Studies on the specificity of the colorimetric assay for sulfamethoxazole

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Summary. Plasma and urine have been analyzed for sulfamethoxazole (SMZ) by a colorimetric and by a TLC method. In special cases we found much higher values with colorimetry than with TLC.

The determination of drug concentrations in biological specimens should be carried out by methods that are specific for the unchanged drug. Unspecific assays, which do still exist, may lead to erroneous results. For instance, with the Bratton-Marshall method[1] for the colorimetric determination of sulfonamides[2], the unchanged drug as well as metabolites with an aromatic amino group are determined together[3]. This sum of colorimetrically determined compounds was defined as 'active sulfonamide' by Rieder[2].

In the urine of subjects with renal disease we found inexplicably high concentrations of the sulfonamide sulfamethoxazole (SMZ) (fig. 1) by the colorimetric method. Therefore, we studied to what an extent interference from metabolites can be measured by this assay.

For this, plasma and urine of subjects with normal and with insufficient renal functions were analyzed by a colorimetric method[2] and by a specific TLC method[4] for their content of SMZ. The results were compared.

Results. 1. Determination of SMZ in plasma. No differences between the 2 methods were found in plasma samples from subjects with normal and with insufficient renal functions (table). This means that the colorimetric assay is sufficiently specific for plasma analysis.

2. Determination of SMZ in urine. When analyzing urine samples of healthy volunteers, to whom a single oral dose of SMZ had been administered, the TLC results were slightly lower than those obtained by colorimetry (table). After spraying the plate with Bratton-Marshall reagent[4], small amounts of a Bratton-Marshall positive metabolite

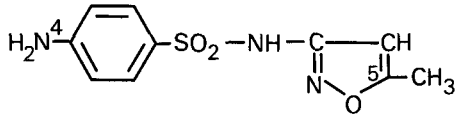

Figure 1. Sulfamethoxazole (SMZ).

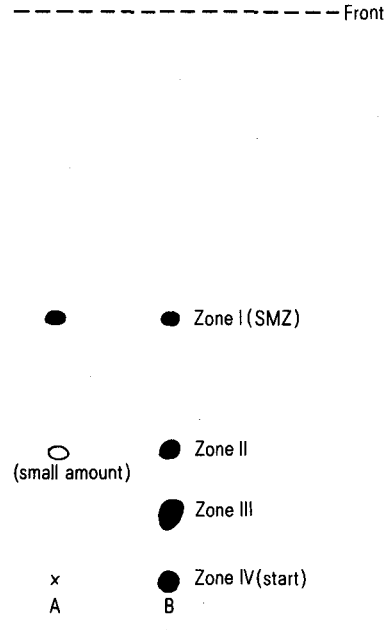

Figure 2. TLC determination of sulfamethoxazole (SMZ) in urine. Eluent: toluene/isopropanol 8:2. Detection after Bratton-Marshall reaction on the plate. (Experimental details in Rieder[3].) *a* Urine of a healthy volunteer; *b* urine of a renal disease subject.

Determination of sulfamethoxazole (SMZ) in plasma and urine. Comparison of results obtained by TLC with those obtained by colorimetry

	Healthy volunteers			Renal disease subjects		
	Sample	TLC µg/ml	Colorimetry µg/ml	Sample	TLC µg/ml	Colorimetry µg/ml
Plasma samples	1	37.7	37.8	9	34.3	34.5
	2	46.1	45.9	10	54.8	56.8
	3	40.3	41.3	11	77.3	76.7
	4	40.3	38.6	12	33.5	33.5
	5	34.3	32.2	13	91.7	92.0
	6	30.4	27.9	14	81.1	82.3
	7	23.0	23.5			
	8	9.7	9.9			
Urine samples	1	23	18	11	37	40
	2	150	122	12	183	2610
	3	436	509	13	137	2740
	4	484	579	14	206	5170
	5	495	533	15	23	4110
	6	370	423	16	18	35
	7	420	524			
	8	211	202			
	9	168	173			
	10	65	91			

could be found in this case (fig. 2). This compound interferes with the unchanged drug when the colorimetric assay is used. But its quantity is small and should be negligible in most cases.

Very different results between the 2 methods were obtained when analyzing urines of subjects with insufficient renal function, to whom multiple oral doses of SMZ had been administered. Here, the results found by the colorimetric assay have in part been much higher than those found by TLC (table).

TLC showed that these urine samples contained a very high quantity of Bratton-Marshall positive metabolites (zones II, III and IV in fig. 2). By TLC the separation of the unchanged drug from these metabolites is possible, but in the colorimetric assay they interfere strongly. Identification of the compounds of zone II was tried by mass-spectrometry. The main compound in this zone could be identified as sulfanilamide, which is reported to be a urinary metabolite[3]. Furthermore, mass-spectrometry gave a hint of the presence of the metabolites 5-hydroxymethyl-sulfamethoxazole[5] and N4-acetyl-5-hydroxymethyl-sulfamethoxazole[3]. The compounds of zones III and IV have not yet been identified.

Our results show that the colorimetric assay for SMZ in urine may lead in special cases to too high a level of sulfonamide being recorded. Therefore a specific chromatographic method is preferable for the determination of sulfonamide concentrations in urine.

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The serum levels of unbound bilirubin that induce changes in some brain mitochondrial reactions in newborn guinea-pigs

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Summary. Bilirubin in different concentrations was injected in newborn guinea-pigs and the following parameters were determined: serum total and unbound bilirubin, whole brain bilirubin content and oxygen consumption, NADH-cytochrome c reductase and ATPase activities in brain mitochondria. The results showed a significant correlation between decreased rates of brain metabolism and the elevation of serum total and unbound bilirubin.

It has long been known that bilirubin can produce kernicterus by interfering with brain metabolism[3]. Electron microscopy has shown that mitochondrial alterations occur early during the development of bilirubin encephalopathy[4,5], although it has not been clearly demonstrated which specific metabolic steps are directly responsible for the chemical pathology of the disease. The specific enzymes or enzyme systems affected by bilirubin have been extensively reviewed[6].

On the clinical side, a point that has puzzled pediatricians is the choice of the blood parameter to follow in handling infants at risk[7]. Up to now, one has had to rely upon either the serum binding capacity or the unbound bilirubin values as determined mainly by the peroxidase method. The indirect bilirubin value alone is viewed as having the most serious drawbacks of all the parameters available.

On the other hand, these parameters could be useful if it were possible to establish a secure relationship between their serum values and early signs of brain damage. In looking for such a correlation, we used an experimental model in which guinea-pigs were made hyperbilirubinemic by i.v. injections of bilirubin solutions. The serum values of unbound bilirubin were compared to the amount of bilirubin entering the brain tissue, as well as to the activities of some mitochondrial systems.

Material and methods. Experimental hyperbilirubinemia. All experiments were carried out on spontaneously (at term) delivered newborn guinea-pigs, weighing 80 (SD ± 16) g, at birth. A PE-10 polyethylene catheter attached to a continuous dropping system was inserted in the superficial vein along the antero-lateral aspect of an upper foreleg for infusion of bilirubin and isotonic saline solutions. The animals were anesthetized with Nembutal® (Abbott Laboratories), 40 mg/kg b.wt i.p. The desired amount of crystalline bilirubin, $\varepsilon_{452\,nm}^{CHCl_3} = 60{,}100$ (Merck), was dissolved in 1 ml of 0.1 N NaOH in the dark, rapidly mixed with 1 ml of adult guinea-pig serum, and the pH adjusted to 8.0 with 1 N acetic acid as described elsewhere[8]. Samples of about 1 ml of blood were drawn from the jugular vein opposite to the site of infusion, after an infusion time of approximately 30 min.

Spectrophotometric determinations of bilirubin in whole brain. At the end of the experiment the animals were decapitated and the whole brain was removed. The brain

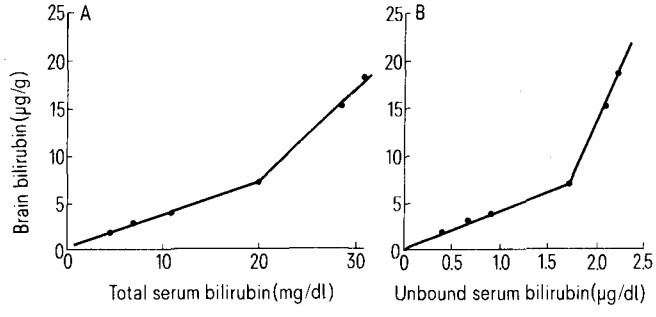

Figure 1 A and B. Relationship of serum bilirubin and brain bilirubin content in hyperbilirubinemic newborn guinea-pigs. *A* Total serum bilirubin vs brain bilirubin; lower slope line: $y = 0.160 + 0.358\ x$; higher slope line: $y = -12.5 + 0.98\ x$. *B* Unbound bilirubin vs brain bilirubin; lower slope line: $y = 1.144 + 4.19\ x$; higher slope line: $y = -30.7 + 22.4\ x$.