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Interference in the fluorometric quantitation of urinary 5-hydroxyindoleacetic acid by aspirin

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IN RECENT years there has been a marked increase in the use of fluorometric procedures for the analysis of biogenic amines and related materials in physiological fluids and tissues. In studies of urinary amines and acids, extreme care must be taken to ensure that the excretion of various drugs and/or their metabolites do not interfere with the various analytical procedures employed. In schizophrenic subjects, the administration of chlorpromazine was shown to interfere with the thin-layer determination of 3,4-dimethoxyphenylethylamine (DMPEA).[1–3] Other drugs, Broncotabs* and Indocin,† have also been found to interfere with this determination of DMPEA.[3] The colorimetric nitrosonaphthol method for the determination of 5-hydroxyindoleacetic acid (5HIAA)[4] has been found to be not as specific as was once held,[5] and recently formaldehydogenic drugs[6] have been added to the list of interfering compounds for this method. The fluorometric procedure for the determination of urinary tryptamine[7] was also subject to interference from thioridazine (Mellaril).‡

In this laboratory, a number of analytical procedures were partially automated to facilitate screening a large number of samples in preparation for a longitudinal urinary excretion pattern study. During the course of this study on normal subjects, an interference was noted in the analysis of 5HIAA. Upon investigation it was determined that the individual had taken aspirin during the collection period. Subsequent trials suggested that this interference is not quenching *per se*, but probably the type of energy transfer investigated by Förster,[8] as cited by Udenfriend.[9]

The extraction procedure for 5HIAA is relatively nonspecific, going from acid media to organic phase and returning to a buffer phase. The procedure of Giacalone and Valzelli[10] was adapted for urine and used in preparation of samples. Two-ml samples of urine were diluted with 3 ml water and mixed with 3 g of NaCl and 5 ml of 0·2 NHCl. This mixture was extracted with 5 ml of washed butyl acetate. Four ml of butyl acetate was transferred to tubes containing 2 ml of 0·5 M phosphate buffer, pH 9·0, and re-extracted into the aqueous phase. The aqueous phase was transferred to sample cups on a Technicon sampler.§ Aliquots were mixed with equal volumes of 6 NHCl using the Technicon proportioning pump§ and the resulting fluorescence read at 300 nm excitation and 540 nm emission, using a quartz flow cell arrangement in a spectrofluorometer‖ with an attached recorder.¶ To facilitate calculations, reagent blanks were used for base-line determinations and the instrument adjusted accordingly. The extraction procedure of Udenfriend *et al.*[11] gave comparable results with analysis of 5HIAA and the interference.

The excitation and emission spectra of 5HIAA have maxima of 300 nm and 540 nm, respectively; whereas, the corresponding values of acetyl salicylic acid (ASA), under the same conditions, are 335 nm and 440 nm. Extracts of urine samples from subjects taking aspirin emitted maximum fluorescence at 440 nm, but the wavelength for maximum excitation varied from 340 to 370 nm, depending upon the subject, the amount of aspirin ingested, and the amount of elapsed time before collection. In general, the interference in the fluorescent assay was detectable within 15 min after ingestion of the aspirin and for at least 24 hr thereafter.

Using the flow-cell arrangement, reading at 300–540 nm (excitation-emission), this energy transfer is apparent in the appearance of a "double" peak on the recording chart (Fig. 1b) when the aspirin is taken during or just before the collecting period begins. Aspirin taken 6–12 hr before the collecting period begins may produce a peak with a flattened appearance (Fig. 1c). This interference can be detected in other ways: (1) by using different volumes of urine for the samples; (2) by using various dilutions of the sample in the sample cup; or (3) by reading the sample at both 300–540 nm and 334–440 nm wavelength settings. With the first two techniques, readings from normal urine will be linear, whereas those from "aspirin" urine will not. With the last technique, normal urine will produce peaks of similar intensities at both settings, while "aspirin" urine will produce an increased peak at

* Ephedrine sulfate, glycerol guaiacolat, theophylline, phenobarbital, thenyldiamine hydrochloride (Broncotabs).

† Indomethacin (Indocin).

‡ Unpublished observations by author.

§ Technicon Instrument Corp.

‖ Farrand Optical Co., Inc.

¶ Heath Co.

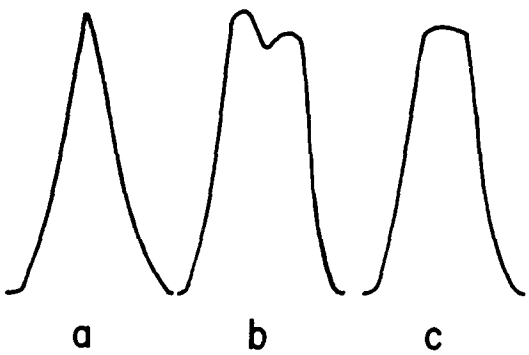

FIG. 1. Illustration of types of peaks obtained from 5-hydroxyindoleacetic acid (5HIAA) analysis using the flow-cell arrangement: (a) normal urine; (b) 0·648 g (10 grain) aspirin given just before collection; (c) urine collected 6–12 hr after 0·648 g aspirin administration.

the 335–440 nm settings, related to the amount of interference (Table 1). Internal standards of 5HIAA are useless when added to "aspirin" urine, but those added to water or normal urine are acceptable, as indicated by the fact that the addition of 5 γ of 5HIAA to normal urine resulted in the same increment of fluorescence (9·0) as the standard reading in water at the 300–540 nm settings, but in "aspirin" urine the increments were much lower, correlating with the interference indicated at the 335–440 nm settings.

An extract of ASA was similarly found to interfere with the fluorescence of urine extracts at the 300–540 nm settings, but did not interfere with the nitrosonaphthol colorimetric procedure for 5HIAA[4] (Table 2). Internal standards of 5HIAA added to normal urine were used for comparisons between the two fluorometric (flow cell and single sample) and the one colorimetric techniques. It was

TABLE 1. RELATIVE FLUORESCENCE OF SAMPLES READ AT TWO WAVELENGTH SETTINGS USING FLOW CELL ARRANGEMENTS*

Sample†		Fluorescence in microamperes × 100	
		300–540 nm‡	335–440 nm‡
Normal urine, 2 ml		32·0	33·5
Normal urine, 1 ml		17·5	17·0
Normal urine, 1 ml + 5 γ 5HIAA		26·5	17·0
"Aspirin" urine, 2 ml	[0·648 g (10 grain) aspirin	9·0	111·7
"Aspirin" urine, 1 ml	taken during the sampling	9·0	75·0
"Aspirin" urine, 1 ml, + 5 γ 5HIAA	period]	11·0	74·0
"Aspirin" urine, 2 ml	[0·648 g aspirin taken	14·0	41·0
"Aspirin" urine, 1 ml	12 hr before sampling	11·0	25·0
"Aspirin" urine, 1 ml, + 5 γ 5HIAA	period]	18·0	24·5
5 γ 5HIAA in water		9·0	0·0

* All readings are corrected for reagent blank.

† Samples were diluted to 5 ml with water, mixed with 3 g NaCl and 5 ml 0·2 NHCl, and extracted with 5 ml washed butyl acetate. Four ml of the butyl acetate phase was transferred and extracted with 2 ml 0·5 M phosphate buffer, pH 9·0. An aliquot of the aqueous phase was placed in Autotechnicon sampler cups and the amount of 5HIAA determined using the semi-automated flow cell fluorometric technique at the indicated wavelengths.

‡ Excitation-emission wavelengths.

TABLE 2. RESULTS OF FLUOROMETRIC AND COLORIMETRIC DETERMINATIONS OF 5-HYDROXYINDOLEACETIC ACID (5HIAA) USING VARIOUS MIXTURES OF BUTYL ACETATE EXTRACTS OF URINE AND ACETYL SALICYLIC ACID (ASA)*

Sample†	Butyl acetate extracts of urine (ml)	Butyl acetate extracts of ASA (ml)	Butyl acetate (ml)	Fluorometric determination of 5HIAA Relative fluorescence in microamperes × 100				Colorimetric determination of 5HIAA Relative optical density × 100
				I Flow cell		II Single sample		III
				300–540 nm	335–440 nm	300–540 nm	335–440 nm	540 nm
Urines								
1	2·0		2·0	2·70	3·03	9·5‡	14·6‡	5·6
2	3·0		1·0	3·98	3·66	12·6‡	17·2‡	8·5
3	3·0	0·5	0·5	2·93	6·90	4·4	19·7‡	8·5
4	3·0	1·0		2·30	9·63	2·2	26·2‡	8·5
ASA only								
5		0·5	3·5	0·45	4·05	2·4	12·2‡	0·3
6		1·0	3·0	0·75	7·29	1·6	19·2‡	0·0
7		2·0	2·0	0·92	12·30	0·4	15·2‡	0·0

* All readings were corrected for reagent blank and the highest reading recorded.

† Three sets of samples were extracted with 2·1 ml 0·5 M phosphate buffer, pH 9·0, and 2 ml of buffer phase was removed. One set was placed in Autotechnicon sampler cups and the amount of 5HIAA determined using the semi-automated flow cell fluorometric technique at 300–540 nm (excitation-emission) settings and repeated at 335–440 nm settings (I). A second set was mixed with an equal volume of 6 NHCl and the amount of 5HIAA determined using the single cell fluorometric technique at 300–540 nm and 335–440 nm settings (II). The final set was mixed with 1 ml 0·1% nitrosonaphthal in 95% EtOH and 1 ml nitrous acid reagent (0·2 ml 2·5% sodium nitrite + 5 ml 2 NH_2SO_4), heated at 55° for 10 min, washed with 10 ml dichloroethane, and the optical density of the aqueous phase read on a spectrophotometer at 540 nm (III).

‡ Indicates emission wavelength was optimum.

determined, by these internal standards, that the quantity of 5HIAA present in the butyl acetate extract of normal urine was identical in all three methods.

Accurate knowledge of all prior medication is a necessity in any study involving urinary metabolites, thus allowing the investigator to choose a method which is not likely to be affected by reported interfering medications.[5,6]

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p-Chloroamphetamine—Species differences in the rate of disappearance and the lowering of cerebral serotonin*†

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THE MAJOR pathway for the metabolism of amphetamine in rats involves *p*-hydroxylation; in mice, however, it appears that deamination is of equal importance.[1,2] Recent studies have shown that the administration of desipramine to rats strikingly prolongs the psychomotor stimulation evoked by amphetamine but not that elicited by its *p*-chlorinated derivative.‡ This prolongation of the central stimulatory action of amphetamine is a consequence of an inhibition of its hepatic metabolism by desipramine which results in a sustained elevation of the levels of amphetamine in both the brains and bodies of rats.[3] The marked prolongation of the half-life of amphetamine which resulted from the inhibition of *p*-hydroxylation suggested that *p*-substituted derivatives, such as *p*-chloroamphetamine, should be metabolized much more slowly than amphetamine in the rat. Moreover, in mice where

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† A preliminary report of this work was presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics, Pittsburgh, Pa. (Pharmacologist **11,** 273, 1969).

‡ F. Sulser and J. V. Dingell, unpublished results.