CLIN. CHEM. 29/6, 1012–1014 (1983)

Measurement of True Salicylate Concentrations in Serum from Patients with Reye's Syndrome

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Patients with Reye's syndrome who have been given aspirin are said to maintain higher-than-anticipated salicylate concentrations in blood, for longer than expected. We explored whether this could be attributed to spurious results from nonsalicylate compounds in the Trinder reaction for salicylates. All of 63 organic acids and amines examined that form colored complexes with Trinder's reagent had detectable absorbance at 540 nm at 0.2 g/L, including some endogenous compounds known to be increased in Reye's syndrome patients and many others endogenous in humans. By subjecting deproteinized sera to thin-layer chromatography and eluting the salicylate fraction before complexing it with ferric ion, true salicylate can be measured quantitatively and differentiated from interfering compounds. In addition, when we examined the effect of salicylate on palmitate binding to serum proteins, we found that salicylate concentrations of 0.2 g/L displaced [16-^{14}C]palmitate binding to protein more in Reye's syndrome patients than in Reye's syndrome survivors or children with influenza. This suggests the presence of atypical binding characteristics for salicylate and palmitate in the acute disorder but not in survivors or children with influenza.

Additional Keyphrases: *analytical error · atypical binding to protein*

As the major drug of choice for antipyresis, acetylsalicylate is commonly used during the prodromal illnesses in Reye's syndrome.

Despite clinical observations in Reye's syndrome of salicylate concentrations that are inappropriately high for the doses given and that persist beyond the expected clearance time, a primary role for aspirin in the syndrome has not been seriously entertained, for several reasons. These include (*a*) there is no history of exposure or detectable salicylate in more than one-third of the cases (*1*); (*b*) there are differences in hepatic pathology in aspirin toxicity from that in the syndrome: focal necrosis, cellular "unrest," ballooning, and eosinophilic degeneration without prominent fatty change (*2*); and (*c*) salicylate toxicity can be reversed by increasing fluid and alkalinizing the urine or by using peritoneal dialysis (*3*), in contrast to Reye's syndrome, where fluid restriction appears to be critical and peritoneal dialysis is apparently contraindicated (*4*).

Recently, considerable interest regarding a possible role for aspirin has re-emerged because of retrospective epidemiological data that seem to demonstrate an association between aspirin ingestion during the prodromal illnesses and the occurrence of Reye's syndrome in several different parts of this country, as compared with children simultaneously ill with a flu-like illness, who did not develop this complication (*5–7*). In addition, Partin et al. (*8*) report higher mean concentrations of salicylate at the time of hospital admission in patients who died of Reye's syndrome or had serious neurological complications than in patients who survived without neurological sequelae. Evaluation of the impact of the latter study requires that the test used for measuring salicylate be highly specific. Thus, we designed this study to determine the specificity of the Trinder method (*9*). Also, salicylates being known to bind to serum proteins, we also studied the effect of salicylate on the binding of a fatty acid, palmitate, in Reye's syndrome.

Materials and Methods

The test widely used for salicylates depends on formation of a ferric ion–salicylate complex, which absorbs light at 500–550 nm (*9*). Ferric ion, however, is known to complex with many other compounds, particularly phenols and aliphatic enols, and some of these complexes absorb light at this end of the visible range (*10*).

We screened 131 organic acids, amines, and other compounds for development of visible color when treated with an acidic 100 g/L solution of $FeCl_3$. About half of them gave colors ranging from deep yellow to purple. Of such compounds, we prepared 0.2 g/L aqueous solutions, treated them with Trinder's reagent to determine their equivalent "salicylate" content, and measured their absorbance at 540 nm with a spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074).

For thin-layer chromatographic separation of sera we used cellulose pre-coated plastic sheets (Brinkmann Instruments, Inc., Westbury, NY 11590; Polygram Cel 300 PE 1, cellulose MN 300 polyethyleneimine impregnated).

Binding studies. Specimens of serum from patients, survivors of Reye's syndrome, and control subjects were extensively dialyzed with a 1000-fold volume of Krebs–Ringer buffer (*11*) at 4 °C, two times. Samples containing 100 μg of protein were diluted to identical volumes and exposed to 1 and 0.5 mmol of [16-^{14}C]palmitate (0.03 μCi), with and without salicylate (1.5 mmol/L). After the samples had stood for 3 h (at 4 °C, to minimize proteolysis) they were washed over a 0.45-μm (av pore size) filter (Millipore Corp., Bedford, MA 01730) with Krebs–Ringer buffer. The radioactivity of the retentate on the filter was measured with a scintillation counter. After determining the recovery of radioactivity in samples with and without salicylate at the two palmitate concentrations, we calculated the percentage of change that resulted from the exposure to salicylate.

Results

Many compounds complex with ferric ion and absorb visible light. We converted absorbances at 540 nm to concentrations of "salicylate" by comparison with a standard curve for salicylate (Table 1). The "percentage of comparability" with salicylate of the compounds under consideration was calculated such that (e.g.) 50% comparability indicates the compound at 0.2 g/L would give half as much absorbance as 0.2 g of salicylate per liter.

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Received Jan. 10, 1983; accepted Mar. 11, 1983.

Table 1. Some Compounds That Complex with Ferric Ion and Absorb at 540 nm

Compound[a]	Absorbance	Apparent "salicylate"	% comparability to salicylate
Tryptophan metabolites			
5-OH-tryptophan	0.116	0.070	35.0
5-OH-tryptamine (serotonin)	0.040	0.023	11.5
Serotonin–creatinine complex	0.107	0.064	32.0
3-OH-kynurenine	0.090	0.054	27.0
Kynurenic acid	0.002	0.001	0.5
Anthranilic acid	0.010	0.005	2.5
3-OH-anthranilic acid	0.170	0.103	51.5
Xanthurenic acid	0.029	0.017	8.5
Indole	0.049	0.028	14.0
3-Indolepyruvic acid	0.028	0.170	85.0
3-Indolelactic acid	0.225	0.130	68.0
3-Methylindole (skatole)	0.314	0.190	95.5
Indican	0.015	0.008	4.1
Melatonin	0.023	0.013	6.5
Phenylalanine and tyrosine metabolites			
3-OH-tyramine (dopamine)	0.019	0.010	5.0
DOPA	0.059	0.035	17.5
Epinephrine	0.048	0.028	14.0
3,4-Dihydroxy-mandelic acid	0.208	0.128	63.0
Homovanillic acid	0.056	0.035	17.5
Protocatechuic acid (3,4-dihydroxy-benzoic acid)	0.059	0.035	17.5
3,4-Dihydroxy-cinnamic acid	0.012	0.005	4.0
3,4-Dihydroxy-phenylacetic acid	0.206	0.125	62.5
Homogentisic acid	0.025	0.013	6.5
Others			
Isocitric acid	0.019	0.010	5.0
α-Ketoisocaproic acid	0.062	0.036	18.0
Acetoacetic acid	0.019	0.010	5.0
Hypotaurine	0.022	0.012	6.0
Pyridoxal-5-phosphate	0.013	0.007	3.5
Tannic acid	0.064	0.038	19.0
Gallic acid (trihydroxybenzoic acid)	0.102	0.061	30.5
ε-Amino-*n*-caproic acid	0.026	0.014	7.0

[a] At 0.2 g/L

β-Hydroxybutyrate, palmitic acid, the cyclic nucleotides, and uric acid, any of which may be increased in the sera of patients with Reye's syndrome, do not form complexes with ferric ion that absorb light at 540 nm.

Compounds with less than 2% comparability to salicylates include kynurenine, tyramine, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpropionic acid, *o*-hydroxyphenylacetic acid, phenylacetic acid, 4(5)-amino-5(4)-imidazole carboxamide, 5-amino-4-imidazole carboxamide riboside, imidazole, imidazole lactic acid, formiminoglutamic acid, lactic acid, α-ketoisovaleric acid, α-ketoglutaric acid, propionic acid, ethyl acetoacetate, acetyl phosphate, L-alanine, pyridoxine, pyridoxal, pyridoxamine, riboflavin 5′-acetylphosphate, pantothenic acid, NAD^+, $NADP^+$, flavin-adenine mononucleotide, FAD^+, coenzyme A, octanoyl coenzyme A, hexanoyl coenzyme A, D-ribose-5-phosphate, and vanillin.

Analytical recovery of salicylate added to serum was also examined. The salicylate concentration in 500 μL of serum from Reye's patients and control subjects, and from normal rabbits, was determined by the Trinder method before and after adding 1.7 μmol of salicylate. Recovery ranged between 86 and 105% (Table 2).

We determined the percentage of "true" salicylate in sera from Reye's patients and controls by separating salicylate from possible interfering compounds in deproteinized sera by thin-layer chromatography (Polygram Cel.300 PE 1) in ethanol/ammonia/water (180/10/10, by vol) before elution with water of the salicylate spot, which we identified by $FeCl_3$ staining of a co-chromatographed standard of salicylate. Salicylate concentrations in sera were compared as calculated before and after chromatography (Table 3). The

Table 2. Recovery of Salicylate Added to Sera

Serum sample[a]	Recovery, %[b]
Reye's patient	93.0
Reye's patient	104.6
Reye's patient	90.2
Patient with flu	93.5
Adult on aspirin	85.9
	Mean 93.4 (SEM 3.1)
Rabbit serum (n = 5)	Mean 100.6 (SEM 1.2)

[a] 500 μL of serum to which was added 1.7 μmol of salicylate.
[b] Salicylate measured by the Trinder method.

Table 3. Salicylate Concentrations before and after Thin-Layer Chromatography of Samples

Salicylate, g/L[a]		
Before TLC (A)	After TLC (B)	(B/A) × 100[b]
Reye's syndrome serum		
0.0065	0.0032	49.2
0.0560	0.0128	22.9
0.0189	0.0010	5.3
Influenza serum		
0.0093	0.0010	10.8
Adult on aspirin		
0.0840	0.0360	42.9
Standard, 50 mg/L		
0.0500	0.0500	100.0

[a] By the Trinder method.
[b] Percent of initially observed value (A) actually due to "true" salicylate.

Table 4. Percent Change in [16-^{14}C]Palmitate Binding to Human Serum Proteins[a] Due to 1–0.5 mmol/L Concentrations of Salicylate

	% change for salicylate concn, mmol/L, of	
	1	0.5
Children with flu (n = 3)	↑ 14.6	↑ 14.9
Reye's survivors (n = 2)	↑ 14.6	↑ 6.6
Reye's syndrome patients (n = 4)	↓ 2.0	↓ 8.4

[a] Serum first extensively dialyzed against Krebs–Ringer buffer.

measured salicylate concentration was much lower in every blood sample after the procedure, whereas a standard of salicylate, treated identically, was entirely recoverable by this technique.

To determine whether salicylates affect palmitate binding in Reye's syndrome, we incubated previously dialyzed sera from patients with labeled palmitate and nonlabeled salicylate. Three children with a flu-like illness and two survivors of Reye's syndrome showed increased palmitate binding to serum proteins (14.6% each) in the presence of 1 mmol of salicylate per liter. Acutely affected Reye's patients showed the opposite trend (2.0% decrease). Thus, the effects of millimolar concentrations of salicylate on palmitate binding are dissimilar between Reye's patients and unaffected children. In Reye's syndrome palmitate is displaced; in unaffected subjects the binding is enhanced.

Discussion

The test commonly used to measure concentrations of salicylate is nonspecific. A color reaction is given with a wide range of compounds found in body fluids, several of which are known to be abnormally increased in Reye's syndrome. Thus, many nonsalicylate compounds could potentially contribute to errors in salicylate measurements of body fluids. Because the additive contributions of these several components could be substantial, interfering compounds should be removed before salicylate is measured in the sample. As indicated here, thin-layer chromatography of deproteinized serum is an effective and inexpensive means of doing so.

Between 50 and 80% of the salicylate in serum is bound to albumin (*3*). It is the unbound salicylate that traverses the plasma membrane of cells and is the major determinant of clinical efficacy and toxicity (*12*). Fatty acids are also known to bind to serum albumin (*13*), the long-chain fatty acids binding to one type of site on the albumin molecule with high affinity (*14*). The site for medium-chain fatty acids is coincident with the lysine 199 residue of fragment C of human serum albumin (*15*)—the same residue that is acetylated by acetylsalicylate (*16*). Because both long- and medium-chain fatty acids are known to be present in increased quantities in sera of patients with Reye's syndrome, implications for possible modulation of the binding of salicylate or fatty acids, or both, are apparent. Salicylate at 0.2 g/L concentration in Reye's syndrome serum displaces palmitate from protein binding, a pattern not seen in survivors and sick controls. Thus, in Reye's syndrome the presence of salicylate may tend to interfere with the binding of fatty acids to albumin and perhaps augment their toxicity.

This study was supported in part by the following grants: NIH HD 11657, NS 16383, USPHS GR AM 16506-10, and USPHS GR AM 07405. We are grateful to Dr. John F. Griffith for his support and Ms. A. Harper for manuscript preparation.

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