


Figure 4. Calibration curve of pipecuronium.

Table I. Precision and accuracy in the determination of pipecuronium.

Compound	Added (ng/ml)	Found (%)	C.V. intra-assay	C.V. inter-assay	n
Pipecuronium	10	96	10.50	16.10	10
	100	98	3.22	5.40	10

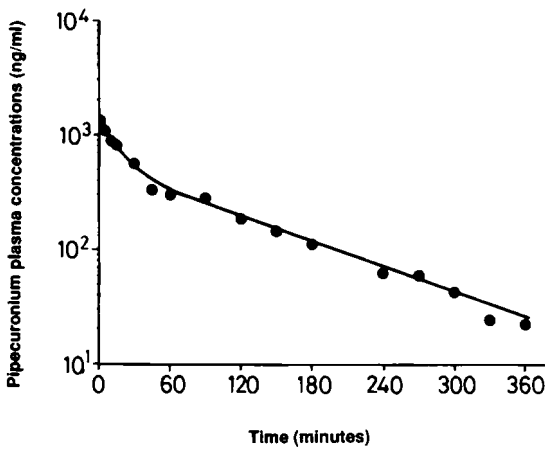


Figure 5. Human plasma levels of pipecuronium after administration of a single i.v. dose of 120 mg/kg of pipecuronium.

DISCUSSION

Since it has been found that the impurities contained in the commercially available rose bengal reduce the labeling yield of the compound (3), purification is necessary before labeling the compound. This can be done by separating the dehalogenated compounds of rose bengal from the parent drug using column chromatography. Using the optimal concentration of ammonia in the solvent is the most important factor in obtaining a good separation. An ammonia concentration that is too weak (10 %) will induce a great difference between the eluting speed of the different components but these will be eluted as broad peaks which would overlap. The use of an ammonia concentration that is too high (25 %), will not allow a good separation of the peaks. The optimum ammonia concentration that will permit a good separation of the compounds without overlapping of the peaks is 13 %.

An important factor in the labeling procedure of the rose bengal is the choice of the isotope of iodine used and this depends principally on availability and physical half-life. There are 11 known isotopes of iodine but most of them can not be used because they have a physical period ranging from only a few minutes to a few hours. The only two isotopes of iodine that are readily available are ^{125}I and ^{131}I which have physical periods of 60 and 8 days respectively. In view of its longer period, ^{125}I is the isotope of choice. It is also safer to work with because the smooth radiations emitted can be easily stopped with 1 mm lead. Furthermore, the radiations emitted by ^{125}I are easily detectable by means of a γ counter.

The previously described methods of labeling rose bengal are based on an exchange reaction. Most of them require a long reaction time (> 24 h) and are performed in an organic solvent under reflux, using an excess of labeled iodine. The use of an oxidizing agent in acidified ethanol described by Hupf (3) make it possible to reduce the reaction time and to perform the reaction at room temperature. This method was therefore used in the present study because it allows for the obtention of goods yields without the necessity of heating the solution, a procedure that can be hazardous when using a labeled substrate, and because the duration of the reaction can be reduced to 45 minutes.

As the labeling technique is based on an exchange reaction, free ¹²⁵I will remain in the solution. Thus a purification step is necessary in order to eliminate this free ¹²⁵I which could interfere with the determination of pipecuronium content. This purification was performed using Sep-Pak C₁₈ cartridges rather than with column chromatography because these cartridges make it possible to confine and then store the labeled substrate in a small volume of eluting solvent.

The purification of plasma samples is necessary to improve the sensitivity of the method by eliminating the endogenous compounds that can interfere with the determination of pipecuronium. If this is not done, the proteins will react with the rose bengal and precipitate in the presence of the organic solvent used to extract the complex. This precipitate can form an aggregate at the interphase of the two solvents which would result in the formation of a stable emulsion which would diminish the precision and sensitivity of the method. The purification methods described in the literature were based either on the precipitation of proteins by an organic solvent (2) or on a dichloromethane extraction and a back-extraction of the drug from serum as iodide ion pairs (5). These methods require a great many operations which can result in an increase of the variability and a decrease of the sensitivity of the method, especially when the pipecuronium content is low. For the purification of plasma samples, we have used a micropartition system which, with a limited amount of handling, allows for a quick purification of the samples by ultrafiltration. The type of membrane used (MW 30,000 cut-off) retains more than 99.9 % serum proteins and less than 3 % pipecuronium. Protein removal by ultrafiltration eliminates the interference and low recovery problems that result from chemical precipitation methods.

Several organic solvents like hydrocarbons, alcohols, ketones and chlorinated solvents have reported to have been used for the extraction of the complex (1). The chlorinated solvents provided the most selective extractions. In the present study, we tested chloroform, dichloromethane and ethylene dichloride. The best results were obtained with chloroform, because it forms less stable emulsions than the other two solvents. Although the ultrafiltration step removes the proteins, it does not remove the free fatty acids, which can increase the stability of the emulsion formed during the extraction step. In order to solve

this problem, small amounts of phenol were added to the organic solvent used for the extraction and by maintaining the phenol concentration at 2.6 % or more, it was possible to break up the emulsion within 30 seconds.

This technique was used for the pharmacokinetic analysis of pipecuronium in normal surgical patients after administration of a dose of 120 µg/kg (2 x ED₉₅) of pipecuronium. After administration, the drug remained detectable in the plasma for 6 hours, a time period sufficient to allow for a precise determination of the parameters governing the pharmacokinetics of pipecuronium.

We have thus developed a simple and sensitive assay for the determination of pipecuronium in plasma samples which is easy to perform and does not require elaborate equipment. It allows for the quantification of pipecuronium at concentrations as low as 5 ng/ml this permitting pharmacokinetic studies for as long as 6 hours after pipecuronium administration. This *in vitro* technique has the advantage of the high sensitivity detection made possible by the use of radioactive compounds while avoiding the necessity of administering these compounds to man.

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