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ACKNOWLEDGMENTS

Presented at the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Chicago meeting, August 1974.

COMMUNICATIONS

Errors in Chlorothiazide Bioavailability Estimates Based on a Bratton–Marshall Colorimetric Method for Chlorothiazide in Urine

Keyphrases □ Chlorothiazide—bioavailability in dogs, colorimetric and high-pressure liquid chromatographic analyses compared □ Bioavailability—chlorothiazide in dogs, colorimetric and high-pressure liquid chromatographic analyses compared □ Colorimetry—analysis, chlorothiazide in urine, bioavailability estimates compared to high-pressure liquid chromatographic analysis in dogs □ High-pressure liquid chromatography—analysis, chlorothiazide in urine, bioavailability estimates compared to colorimetric analysis in dogs □ Diuretics—chlorothiazide, bioavailability in dogs, colorimetric and high-pressure liquid chromatographic analyses compared

To the Editor:

Chlorothiazide (6-chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) is a weakly acidic (pKa_1 = 6.7 and pKa_2 = 9.5) and poorly absorbed (1, 2) diuretic used in the treatment of hypertension, congestive heart failure, and other edematous conditions in humans and dogs. The pharmacokinetics of chlorothiazide in both species are not well characterized, and the potential use of the dog as an animal model in comparative bioavailability studies on various commercial chlorothiazide tablet and aqueous suspension products remains unexplored.

Based on comparisons of urinary excretion data obtained with a high-pressure liquid chromatographic (HPLC) method (3, 4) and several Bratton–Marshall-based colorimetric methods (2, 5, 6) for chlorothiazide in urine, the Food and Drug Administration tentatively concluded that the appreciable intersubject variation in chlorothiazide tablet bioavailability observed in human studies employing a colorimetric method of analysis was due, in part, to variable assay interference by urine constituents (3, 4). During oral and intravenous studies on the effect of dose on the pharmacokinetics of chlorothiazide absorption and disposition[1], we assessed whether chlorothiazide bioavailability estimates in male mongrel dogs were also a function of the assay used to determine unchanged chlorothiazide in urine. This communication reports the results of these determinations.

In two complete crossover studies, four fasting male mongrel dogs received 125–750 mg po[2] and 250 mg iv[3] of chlorothiazide. Urine samples were collected at predetermined intervals for 48–72 hr and assayed by both a slight modification[1,4] of a specific HPLC method (7) and a modified Bratton–Marshall method purported to reduce assay interference by urine constituents (2).

The original colorimetric method (2) was slightly modified to allow for quantitative recovery and to eliminate turbidity in samples subjected to the Bratton–Marshall reaction for color development. Chlorothiazide was hydrolyzed quantitatively to 6-amino-4-chlorobenzene-1,3-disulfonamide by heating a mixture of 1.0 ml of 3.75 *N* NaOH and 5.0 ml of diluted urine (1:25 or 1:50), containing 6.25–37.5 μg of chlorothiazide, at 100° for 1 hr. The solution was cooled to room temperature and extracted three times with 4.0 ml of ethyl acetate.

The combined ethyl acetate extracts were evaporated to dryness at 60–70° under a gentle stream of nitrogen, and the residue was reconstituted with 0.5 ml of 0.5 *N* NaOH and 5.5 ml of distilled water. Then the solution was filtered[5], and a 5.0-ml aliquot of the filtrate was acidified with 1.0 ml of 6 *N* HCl and mixed with 0.5 ml of 0.1% sodium nitrite. After 3 min, 0.5 ml of 0.5% ammonium sulfamate was added, and the mixture was vortexed[6]. Five minutes later, 0.5 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride was added, and the reaction mixture was stored in the dark for 10 min to allow for maximum color development.

The absorbance of the colored solution was measured[7] (within 2 hr of color development) at 518 nm. The coefficient of variation of the modified method, based on 10 replicate determinations of a standard urine sample containing 60 μg of chlorothiazide/ml, was 7.6%. Twenty-four hour blank urine specimens subjected to this procedure yielded a mean "apparent chlorothiazide" excretion value (n = 4, $\pm SD$) of 0.63 ± 0.2 mg or 0.037 ± 0.01 mg/kg.

Although there was an apparent linear relationship (Fig. 1) between the cumulative urinary excretion values based on the HPLC and Bratton–Marshall assay results, only eight of 24 urinary excretion values determined by the colorimetric method were within ±10% of the values determined by the specific HPLC method. The interanimal variability in chlorothiazide excretion was greater after oral

[1] To be published.
[2] One-half, one, two, or three 250-mg Diuril tablets (lot V4092, Merck Sharp and Dohme), purchased on the open market.
[3] Aqueous solution of the sodium salt.
[4] The original HPLC method (7) was modified to include an ethyl acetate extraction step and sulfadiazine as the internal standard. An HPLC unit (model 204) was equipped with a μBondapak C_{18} reversed-phase column and a UV monitor (model 440) operated at 280 nm (Waters Associates, Milford, MA 01757). The mobile phase was 10% acetonitrile in 0.01 *M* phosphoric acid at a constant flow of 2.0 ml/min. The retention times for sulfadiazine and chlorothiazide were 5.61 and 6.97 min, respectively. No interference by urine constituents was observed. The coefficient of variation of the modified method, based on 10 replicate determinations of a standard urine sample containing 60 μg of chlorothiazide/ml, was 1.2%.
[5] Swinnex-13 filter unit with 0.45-μm pore size filters (type HA), Millipore Corp., Bedford, MA 01730.
[6] Vortex-Genie, Fisher Scientific Co., Rochester, NY 14624.
[7] Beckman DB-G spectrophotometer, Beckman Instruments, Mountainside, NJ 07091.

0022-3549/79/0100-0126$01.00/0

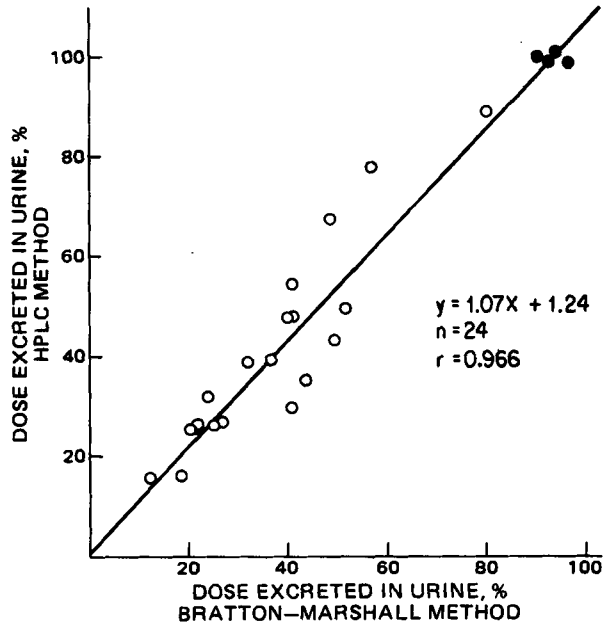

Figure 1—*Relationship between the percent of chlorothiazide dose excreted in the urine of four dogs as determined by HPLC and Bratton–Marshall methods. Key:* ○, *125–750 mg po; and* ●, *250 mg iv.*

administration of chlorothiazide (coefficient of variation of 26–50% for the various doses irrespective of analytical method) than after intravenous administration (coefficient of variation was 1.0% by the HPLC method and 2.5% by the Bratton–Marshall method).

These results clearly indicate that interferences by urinary constituents, which vary during the experimental period and with the extent of dilution of the urine specimen (3, 4, 8), can cause appreciable errors in chlorothiazide bioavailability estimates based on the colorimetric procedure and that HPLC should be the analytical method of choice in all future urinary excretion-based bioavailability studies on chlorothiazide and, perhaps, other thiazide diuretics.

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Received July 20, 1978.

Accepted for publication October 17, 1978.

Presented in part at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Hollywood, Fla., meeting, November 1978.

Supported in part by Grants GM-20852 and GRS-RR0545415 from the National Institutes of Health.

Localization of Isoproterenol-Induced Contractions of Canine Small Intestine

Keyphrases □ Isoproterenol—*in vivo* effects on small intestinal motor activities, dogs □ Motor activities *in vitro*—effect of isoproterenol on muscularis mucosa and muscularis externa motility

To the Editor:

The proposition is generally accepted that the catecholamines produce relaxation of the small intestinal musculature of a number of species *via* α- and β-receptor mechanisms. This acceptance was gained because exceptions to this rule were rarely noted and were usually inconsistent or segment related. These scattered observations have not been systematically analyzed to determine what possible common threads connected them.

For all practical purposes, most experimental evidence for the small intestinal relaxatory activities of the catecholamines has derived from *in vitro* and *in situ* studies involving dogs, cats, rats, rabbits, mice, and guinea pigs. The only serious disagreement arises with the canine ileum. Some years ago, it was reported (1, 2) that occasionally high dosages of isoproterenol produced marked stimulation of the ileum of the anesthetized dog *in situ*, but no explanation for this phenomenon was advanced. Since that time, we have observed similar anomalous effects of isoproterenol stimulation on the canine small intestine. Thus, the purpose of this communication is to propose a unifying theory with respect to the excitatory activities of catecholamines on the mammalian GI tract.

Kokas and Gordon (3) reported that the villi of the canine small intestine were specifically stimulated by β-adrenergic agents and that this effect was enhanced by α-adrenolytics. Later, we also observed that stimulatory effects of β-adrenergic agents on canine ileal segmenting activities in the chloralose–urethan anesthetized animal became predictably reproducible following pretreatment by α-adrenolytics. Intravenous doses of isoproterenol usually produced marked jejunal and ileal segmenting activity. Both bilateral vagotomy and atropine were ineffective in blocking this small intestinal response. Prior administration of the α-adrenolytic phenoxybenzamine potentiated the response (Fig. 1), which was blocked by dichloroisoproterenol (not depicted).

We subsequently decoupled the canine ileal tunica muscularis from the muscularis mucosa (4) and demonstrated that the β-excitatory effect upon the latter occurs *pari passu* with the inhibitory effect on the former. Again, the excitatory effects of the β-agonists are enhanced by pretreatment with α-adrenolytics. In experiments using the classical tissue bath technique, contractions of the circular and longitudinal components of the muscularis were recorded (Fig. 2). Isoproterenol had opposite effects in different parts of the ileum of the same animal. The tunica muscularis portion of the ileum exhibited a relaxation response to a fixed dose of isoproterenol, whereas the muscularis mucosa portion exhibited only a tonic contractile response to the same dose of the β-agonist. These observations using mechanically decoupled effector systems are identical to the results obtained by Kokas and Gordon (3) without decoupling but by specific observations of the motility of the villi.

0022-3549/79/0100-0127$01.00/0