Phosphotungstic Acid Eliminates Uranyl Nitrate as a Sensitizer Before the Silver Impregnation of *Helicobacter pylori*

Rene J. Buesa
Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center of Greater Miami, Miami Beach, FL

Abstract

Five different oxidizers were tested as "sensitizers" for the silver impregnation used to screen for the presence of *H. pylori* in gastric biopsies. Only phosphotungstic acid (0.01% aqueous solution) proved to be as reliable as the conventional 1% aqueous solution of uranyl nitrate. The organisms stained deep black and could be detected in all the samples where present. This procedure reduces the health hazards existent in laboratories where the radioactive uranyl nitrate is used. (*The J Histotechnol* 24:113, 2001)

Submitted: January 23, 2001; Accepted with revisions: March 6, 2001

Key words: gastric biopsies, phosphotungstic acid, radioactive reagent, Steiner method

Introduction

The detection of *Helicobacter pylori* (*H. pylori*) in gastric biopsies has been a standard procedure for almost 20 years in the practice of pathology, and several methods are used for that purpose. Sometimes the weak pink staining of the organisms with the eosin in the routine hematoxylin and eosin procedure is used as the screening method; other practices rely on the Diff-Quick procedure, on modifications of the Wright stain, or on a combination of periodic acid and Feulgen reaction (1–3). The preferred method in many laboratories however, is the modified Steiner procedure (4).

The drawback of the Steiner method is that it requires uranyl nitrate as the sensitizer. This is not only highly toxic, but a source of both alpha and gamma radiation (5).

Commercially available lots of uranyl nitrate (Fluka Chemie AG or Mallinckrodt) have specific activities of up to 0.33 μCi per gram, equivalent to 0.26 μg radium per gram of uranyl nitrate (6). That level of radiation means that 100 mL of a 1% aqueous solution of uranyl nitrate undergoes about 12,000 disintegrations per second (123 Bq/mL), something that should be avoided.

Faced on the one hand with the fact that the silver impregnation of *H. pylori* greatly facilitates the screening, and on the other with the radioactive nature of uranyl nitrate, we conducted a series of tests during December 1997 and October 1998 with 5 other chemicals aimed at eliminating uranyl nitrate from the procedure.

After the results were evaluated, our laboratory adopted a 0.01% aqueous solution of phosphotungstic acid as the sensitizer in the Steiner silver method. This has resulted in a less hazardous environment for personnel, reduced costs for disposal of toxic waste, and a savings of ~$1.20 per gram of reagent: uranyl nitrate = $2/gm vs phosphotungstic acid = $0.80/gm.

Uranyl nitrate has been considered both a "sensitizer" and an "accelerator", although chemically it is an oxidizer with known reactive equivalences with other oxidizers (5,7). Therefore, our first approach was to substitute other oxidizers for the 1% aqueous solution of uranyl nitrate used in the Steiner procedure. We tried different concentrations and incubation periods. The tested reagents were used as the first step (sensitizer) in the procedure, leaving the rest of the procedure unaltered. A total of 156 experiments using 5 different reagents were performed during December 1997 and October 1998.

Materials and Methods

Sections

Serial sections of strongly positive gastric biopsies were always used and with each experiment 1 positive control (also a gastric biopsy) was run with uranyl nitrate.

Evaluation

The result of each experiment with different sensitizers was compared with that using uranyl nitrate for both intensity of the silver impregnation (of an ideal dark black) and the numbers of organisms impregnated.

Address reprint requests to Rene J. Buesa, Department of Pathology and Laboratory Medicine, Mt. Sinai Medical Center, 4300 Alton Road, Miami Beach, Florida, 33140.

Because it is difficult to count bacteria in tissue sections when serial sections are used, the reactive areas within each section with any given chemical tested were compared with the same areas in the section treated with uranyl nitrate, allowing a quantification of the results with regard to the detection of positive areas.

The test results are summarized in Table 1.

Reagents

Phosphotungstic Acid

1% Stock solution:
- Phosphotungstic acid ..... 1 gm
- Distilled water (DW) ..... 100 ml

0.01% Working solution:
- 1% Stock solution ..... 1 ml
- DW ..... 99 ml

1% Silver nitrate
- Silver nitrate ..... 1 gm
- DW ..... 100 ml

2.5% Gum mastic (alcoholic):
- Gum mastic ..... 2.5 gm
- Absolute ethanol ..... 100 ml

Developing solution:
- Hydroquinone ..... 0.5 gm
- 2.5% alcoholic gum mastic ..... 10 ml
- Absolute ethanol ..... 5 ml
- DW ..... 25 ml

Add 1 ml of 1% aqueous silver nitrate before using.

Staining Protocol

1. Dewax and hydrate as usual (xylene – ethanol – water).
2. Rinse with DW twice.
3. Place slides in the phosphotungstic acid 0.01% working solution. Heat in the microwave to an equivalent of 5.1 kcal (to 60°C) and incubate for 10 min at 60°C in a water bath.
4. Rinse with hot tap water, then rinse twice with DW.
5. Place slides in 1% aqueous silver nitrate solution. Heat and incubate as in Step 3.
6. Wash as in Step 4. Rinse twice with absolute ethanol.
7. Add the 2.5% alcoholic gum mastic solution to the Coplin jar with the slides. Place in the microwave and incubate 45 seconds at power 5 (@2.6 kcal). The lower power level is required to prevent the ethanol from boiling. Incubate for 10 min in a water bath at 60°C.
8. Prepare the developing solution: In the microwave (5.1 kcal) heat 25 ml DW. Once heated, add 0.5 gm of hydroquinone; mix well. Once dissolved, add 10 ml of the 2.5% alcoholic gum mastic and 5 ml absolute ethanol. Stir and filter once. The solution will be filtered when Step 7 is completed. Just before using this solution, add 1 ml of 1% aqueous silver nitrate solution. Reheat the solution to ~60°C.
9. Discard the gum mastic of Step 7 and, without washing, add the reheated developing solution. Return the slides to the 60°C water bath.
10. Leave the slides in the developing solution until the overall color is golden brown (like a Manila envelope). After 4–5 min check the control slide microscopically for the stained bacteria and the cellular staining.
11. Discard the developing solution, and wash the slides in hot running tap water, followed by absolute ethanol to interrupt the developing of the impregnate silver.
12. Complete the dehydration, clear with xylene, and mount sections.

Procedural Note

The microwave oven used in the staining protocol was experimentally calibrated for each power level, and its energy output (in Watts) times the duration of each boost (in seconds) times 0.2390×10^{-3} $kcals^{-1}/W^{-1}$, expresses the energy the slides received (8).

By presenting the energy this way, the exact duration of the boost using another microwave oven could be calculated and would be inversely proportional to its energy output.

Results (Table 1)

Phosphotungstic Acid

All bacteria were stained pitch black; epithelial cell cytoplasm was golden brown to brown; all nuclei were dark brown to brown-black.

Phosphotungstic (phosphowolframic) acid was tested in 13 different concentrations (0.0025 to 1%) with a detection rate of 92% of organisms for all the tests. The best concentration (0.01% aqueous solution) detected all the organisms with results equal to those obtained with uranyl nitrate. There was a microwave boost (5.1 kcal) for 45 sec and an incubation for 10 min at 60°C in a water bath.

Table 1. Summary of the Experiments With Five Oxidizers

	Hydrogen peroxide	*Potassium nitrate*	*Potassium permanganate*	*Lead nitrate*	*Phosphotungstic acid*
Experiments	2	27	92	15	20
Overall detection	0	0	46%	70%	92%
Best concentration	—	—	0.01%	0.025%	0.01%
Microwave boost of 5.1 kcal	—	—	Yes	No	Yes
Minutes at 60 °C	—	—	5	5	10
% Detection with best concentration	—	—	80	83	100

Control: Serial sections stained with uranyl nitrate.

This was the method adopted on October 23, 1998 and used since in our laboratory for the detection of *H. pylori* in gastric biopsies.

Hydrogen Peroxide

Hydrogen peroxide was tried in 1 and 3% concentrations, respectively, both with negative results.

Potassium Nitrate

Similarly, all the results for the 27 experiments with potassium nitrate (3 different concentrations from 0.5 to 3%, with 7 different incubation periods and 2 concentrations of silver nitrate in the developer) were all negative.

Potassium Permanganate

Potassium permanganate was tried in a total of 92 experiments: a total of 11 concentrations (from 0.01 to 0.5%) with 10 different incubation times, microwave heating, and 2 concentrations of oxalic acid (1 and 3%) to decolorize the potassium permanganate.

There were positive results with an overall detection rate of 46% and a best combination of 0.01% heated for 45 sec, (5.1 kcal) incubated at 60°C in a water bath for 5 min, and decolorized with 1% oxalic acid.

This procedure produced dark black impregnation of 80% of the organisms, meaning that there was an unacceptable risk of a false negative in cases with few organisms.

Lead Nitrate

Lead nitrate (10 concentrations from 0.025 to 0.5%) permitted a detection rate of 70% of organisms for all the experiments, with a best concentration of 0.025%, a microwave boost of 5.1 kcal, and incubation at 60°C for 5 min.

This procedure rendered the organisms dark black with a detection rate of 83% of all, which amounted also to an unacceptable false negative rate.

Discussion

In spite of its extreme toxicity (kidney and liver damage and accumulation in growing areas of bone), uranyl nitrate has been in use in histological technique for more than a century, especially in those early techniques dealing with the detection of the Golgi apparatus (9–11).

Starting with the Kahlden and Laurent formula for uranium-carmine from 1896, uranium nitrate has been used as a component of accelerators, developers, fixatives, preservatives, and stains, especially popularized by pioneer histologists like Golgi and Cajal (7). Uranyl nitrate has been categorized as a "sensitizer," an "enhancer," and other roles of obscure chemical meaning.

Because of the health risks associated with its use, there have been attempts at substitution of uranyl nitrate with special fixatives, with other oxidizers like lead nitrate, or simply by creating alternative procedures that totally eliminate its use for the detection of *H. pylori* (1–3, 12–14).

Of the 5 oxidizers tested, 2 of them (hydrogen peroxide and potassium nitrate) failed completely. Another 2 (potassium permanganate and lead nitrate) produced positive results, yet were inconsistent, both in the intensity of the silver impregnation of the organisms and in their detection rate.

Only phosphotungstic acid proved to be as good as uranyl nitrate for the detection of *H. pylori,* implying that there is more involved than just oxidation when the substitution of uranyl nitrate is the objective. Of all the tested chemicals, phosphotungstic acid is, besides being an oxidizer, a polyvalent acid. As such, its solutions have a different pH with relation to its concentration, from pH 2.4 at a 2% aqueous solution, to pH 4.7 at a 0.0025% concentration. At the best (selected) working dilution (0.01%), the pH is 4.1, and perhaps it is not a coincidence that this is the pH of the buffer used in the Warthin-Starry procedure for silver impregnation of spirochetes (7).

Perhaps the fact that phosphotungstic acid is a conventional acidifying agent is a significant factor in its ability to mimic the somewhat obscure role of the uranyl nitrate in the Steiner procedure (15).

Phosphotungstic acid has been used since 1900 as a fixative and differentiating agent, as part of complex stains, and as a hematoxylin mordant with roles almost as obscure as those assigned to uranyl nitrate (7).

Besides some similar histological functions, both uranyl nitrate and phosphotungstic acid are oxidizers of high molecular weight that belong to VIn group of the elements, have 2 electrons in the outer shell, and facilitate silver impregnation. Phosphotungstic acid also promotes the formation of cationic complexes.

The chemistry behind the silver impregnation of fixed organisms has been a source of speculation for years, and the actual mode of action of the different components of any silver stain is an ongoing source of debate (5). The reason for the ability of phosphotungstic acid to substitute for uranyl nitrate in the silver impregnation of *H. pylori* and any other microorganisms found in the digestive tract may simply fall in the group of incompletely understood silver stains.

As a final note, although the use of phosphotungstic acid assures a remarkable staining of *H. pylori* and the cells of stomach mucosa, as well as any other intestinal bacteria, it will not allow the staining of spirochetes. Uranyl nitrate cannot be substituted by any other chemical if spirochaete are to be stained, as has been pointed out elsewhere (14).

Why? That is a riddle that remains to be solved, and the answer may lie in the surface chemical composition of the spirochaetes, which have been known to be extremely elusive to staining since their discovery (7).

Conclusions

After using 5 different oxidizers (hydrogen peroxide, potassium nitrate, potassium permanganate, lead nitrate, and phosphotungstic acid) in an attempt to eliminate the radioactive uranyl nitrate used in the Steiner procedure to detect the presence of *H. pylori* in gastric biopsies, only phosphotungstic acid proved to be as consistently reliable as uranyl nitrate.

A very weak (0.01%) aqueous solution of the acid can be used as a sensitizer in the Steiner procedure. The resulting staining of the organisms is pitch black and the cells of the gastric mucosa stain in a remarkable manner in tones from gold to deep/dark brown. The detection rate is 100% of all the organisms present, eliminating false negatives.

This modification allows the elimination of the highly toxic uranyl nitrate, which is very expensive to dispose of because of radioactive levels that may pose environmental problems and that are hazardous to histology personnel.

There is also a favorable cost effect in the substitution since the cost of uranyl nitrate is about $2/gm in contrast to about $0.80/gm for phosphotungstic acid.

Acknowledgments

The author expresses his gratitude to Mr. Daniel Sorace, HT, for performing the experiments while working at the Histology laboratory of Mount Sinai Medical Center. Special thanks are given to Dr. John Alexis, pathologist in charge of the histology laboratory for his involvement in the final steps of this research, for the selection of the working concentration of phosphotungstic acid adopted as our procedure, and for reading and correcting the manuscript.

References

1. Skipper R, DeStephano BD: A rapid stain for *Campylobacter pylori* in gastrointestinal tissue sections using Diff-Quick. *J Histotechnol* 12:303–304, 1989
2. Buttler GD: Buttler modified Wright stain for demonstration of *Campylobacter pylori. J Histotechnol* 13:109–111, 1990
3. Cohen LF, Sayeeduddin M, Phillips C, Shahab I: A new staining method for identification of *Helicobacter pylori* and simultaneous visualization of gastric morphologic features. *Mod Pathol* 10:1160–1163, 1997
4. Steiner G, Steiner G: New Simple stain for demonstration of bacteria, spirochetes, and fungi in sections from paraffin embedded tissue blocks. *J Lab Clin Med* 39:868–871, 1944
5. Thompson SW: *Selected Histochemical and Histopathological Methods.* CC Thomas, Springfield, IL, 1966, pp 1,639
6. Illingworth V (ed): *Dictionary of Physics.* Penguin Books, London, 1991, pp 544
7. Gray P: *The Microtomist's Formulary and Guide.* The Blakiston Co, New York, 1954, pp 794
8. Login GR, Dvorak AM: *The Microwave Book. A Practical Guide for Microscopists.* Beth Israel Hospital, Boston, MA, 1994, pp 164
9. Dempsey EW: Current concepts of cellular structures. In: *Frontiers in Cytology,* Palay SL (ed). Yale University Press, New Haven, 1958, pp 8–18
10. Scott KG, Axelrod D, Crowley J, Hamilton JG: Deposition and fate of plutonium, uranium and their fission products inhaled as aerosols by rat and man. *Arch Pathol* 48:31–54, 1949
11. Cajal SRY: Formula de fijacion para la demostracion facil del aparato reticular de Golgi y apuntes sobre la disposicion de dicho aparato en la retina, en los nervios y en algunos estados patologicos. *Trab Lab Invest Biol Univ Madrid* 10:209–220, 1912
12. Margeson LS, Chapman CM: Use of zinc formalin as a sensitizer in silver stains for spirochetes. *J Histotechnol* 19:135–138, 1996
13. Elias JM, Greene C: Substitution of lead nitrate for uranium nitrate as used in the Steiner silver method. Basic Health Sciences Center, SUNY @ Stony Brook, New York, p 5 (undated, mimeographed MS)
14. Garvey W, Fathi A, Bigelow F: Modified Steiner for the demonstration of spirochetes. *J Histotechnol* 8:15–17, 1985
15. Bancroft JD, Stevens A: *Theory and Practice of Histologic Techniques.* Churchill Livingston, London, 1990, pp 726