Validity of Oral Bioavailability Estimates of Phenolsulfonphthalein Based on Total Urinary Excretion from Rats

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Received April 11, 1977, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Amherst, NY 14260.* Accepted for publication May 5, 1977.

Abstract □ Urinary recovery of phenolsulfonphthalein from rats was determined after intracardial (0.075 mg) and oral (1.5 mg) doses. Although trace quantities of conjugated metabolites could be identified by TLC, the levels present did not introduce significant error into estimates of total phenolsulfonphthalein excretion if samples were assayed directly by colorimetric methods for only unchanged dye. The absolute availability of phenolsulfonphthalein based on urinary recovery under the present experimental conditions was estimated at 10.6%.

Keyphrases □ Phenolsulfonphthalein—urinary excretion from rats, validity of oral bioavailability estimates □ Excretion, urinary—phenolsulfonphthalein in rats, validity of oral bioavailability estimates □ Bioavailability, oral—phenolsulfonphthalein in rats, based on urinary excretion data □ Dyes—phenolsulfonphthalein, urinary excretion from rats, validity of oral bioavailability estimates

Phenolsulfonphthalein (I), a strong acid, is completely ionized at a pH above 1. Because of its ionic nature and poor lipid solubility, the absorption of this dye from the rat small intestine is relatively poor (1). Mechanistically, I is absorbed from the rat small intestine in part by an active transport process of low capacity (2, 3); its percentage of absorption from the perfused rat intestine was found to decrease as the dye concentration was increased (4).

Because I can be easily detected in urine by simple colorimetric methods (5, 6) and its absorption characteristics are well known, the dye is often used as a marker to monitor absorption changes in the small intestine. The effects of surfactants (7, 8), vehicle viscosity, and an anticholinergic agent (9) on absorption were studied using I. Conclusions regarding absorption differences were based on the total urinary excretion and/or the excretion rate of I as determined by direct colorimetry.

Recently, Conway *et al.* (10) reported the identification of I sulfate and glucuronide conjugates in the urine and bile of rats after 15-mg/kg iv administration. No estimate of the amounts of metabolites excreted was given. Since conjugation of the phenolic moiety suppresses the acid–base color change that is the basis of the colorimetric assay used in all absorption studies, urinary excretion of I in rats may be underestimated by not accounting for both free and conjugated materials.

The purpose of this study was to investigate the potential for error in analyzing only unconjugated I as an estimate of total oral absorption. The absolute oral bioavailability of I in the rat based on total urinary excretion also was determined.

Table I—Percent of Administered I Recovered in the Urine of Rats (0–24 hr) before and after Hydrolysis Treatment

	Unhydrolyzed		Hydrolyzed	
Rat	Oral	Intracardiac	Oral	Intracardiac
1	8.8	66.6	8.9	58.6
2	4.3	64.5	3.8	72.6
3	6.1	50.4	7.0	68.0
4	7.7	81.2	6.3	51.9
5	7.3	78.5	5.4	61.1
6	5.5	43.1	6.0	54.1
Mean (*SD*)	6.6 (1.6)	64.1 (15.1)	6.2 (1.7)	61.1 (8.0)
Paired *t* test	n.s. ($p > 0.80$)			
			n.s. ($p > 0.75$)	

EXPERIMENTAL

Materials—All chemicals were reagent grade. Solutions were prepared with distilled water. Compound I was obtained commercially as the sodium salt[1]. Silica gel plates[2] were used for TLC analysis.

Animal Experiments—Male Sprague–Dawley rats[3], 290–350 g, were fasted overnight (16–18 hr) with free access to water. Compound I was administered either intracardially (0.5 ml of 0.15 mg/ml equivalent free acid) or by gastric intubation (1 ml of 1.5 mg/ml equivalent free acid) to each rat in a randomized crossover design.

A 1-week recovery period was allowed between administrations. Animals were placed in metabolism cages with free access to water only. Urine was collected from 0 to 24 hr and centrifuged to remove stray hair contamination, and the volume was recorded.

Analysis of Unconjugated I in Urine—Aliquots of 2.0 ml of urine were pipetted into each of two test tubes; 3.0 ml of 0.1 *N* HCl was added to one tube, and 3.0 ml of 1 *N* NaOH was added to the second. Both tubes were centrifuged, and the supernates were decanted into 1-cm glass spectrophotometer cells. The acid solution served as the reference material.

The absorbance of the basic solution *versus* the reference was recorded on a double-beam spectrophotometer[4] from 530 to 650 nm. The absorbance value at the maximum of 560 nm was noted, and the concentration of I in the sample was calculated. Standard solutions were prepared by adding 1.0 ml of a known I solution (2.4–20 μg/ml) to 2.0 ml of blank urine and 2.0 ml of 1 *N* NaOH or 0.1 *N* HCl.

Analysis of Free and Conjugated I in Urine—Aliquots of 5.0 ml of urine were pipetted into test tubes with polytef-lined screw caps followed by the addition of 0.5 ml of concentrated hydrochloric acid. Loosely capped tubes were heated in an oil bath at 96–97° for 90 min, removed, and allowed to cool to room temperature. After centrifugation, 2.0 ml of urine was transferred to each of two 10-ml volumetric flasks. Samples were neutralized by dropwise addition of 10 *N* NaOH. Solutions were brought to volume with either 0.1 *N* HCl or 1 *N* NaOH. After centrifugation, absorbance values were recorded as described.

Standards were prepared by adding 1.0 ml of a known I solution (20–80 μg/ml) to 4.0 ml of blank urine and 0.5 ml of concentrated hydrochloric acid and were treated in an identical manner.

Qualitative TLC Analysis—Aliquots of 50 μl of sample were spotted on silica gel plates. Elution was carried out in 1-butanol–acetic acid–water (40:12:34) as described by Hart and Schanker (11). Compound I was visualized by exposing the plates to ammonia vapor.

RESULTS

Table I shows the percent of administered I recovered in the 0–24-hr urine samples of rats after oral or intracardiac administration and the

[1] Lot 323607, J. T. Baker, Phillipsburg, N.J.
[2] No. 5762-9H, E. M. Laboratories; supplied through VWR Scientific, Buffalo, N.Y.
[3] Blue Spruce Farms, Altamont, N.Y.
[4] Hitachi Perkin-Elmer model 124.

Table II—Percent Oral Availability of I in Rats Based on Total Urinary Excretion

Rat	Unhydrolyzed	Hydrolyzed
1	13.2	15.2
2	6.7	5.3
3	12.1	10.3
4	9.5	12.1
5	9.3	8.8
6	12.8	11.8
Mean for pooled data	10.6%	
95% confidence interval	5.5–18.1%	

effect of hydrolysis treatment. Qualitative TLC of unhydrolyzed urine revealed two spots with R_f values similar to those reported previously (12) for free and conjugated I, but the latter spot was present only in trace quantities. Only one spot corresponding to unconjugated I was detected in hydrolyzed urine.

Hart and Schanker (11) previously demonstrated the effectiveness of the hydrolytic procedure by comparison with results after glucuronidase treatment. Preliminary studies also indicated no loss of I as a result of the deconjugation process. No drug was found in the 24–48-hr urine samples, in agreement with findings by Feldman *et al.* (7) at the same oral dosage level.

Paired t test comparison of the unhydrolyzed *versus* hydrolyzed samples showed no significant difference for oral ($p > 0.80$) or intracardiac ($p > 0.75$) administrations. It can be concluded that no substantial error is introduced in measuring only free I in the urine of rats after oral or intracardiac administration at doses used in this study. The absolute oral availability, A, of I based on total urinary excretion was calculated as:

$$A = \frac{\%\text{ of oral dose recovered}}{\%\text{ of intracardiac dose recovered}} \times 100 \qquad \text{(Eq. 1)}$$

The results are shown in Table II. Data from unhydrolyzed and hydrolyzed samples were also pooled since the two treatments were not different from each other. The 95% confidence interval for the ratio of two normally distributed variables was calculated using Fieller's theorem as outlined by Colquhoun (13).

DISCUSSION

Compound I is a sulfonic acid dye with pKa's of 1 (sulfonic acid) and 7.9 (phenolic) and is completely ionized at gastric and intestinal pH's. A characteristic yellow (acid) to red (base) color change makes it widely used as an acid–base indicator. The development of simple assays in biological fluids based on this color change has made I an attractive compound to study in animal species (7, 8, 10–12) as well as in humans (6, 9). About 3–5% of orally administered solutions are recovered as unchanged drug in the 0–24-hr urine of rats (7, 8).

Conway *et al.* (10) identified conjugated metabolites of I in the urine and bile of rats after intravenous administration of a dose 3.3 times higher than the dose used in absorption studies. The fraction of the dose excreted as metabolites was not given. If metabolites are present in sufficient amounts, it is possible that the recovery of I reported previously may be an underestimate of total urinary excretion. Results reported here show this not to be the case after either oral (1.5 mg) or intracardiac (0.075 mg) administrations; there is no significant difference in the recovery of I in urine before and after hydrolysis.

The absolute oral availability of I based on total urinary excretion was estimated to be 10.6% (95% confidence interval of 5.5–18.1%). This information has not been reported previously.

An average of only 62.6% of an intracardiac dose was recovered in the urine. Hart and Schanker (11) reported the active secretion of I in the bile of rats. This interpretation is complicated by the fact that urinary excretion was prevented by ligation of the renal pedicles. Feldman *et al.* (7) recovered about 42% of an intraperitoneal dose of I as unchanged drug in rat urine. If biliary excretion is an important pathway for the elimination of I in rats, the potential for saturation of this pathway must be considered in interpreting urinary excretion data at different dose levels. An example of this phenomenon was reported by Axelson and Gibaldi (14), who showed that the biliary excretion of riboflavin in rats increases disproportionately with increasing body levels, thus affecting the total urinary recovery of the vitamin.

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ACKNOWLEDGMENTS

Supported in part by Grant 5S07RR05454-14.

S. T. Horhota is a Fellow of the American Foundation for Pharmaceutical Education.

The authors acknowledge John Lettieri for technical assistance.