

## Decreased Signal in Emit Assays of Drugs of Abuse in Urine After Ingestion of Aspirin: Potential for False-Negative Results

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During routine drug analysis with the Syva d.a.u. Emit immunoassays we observed a high frequency of urines with lower rates of changes in absorbance ( $\Delta A R$ ) than the rate for a drug-free urine calibrator. Many of these urines contained salicylates. Among 40 urines with apparent salicylate concentrations between 15 and 420 mg/dL tested for benzoylecgonine (BE), 20 had  $\Delta A R < -4$  (range +2 to -28 mA/min). The rates decreased with increasing salicylate:  $\Delta A R = -0.057 \times (\text{salicylate, mg/dL}) - 0.22 \text{ mA/min}$  ( $r = 0.85$ ,  $n = 40$ ,  $P < 0.01$ ). Urines from 100 control subjects (no salicylate) had mean  $\pm$  SD  $\Delta A R$  values of  $-1.05 \pm 2.2 \text{ mA/min}$  (range +3 to -7; only two were  $< -4 \text{ mA/min}$ ). Although direct addition of salicylic acid (200 mg/dL) to urine specimens did not reproduce the negative bias, ingestion of aspirin (acetylsalicylic acid) did by  $-0.09 \text{ mA/min}$  per 1 mg/dL (72.4  $\mu\text{mol/L}$ ) salicylate. Negative biases observed for other Emit d.a.u. assays after salicylate ingestion lead us to conclude that ingestion of therapeutic doses of aspirin may cause false-negative results for drug screens in urines by this technology.

**Indexing Terms:** enzyme immunoassay/acetyl salicylate/drug metabolism/variation, source of/screening

Enzyme immunoassay for drug of abuse testing provides rapid and reliable detection of drugs in urine. It is perhaps the most widely used immunoassay screening method for drugs in both forensic and emergency medical situations (1). In drug screening protocols, positive results may be subsequently confirmed by other more rigorous and specific techniques, whereas negative initial screening results warrant no follow-up. Users of illicit drugs have attempted to obtain false-negative enzyme immunoassay results by altering urines with a variety of chemical agents such as: sodium chloride (2); liquid drain cleaners (3); handsoaps (3); Visine eye drops, vinegar, tea, ibuprofen, and water (4). Most of these adulterants can be detected by observing the pH, appearance, creatinine concentration, or relative density of the urines (4). Substances that cause false-positive assay responses can also be used to alter drug immunoassay results (5).

In the Emit d.a.u. method (Syva Co., San Jose, CA) a cutoff concentration (that of a one-point reference calibrator called a "low calibrator") is used to determine the

presence of a drug in the sample being tested. If the observed signal in the assay is greater than the signal obtained at the cutoff concentration, the results are considered screen-positive; those with a lower signal are screen-negative.

During routine drug analysis for benzoylecgonine (BE; the major metabolite of cocaine) with Syva Emit and Emit II d.a.u. reagents, we observed several urines showing negative absorbance rates in comparison with that for a drug-free urine calibrator (6, 7). Most of these urines tested positive for salicylates. We postulated that the presence of salicylate or related metabolites in urine was responsible for the negative bias in the Emit assays. To test this hypothesis, we performed recovery experiments by adding BE to urines with and without salicylates present, compared the magnitude of the bias in the cocaine assay with the salicylate concentrations in the urine, and studied the effects of aspirin ingestion on salicylate excretion in urine and the negative bias produced in the Emit d.a.u. assays. Our data demonstrate that ingestion of therapeutic doses of aspirin can decrease the signal in urine drug analysis by Emit technology.

### Materials and Methods

**Emit assays.** Emit d.a.u. and Emit II d.a.u. reagents were obtained from Syva Co. Emit assay protocols were performed according to manufacturer's instructions on the Hitachi 704 (Boehringer Mannheim, Indianapolis, IN). The assays were calibrated with a drug-free (negative) urine calibrator supplied with the Emit assay kits. The absorbance rate (mA/min at 340 nm) for the negative calibrator was set as the zero point. The absorbance difference for the 60-s interval from the 19th to the 22nd data point was used to determine the absorbance rate. We used two additional Syva calibrators, a cutoff concentration calibrator (300 ng/mL for the cocaine assay) and a high-concentration calibrator.<sup>3</sup> Differences between the absorbance rates for the calibrators were used as quality-control indicators for the assay. To verify that all urines tested in this study had pH values between 5 and 8 as required in the Syva protocol, we used pHDrion paper (Micro Essentials Lab., Brooklyn, NY).

**Salicylate assays.** A modified Trinder protocol (8) was used for measuring salicylates (9, 10). Sodium salicylate was obtained from Sigma (St. Louis, MO). Reagents were analytical grade. Standards were prepared by di-

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<sup>3</sup> SI conversion factors: cocaine, 1 ng/mL = 3.30 nmol/L; benzoylecgonine, 1 ng/mL = 3.46 nmol/L; salicylate, 1 mg/dL = 72.4  $\mu\text{mol/L}$ .

luting a salicylic acid stock solution (232 mg of sodium salicylate dissolved in 100 mL of water) with deionized water. We measured the absorbance at 540 nm (referenced to a reagent blank) with a Gilford System 102 spectrophotometer (Gilford Instruments, Oberlin, OH).

**Selection of urine specimens.** Salicylate-containing urines were obtained from routine specimens submitted to our toxicology laboratory. Urines were identified as positive for salicylates by a ferric chloride spot test (visual determination) for which the minimum detectable salicylate concentration determined in our laboratory was 5 mg/dL.

**Aspirin ingestion experiments.** Aspirin tablets (325 mg of acetylsalicylate) were obtained at a local pharmacy. Six volunteers (five men, one woman) ingested four tablets each. Neither time of ingestion nor eating habits were discussed with the volunteers. Volunteers were instructed to obtain one urine sample immediately before ingestion of aspirin and about every 2 h thereafter for various times up to 20 h. Urines were stored at 2–6°C until analyzed.

**BE recovery and enzyme inhibition experiments.** Urines (negative calibrator urine provided by Syva and patients' urine samples with and without salicylate) were mixed with the Syva high calibrator (containing BE) to yield samples with various concentrations of BE. We analyzed these samples with the cocaine Emit d.a.u. assay and plotted the standard concentration–response curves as BE concentration vs the delta absorbance rate,  $\Delta A R$  (mA/min of sample minus mA/min of the negative calibrator).

To test the potential inhibition by salicylate-containing urines of the enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49) used in the Emit reagents, we concentrated a urine specimen containing 84 mg/dL of excreted salicylate (Trinder method) and a urine specimen from the same individual collected before the ingestion of aspirin. We used a Speed-vac (Savant Instruments, Farmingdale, NY) to concentrate both urines to the same extent; the final salicylate concentration in the salicylate-containing urine was 672 mg/dL. The reagents (R1 and R2) from two different Emit drug kits (amphetamine and BE) were interchanged to maintain the chemical integrity of the reagent mixtures. After diluting the concentrated salicylate-containing urine with the salicylate-free concentrated urine, we assayed the resulting samples by using R1 for amphetamine and R2 for BE and then using R1 for BE and R2 for amphetamine. This was done to test the inhibition of the enzyme after accounting for any effects of antibody and (or) drug concentration. Reagent R1 contains glucose 6-phosphate, NAD<sup>+</sup>, antibody, and stabilizing agents; reagent R2 contains glucose-6-phosphate dehydrogenase covalently attached to the specific drug and stabilizing agents (Syva package insert). In the absence of the appropriate antibody, the assay should proceed at maximal velocity; a decrease in the observed rate should be attributable to inhibitor(s) present in the urine sample.

## Results

**Reduced delta absorbance rates in Emit cocaine assay.** Fig. 1 shows the relationship between salicylate concentration in urine and reduced delta absorbance rate ( $\Delta A R$ ) measured with the Emit cocaine assay in 40 salicylate-containing urine specimens. Twenty (50%) of these samples gave negative absorbance rates < -4 mA/min (range -6 to -28 mA/min). In contrast, the mean  $\Delta A R$  for a series of 100 control subjects (not taking drugs or salicylates) requiring drug screening in our Emergency Department was -1.05 mA/min (range +3 to -7 mA/min, SD = 2.18, SE = 0.22). Only two of the controls had rates < -4 mA/min.

**Recovery experiments with BE and salicylate.** To determine the magnitude of negative bias in the measured concentration of BE caused by various amounts of salicylate in urine, we added BE concentrations of 300, 600, and 900 ng/mL to two control urines (not containing salicylates) and to 16 urines (salicylate concentrations 15–420 mg/dL) from salicylate-positive subjects submitted for routine drug screening. This established a four-point standard calibration curve for each of these 18 urines, from which we wanted to determine how much BE was needed to exceed the  $\Delta A R$  of the 300 ng/mL calibrator. Fifteen (94%) of the 16 urines containing salicylates required >300 ng/mL of BE before they produced a positive screening result by this assay. Fig. 2 shows the correlation between the BE concentration needed to give a positive screening result (i.e., >300 ng/mL cutoff) and the measured salicylate concentration in the urines. In the Hitachi analyzers high initial absorbance (>1.0 A) can be used as an indicator to flag problem specimens undergoing Emit urine screens. Although the initial absorbances measured in these urines were all in the range 0.14 to 0.90 A and were positively correlated with salicylate concentration [initial absorbance = 0.0016 × (salicylate, mg/dL) + 0.176 A;  $r = 0.94$ ,  $n = 18$ ,  $P < 0.01$ ], the absorbance rates (mA/min)

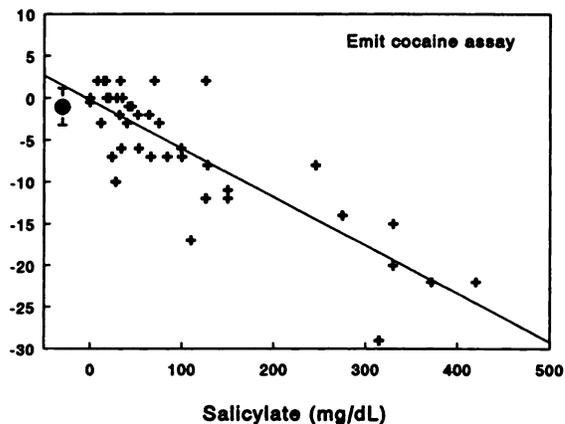


Fig. 1. Plot of salicylate concentrations vs delta absorbance rates in 40 salicylate-positive urine samples measured with the Emit d.a.u. cocaine assay.

The line represents the linear regression correlation:  $\Delta A R$  (mA/min) =  $-0.057 \times$  (salicylate, mg/dL)  $- 0.22$  mA/min;  $r = 0.85$ ,  $n = 40$ ,  $P < 0.01$ . The solid circle with error bars represents the mean  $\pm$  SD  $\Delta A R$  measured for 100 control subjects (drug-free and no salicylates), -1.05 mA/min (range +3 to -7, SD = 2.18, SE = 0.21).

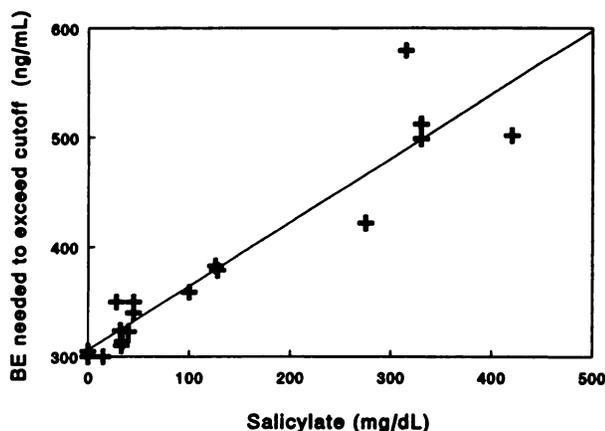


Fig. 2. Plot of correlation between measured salicylate concentration in urine and the concentration of BE needed to give a test result that meets or exceeds the 300 ng/mL BE cutoff value.

The line is the linear regression: BE needed (ng/mL) =  $0.58 \times (\text{salicylate, mg/dL}) + 306 \text{ ng/mL (BE)}$ ;  $r = 0.94$ ,  $n = 18$ ,  $P < 0.01$ . For each urine a standard curve was constructed by adding BE (see text).

were negatively correlated with salicylate concentration [ $\text{mA/min} = -0.058 \times (\text{salicylate, mg/dL}) - 0.71 \text{ mA/min}$ ;  $r = 0.89$ ,  $n = 18$ ,  $P < 0.01$ ]. All urines tested in this study measured between pH 5 and 8, the acceptable range for d.a.u. testing.

**Ingestion of aspirin and excretion experiments.** To demonstrate that a negative bias for cocaine measurement in urine resulted from ingestion of acetylsalicylic acid, six subjects (drug-free) ingested four 325-mg tablets of acetylsalicylate and collected urines at  $\sim 2$ -h intervals after ingestion. Fig. 3 shows the inverse relationship between the measured salicylate concentrations in urines and the  $\Delta A R$  measured by the Emit cocaine assay for the individual patients. Note that the magnitude of the peak urine salicylate concentrations and the time to reach the peak concentrations vary between subjects, and that in

each case the maximum salicylate concentration corresponds to the maximum negative  $\Delta A R$ . Regression of the data points from all subjects combined and randomized with respect to time gave:  $\Delta A R (\text{mA/min}) = -0.092 \times (\text{salicylate, mg/dL}) - 0.32 \text{ mA/min}$  ( $r = 0.79$ ,  $n = 37$ ,  $P < 0.01$ ). This establishes correlation parameters useful in predicting the average assay bias introduced by a given amount of salicylate in urine for this assay.

Salicylic acid (sodium salicylate) added at a final concentration of 200 mg/dL to urines from four different individuals (one without and three with BE) did not produce a bias in the recovery of cocaine. The  $\Delta A R$  values with and without added salicylate were  $-2$  and  $-3 \text{ mA/min}$  for the urine without BE and  $+68$  and  $+63$ ,  $+172$  and  $+171$ , and  $+196$  and  $+198 \text{ mA/min}$  for urines with BE, respectively. This suggests that physiological metabolism of aspirin is needed to induce the negative bias in the Emit assay.

**Effects of urine salicylates on enzyme activity.** To determine whether the bias was due to influences on the antibody component or the reaction indicator component of the Emit reagents, we exchanged reagents from two different Emit assays to eliminate the effects of antibody binding to the enzyme. The matrix of the concentrated urine was held constant and thus compared with itself but containing no salicylate. Fig. 4 shows that increasing the concentrations of measured salicylate in urine from one individual proportionately reduced the apparent enzymatic activity of the glucose-6-phosphate dehydrogenase. Under these experimental conditions a decrease in the observed enzymatic rate would be due to inhibition of the enzyme or inhibition of the apparent rate of the reaction by substances present in the urine sample. The results demonstrate that the bias is not an antibody-related phenomenon and suggest that the interference acts by reducing the signal of the indicator reaction.

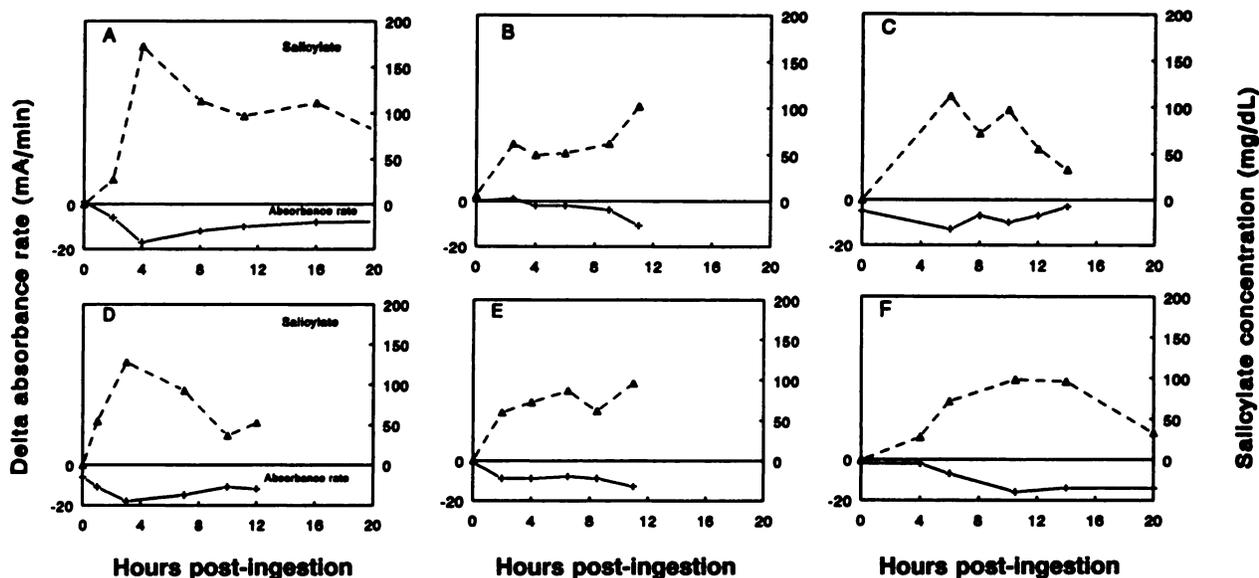


Fig. 3. Relation of salicylate concentrations (upper panels) in urine samples collected from six subjects (A-F) after ingestion of four 325-mg tablets of aspirin (at time zero) to  $\Delta A R$  (lower panels) measured for each urine sample with the Emit cocaine assay.

Subject A had one additional data point (not shown) at 28 h post-ingestion: 24 mg/dL,  $-5 \text{ mA/min}$ .

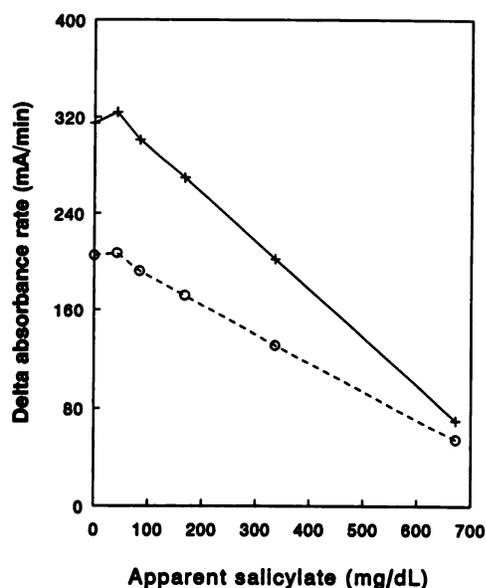


Fig. 4. Apparent inhibition of glucose-6-phosphate dehydrogenase activity in the Emit II reagent kit as a function of increased salicylate concentration.

Reagent 1 from the amphetamine and cocaine assays were exchanged to eliminate effects of drug concentration and antibody binding to the enzyme on the assay. Reagent 2 from cocaine (O) and from amphetamine (+) Emit assays were used in each dilution experiment. The delta absorbance rate at 0 mg/dL salicylate refers to the rate determined for the drug- and salicylate-free urine from the same individual, to illustrate maximal enzyme activity.

**Effects of urine salicylate on other Emit assays.** Using seven additional Emit d.a.u. assays—amphetamines, barbiturates, opiates, benzodiazapines, phencyclidine, propoxyphene, and tetrahydrocannabinol—we measured four urine specimens with salicylate contents ranging from 0 to 100 mg/dL obtained from one of the volunteers who ingested aspirin. Reduced signals (mA/min) were observed in all cases, paralleling the increased salicylate concentrations in the urine specimens (data not shown).

## Discussion

Apparently, ingestion of therapeutic doses of acetylsalicylic acid causes a reduction in the signal of the Emit d.a.u. assays with urine samples. Although direct addition of salicylic acid to urine did not produce the negative bias, ingestion of aspirin did. Our data suggest that either metabolic products of acetylsalicylic acid produce the decreased signal in urine, or ingestion of aspirin induces excretion of some other substance(s) whose presence similarly affects the Emit methodology. The hypothesis implicating the metabolism of salicylate is the simplest explanation.

Aspirin is converted to acetic acid and salicylic acid in the stomach. A major portion is also metabolized by microbial esterases in gut and by esterases in blood and liver. Salicylates are excreted in urine as various conjugated species (11, 12): free salicylic acid (10%), salicyluric acid (75%), salicylic phenolic (10%) and acyl (5%) glucuronides, and gentisic acid (<1%). The excretion of free salicylate is variable and depends on both the dose ingested and the urinary pH. In alkaline urine, >30% of

the ingested aspirin may be excreted as free salicylate, whereas in acidic urine this may be as low as 2% (for more detail see 11, 12).

The extent to which metabolic products of salicylates are measured by the Trinder method is not well defined. The scatter in the correlations plots between measured salicylates and magnitude of decreased signal for different subjects (Fig. 1) may be due to interindividual differences in metabolism of salicylates combined with the undefined specificity of the Trinder method for measuring the excreted products of salicylate metabolism. This possibility is consistent with the linear relation between enzyme inhibition and apparent salicylate concentration seen in urine from one subject (Fig. 4). In that experiment the ratio of the salicylate metabolic species remained constant throughout the dilution.

In placing our findings into perspective, three major considerations are important: the prevalence of aspirin ingestion in the general population, the amount of aspirin ingestion required to create the problem noted, and the potential number of false-negative Emit d.a.u. results caused by ingestion of salicylate.

In our hospital setting (primarily emergency clinical toxicology and obstetrics screening) the prevalence of salicylate detection in urines from toxicological work-ups is ~2%. However, the prevalence of salicylate use in a population expecting to be screened, such as in employment or pre-employment screening (i.e., persons trying to avoid detection) may potentially be considerably higher. Aspirin is widely used, easy to acquire, can be taken liberally, and is found in many over-the-counter pharmaceutical preparations (12).

This leads to the second point, the amount of aspirin ingestion required to induce the bias observed in our studies. Ingestion of four 325-mg tablets of aspirin was easily tolerated by our subjects. The magnitude of peak salicylate concentrations in urine and the time to reach the peak varied considerably between subjects ingesting aspirin (Fig. 3). According to medical texts (13), daily doses of up to 3 to 7.5 g of aspirin per day (about 10 to 25 325-mg tablets) are prescribed for patients being treated for rheumatoid arthritis. Salicylate concentrations would be considerably higher in urine than in serum depending on (e.g.) the time of urine collection and the state of hydration of subject. Thus, concentrations of salicylate metabolites in urine greater than those tested in this study can be easily achieved.

The third consideration is the extent to which the observed bias in the absorbance rate creates problems in screening. This depends largely on three variables: the reproducibility of assay response for subjects not taking cocaine; the distribution of  $\Delta$ mA/min values for the population of individuals screened who are using cocaine; and the apparent salicylate concentrations achievable in urine. The mean  $\Delta$ AR in our Emit cocaine d.a.u. assay was  $-1.05$  mA/min (range  $+3$  to  $-7$ , SD = 2.18, SE = 0.21) for 100 subjects (drug-free, including no salicylates) who presented to our Emergency Department requiring drug screens (Fig. 1). Thus, in our hands, the  $\Delta$ mA/min variability in urines from these

subjects is well within the limit of  $\pm 5$  mA/min variation as a routine quality-control criterion. Fig. 1 shows that apparent salicylate values as low as 50 mg/dL may shift the baseline to  $< -5$  mA/min. Thus, the probability of obtaining a false-negative result will depend on the actual concentrations of BE and of apparent salicylates in the urine being tested.

Figure 5 (top panel) shows the broad distribution of the signal in the Emit assay for positive cocaine results obtained in our hospital from a series of 186 subjects who tested positive for cocaine (confirmed by gas chromatography/mass spectrometry). The positive cutoff (BE 300 ng/mL) measured an average  $\Delta A R$  of 52 mA/min. The net effect of salicylate ingestion on the measured absorbance rate would be to shift this distribution towards the left (lower mA/min). The magnitude of the shift can be estimated using the correlation parameters detailed above. The bottom panel of Fig. 5 shows the projected frequency of expected false-negative cocaine results as a function of measured salicylate concentrations in urine. This model is a first-order approximation and assumes a linear response in the negative bias over the range of concentration indicated. Whether or not

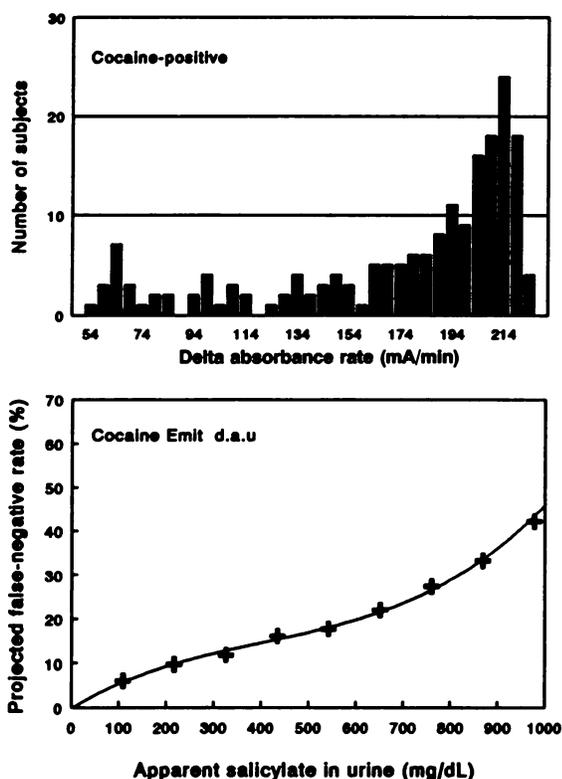


Fig. 5. (Top) Actual distribution of Emit signals ( $\Delta A R$ ) from 186 subjects testing positive for cocaine (the positive BE cutoff, 300 ng/mL, measured  $+52$  mA/min); (bottom) projected frequency of false-negative cocaine results calculated by combining the above actual frequency distribution with the salicylate linear regression parameters from the volunteers ingesting aspirin:  $\Delta A R$  (mA/min) =  $-0.092 \times$  (salicylate, mg/dL)  $- 0.32$  mA/min ( $r = 0.79$ ,  $n = 37$ ,  $P < 0.01$ ).

this is exactly the case, each individual laboratory should determine the impact, if any, of this salicylate-induced phenomenon on their own drug screening protocol.

From Fig. 4 and our preliminary experiments with other Emit d.a.u. assays, the salicylate-related negative interference (negative bias) is not specific to the assay for cocaine. The negative bias in the Emit d.a.u. and Emit II assays appears to be a phenomenon not related directly to the analyte being measured but rather to the reagent system. Inhibition of apparent enzyme activity is a probable explanation. Studies to identify the substance(s) in urine and the mechanism causing this phenomenon are under way. Meanwhile, it may be prudent to test urines for the presence of salicylates (e.g., spot test) before screening them by Emit methods. Then, either urines positive for salicylates at predetermined concentrations can be analyzed for drugs of abuse by an alternative methodology or at least the potential for this negative bias can be taken into consideration. Our preliminary data suggest that the negative bias is a general phenomenon in other Emit d.a.u. immunoassay analyses.

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