Removal of Interference by Erythromycin, Phenazopyridine, and Methenamine Mandelate in the Porter–Silber Reaction

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Erythromycin, phenazopyridine, methenamine mandelate, acetazolamide, and ascorbic acid reportedly interfere with the Porter–Silber reaction in the determination of urinary 17-hydroxycorticosteroids. Sodium bisulfite, added to the urine after hydrolysis with gluconoridase, removed almost all nonsteroidal interferences in urine from healthy adults medicated with the first three drugs, but such studies proved that acetazolamide and ascorbic acid do not interfere with the Porter–Silber reaction, whether or not sodium bisulfite is added.

Additional Keyphrases: sodium bisulfite • ascorbic acid • acetazolamide

In my previous paper (1), a new method for determination of urinary 17-hydroxycorticosteroids (Porter–Silber chromogens) was described: After hydrolysis with β-glucuronidase, NaHSO₃ is added to the urine, followed by extraction with methylene chloride. This results in a remarkable decrease in the blank without loss of Porter–Silber chromogens in urine, owing to the removal of non-tetrahydro compounds of steroids and nonsteroidal impurities (2)—such as acetylsalicylamycin, triacycloleandomycin, and rifampicin—that interfere with the subsequent Porter–Silber reaction.

This paper deals with the effect of NaH₂SO₃ on the removal of interferents in the urines of healthy adults medicated with erythromycin, phenazopyridine, acetazolamide, methenamine mandelate, and ascorbic acid, all of which reportedly interfere with the above-mentioned reaction.

Method

Pipet 5 ml of urine,¹ adjusted to pH 5.0, into a glass-stoppered test tube containing 0.5 ml of acetate buffer (2 mol/liter, pH 5.0), dissolve 0.5 g of Na₂SO₃ in the urine, and add 2500 Fishman units of β-glucuronidase (EC 3.2.1.31, bovine liver origin; Hokuiken, Sapporo, Japan). After incubation at 48 °C for 18 h, add 1.5 g of NaH₂SO₃ (in the case of high concentration of interferant, 3 g) and dissolve. Shake the urine for about 40 s (four or five times per second) with 35 ml of methylene chloride. Aspirate the urine layer and filter the extract through fluted paper into another test tube. Wash the extract, first with 3 ml of 0.25 mol/liter Na₂CO₃, then with 3 ml of 0.05 mol/liter NaOH, and finally with 3 ml of 0.05 mol/liter H₂SO₄ (each for 5 s); all of these solutions are prepared in a 10 g/liter solution of Na₂SO₄. After the last washing, centrifuge the extract and remove the aqueous layer. Add 10 ml of the methylene chloride extract to 1.0 ml of ethanol, followed by shaking with 1.5 ml of phenylhydrazine/sulfuric acid solution² for 30 s. For the sample blank, instead of phenylhydrazine/sulfuric acid solution, use 1.5 ml of the diluted acid.³ After removing almost all the methylene chloride layer (upper layer), incubate the reagent layer at 60 °C for 30 min, keeping the surface of the layer 1 to 2 cm lower than that of the water bath. During the incubation the remaining methylene chloride is vaporized. Measure the absorbance of the sample against the blank at 410 nm in 10-mm cuvettes. Take a reagent blank, without water instead of urine, through the same procedure without β-glucuronidase. Subtract its reading from that of the sample. This yields the true absorbance value for the sample.

Use cortisol as a standard: Take 5 ml of the working standard solution (10 mg of cortisol per 1000 ml of water) plus 0.5 ml of acetate buffer without β-glucuronidase through the procedure, starting with methylene chloride extraction in the presence of 1 g of NaH₂SO₃ instead of NaH₂SO₄. This is reported in detail in previous papers of this series (1–3).

Results and Discussion

Erythromycin. The administration of erythromycin (4), as well as of the closely related drug, triacycloleandomycin (5), has been reported to result in a false increase in apparent 17-hydroxycorticosteroids in urine.

Figure 1 shows the spectra of methylene chloride extracts of β-glucuronidase-hydrolyzed urine from a healthy adult on the second day of erythromycin administration (4 × 200 mg/day).

The spectra on the left were obtained using the no-NaH₂SO₃ (original) method, where 0.5 g of NaH₂SO₃ instead of 1.5 g of NaH₂SO₃ is added to the hydrolyzed urine before methylene chloride extraction. The spectrum for the Porter–Silber chromogen shows a negative absorbance maximum at 483 nm because the blank has a positive maximum at the same wavelength. The absorption value for the sample blank at 410 nm is remarkably high.

¹ Twenty-four-hour urine samples, with 5–7 g of NaH₂SO₃ added, are collected in glass bottles at room temperature. This quantity of NaH₂SO₃, determined in cooperation with Drs. Graef, Hansten, and Barclay (see acknowledgments), results in urinary 17-hydroxycorticosteroids being stable for one month at 5 °C. The urine samples are adjusted to pH 5.0 (pH 4.8–5.2) with 100 g of Na₂CO₃ per liter.

² Diluted sulfuric acid: 64 ml of H₂SO₄ ("super special" grade) plus 36 ml of water, or 66 ml of H₂SO₄ (concentrated, analytical grade) plus 34 ml of water. Phenylhydrazine/sulfuric acid solution: 100 mg of phenylhydrazine sulfate (recrystallized three times from water–ethanol) plus 100 ml of diluted sulfuric acid.

³ Received July 13, 1977; accepted Aug. 16, 1977.
The spectra on the right, however, derived from application of the present method, shows no interference from erythromycin. The form of the spectrum was exactly the same as that obtained from the same adult when not medicated.

The 17-hydroxycorticosteroid values calculated from the absorbances at 410 nm in both spectra “a” (Figure 1) are, expressed as cortisol, 11.6 mg/liter and 8.7 mg/liter, respectively; thus the former is 30% higher than the latter.

Phenazopyridine also reportedly interferes with urinary steroid determinations (6, 7). Figure 2 shows the spectra of methylene chloride extracts of a healthy adult’s β-glucuronidase-hydrolyzed urine collected on the second day of phenazopyridine administration (3 × 200 mg/day) with, again, the left-hand curves being for the original method and the right-hand curves those for the present method. In the present method, in connection with methylene chloride pretreatment: To 5 ml of the urine with 0.5 ml of acetate buffer add 0.5 g of Na₂SO₄, and extract the mixture once with 30 ml of methylene chloride. Incubate the urine layer with β-glucuronidase. This procedure is often conducted to remove free steroids and nonsteroidal lipophilic impurities. All the absorbances in the spectrum “c” are lower than those in the same (present) method without the methylene chloride pretreatment, but the shapes of both spectra are the same. A spectrum of exactly the same shape has also been obtained in the case of urine extracts from healthy adults without medication. The 17-hydroxycorticosteroid values calculated from the absorbances at 410 nm in both spectra “a” are 8.3 and 8.9 mg/liter (expressed as cortisol). Taking into account the decrease of about 5% as a result of the removal of free 17-hydroxycorticosteroids by means of methylene chloride pretreatment, it can be concluded that the value in the present method is reasonable and that the 10.6 mg/liter value in the original method must be false, i.e., phenazopyridine interferes in vivo with the absorbances in the original method, but not in the present method.

Acetazolamide reportedly (6, 7) interferes with urinary 17-hydroxycorticosteroid determination, resulting in an increase in absorbance at 410 nm in the method (in vitro study). Thus the effect of administered acetazolamide remains to be determined.

Figure 3 (upper part) shows the effect of acetazolamide administration (3 × 250 mg/day) on the Porter–Silber chromogens spectra of the methylene chloride extracts of β-glucuronidase-hydrolyzed urine in the original and present methods. In this experiment we also used a healthy adult’s

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3 Using 29 urine samples from 29 healthy adults on no medication, I found the blank values (indicated as the ratio of the blank to the true absorbance at 410 nm, multiplied by 100) in the original, present, and present (with methylene chloride pretreatment) methods to be 25 ± 4.2 (18–33), 20 ± 4.6 (11–29), and 12 ± 3.6 (11–22); P < 10⁻⁴ between the former two, and P < 10⁻⁶ between the latter two. On the other hand, using 69 urine samples from patients of our university hospital, I found the blank value in the original and present methods to be 33 ± 8.5 (19–56) and 20 ± 5.1 (10–36) (P < 10⁻⁶). The difference between the two figures, 25 ± 4.2 and 33 ± 8.5, is highly significant (P < 10⁻⁶). This seems to reflect the impact of medication. As for urine from hospitalized patients, the likelihood of the Porter–Silber reaction being affected seems to be high in the original procedure, but low in the present method.
urine collected on the second day of the administration of the drug. As seen in this figure the form of the spectra "c" in both methods are the same (these spectra were again exactly the same as those obtained from the same adult without medication), and the values calculated from the absorbances at 410 nm are similar (6.4 and 6.1 mg/liter), even if the absorbances in the range studied are higher in the original method than those in the present method. From this in vivo experiment I conclude that neither method is affected by acetazolamide administration.

Methenamine mandelate administration (8) has been shown to interfere with the Reddy (butanol) method (9) for urinary 17-hydroxycorticosteroids determination, apparently because the formaldehyde in the urine, originating from the methenamine compound, falsely increases the assay value—an increase reportedly eliminated when Allen's correction is applied.

Figure 3 (lower part) shows the spectra of methylene chloride extracts of β-glucuronidase-hydrolyzed urine from a healthy adult on the second day of methenamine mandelate administration (4 × 1 g/day). In this experiment, even if the absorbances of the spectrum "c" in the wavelength studied are higher in the original method than those in the present method, the forms of both spectra are the same and the 17-hydroxycorticosteroid values are almost equal (7.9 and 7.3 mg/liter). After making Allen's correction at three wavelengths (370, 410, and 450 nm) the values are 7.2 and 7.2 mg/liter, respectively. This means that the value obtained at 410 nm in the original method contains the value caused from the drug or its metabolites or both, showing a positive contamination and a concave spectrum in the wavelengths studied. From this experiment I conclude that the present method is unaffected by this drug and it is not necessary to make the Allen correction.

Ascorbic acid. I also conducted an experiment with ascorbic acid (4 × 1 g/day), which reportedly interferes with 17-hydroxycorticosteroid determination by the Reddy, Jenkins, Thorn procedures. The spectra obtained in the original and present methods were almost the same as those obtained in the above experiment with acetazolamide, showing that neither is affected by ascorbic acid administration.

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References


4 Forsom, P. H., Metabolic steroid laboratory requisition. Metabolic Steroid Laboratory, University of California at San Francisco, San Francisco, Calif.