FLUOROPHOTOMETRIC ESTIMATION OF STILBAMIDINE IN URINE AND BLOOD

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(Received for publication, March 14, 1947)

Stilbamidine (4,4′-diamidinostilbene) has been introduced by Yorke and his colleagues (1) as a powerful chemotherapeutic agent for the treatment of trypanosomiasis, kala-azar, and babesiasis. It has also been experimentally used in the therapy of purulent arthritis (2), simian malaria (3), and most recently in multiple myeloma (4). A method for the estimation of this drug in biological fluids is of importance and is the basic requirement of any study with stilbamidine. Direct application of physical methods, *i.e.* fluorescence or ultraviolet absorption, while highly desirable because of their sensitivity and specificity, results in inaccurate readings because of the presence in biological fluids of interfering substances. In the method to be presented stilbamidine is first separated quantitatively by an ion exchange column of Decalso (suggestion of Dr. Oliver Lowry), and then eluted and measured with a fluorophotometer. The method also has the advantage that a preliminary precipitation of plasma proteins is not necessary.

Principle—Stilbamidine exhibits a brilliant blue fluorescence when exposed to ultraviolet light and this property can be used for its estimation. The fluorescence is measured by a photometer after separation from other fluorescent materials. The fluid to be examined is placed on a column of Decalso for adsorption. The column is washed free of impurities with large volumes of hot water. Elution is carried out with a 0.2 N HCl-50 per cent ethanol mixture, which is more efficient than the usual HCl-KCl mixture. The small blank value for the urine is then reduced by strong acidification of the eluate. After a specific time interval the eluate is measured in a fluorophotometer.

Reagents—

1. Decalso, 50 to 80 mesh, washed before use with 3 per cent acetic acid followed by distilled water and drying.
2. Silica sand, fine granular.
3. Eluting mixture, equal parts of 0.4 N HCl and 95 per cent ethanol.
4. Hydrochloric acid, C.P., concentrated.

*Assisted by a fellowship from the Emanuel Libman Fellowship Fund.

Method of Analysis

Stilbamidine is photolabile in aqueous solution, necessitating the performance of all stages of the method in dim artificial light or in the dark. Adsorption columns were made from ordinary glass tubing 9 mm. in diameter, with a 0.5 mm. hole at the bottom. They should be at least 10 inches long and preferably with a funnel top. A few grains of silica sand are added to the column which is wetted with a drop of water applied to the bottom. Decalso is added until a column 6 inches in length is formed. Gentle tapping of the tube assures uniform distribution of the Decalso.

Urine samples are collected in dark bottles. Measure 5 cc. of urine (containing 1 to 30 γ of stilbamidine per cc.) into the column and allow adsorption by gravity. It is advisable to check adsorption by a momentary exposure to a weak source of ultraviolet light.

Wash the column with 60 cc. of hot water, using gentle suction. Discard the water. Elute with three portions of 4 cc. of eluting mixture and collect in a 25 cc. volumetric flask. This step is done without suction.

Add 1 cc. of concentrated HCl to the solution in the flask, dilute to the mark with distilled water, mix, and place in the dark for 15 minutes before measuring the fluorescence.

Standard Solutions—The stock standard is a 30 mg. per cent aqueous solution of stilbamidine isethionate. This is stable in the dark at room temperature, losing only 2 per cent per week. Of this solution 0.1, 0.3, and 0.5 cc. are carefully measured into 25 cc. volumetric flasks. 12 cc. of elution mixture are added to each flask and mixed. Then 1 cc. of concentrated HCl is added and the solution made up to the mark with distilled water. After mixing, exactly 15 minutes should elapse before the fluorescence is measured. Standard solutions in water are not comparable, as they have only two-thirds of the fluorescence of those containing alcohol.

Reagent Blank—12 cc. of elution mixture and 1 cc. of concentrated hydrochloric acid are made up to 25 cc. with distilled water.

Fluorometric Measurement—An instrument containing a balance photocell and bridge circuit is required for stable, reproducible readings and linear response (Lumetron[1]). The fluorescence is measured with the usual primary and secondary vitamin B_1 filters, and a wire screen which cuts down the intensity of the measuring beam about 70 per cent. After the Lumetron has warmed up, the secondary standard (1 γ per cc. of quinine sulfate in 0.1 N sulfuric acid) is set at 50 and the reagent blank at 0 with the zero suppressor control. If the 15 cc. sample holder is used, 12 cc. aliquots are pipetted into the sample holder.

[1] Photovolt Corporation, New York.

EXPERIMENTAL

The fluorescence of stilbamidine deteriorates rapidly during measurement, probably owing to saturation of the ethylene linkage (5). The deterioration experienced under our conditions of measurement is given in Fig. 1. It is seen that the greatest rate of change of fluorescence is in the 1st minute. If one exposes the solution to be examined for exactly 1 minute before taking a reading, reproducible measurements can be made with a variation of 2 per cent or less. The disadvantage of reading the

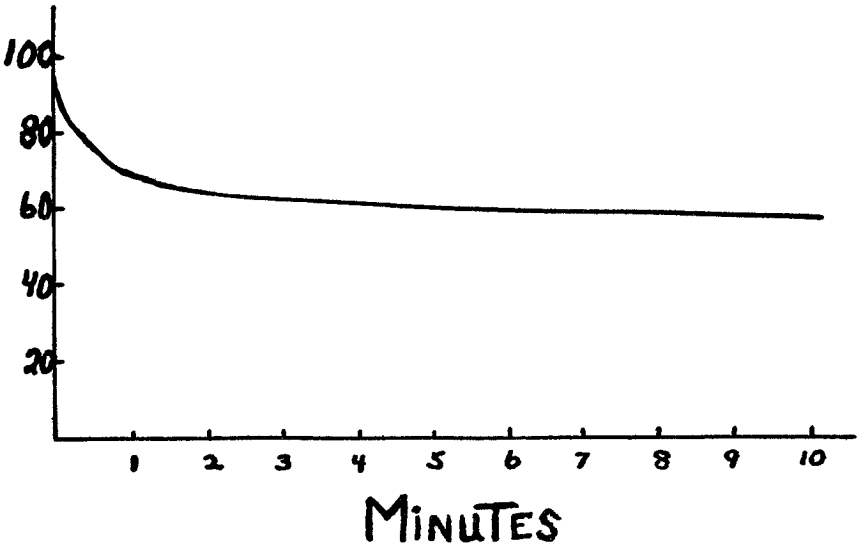

FIG. 1. Galvanometer readings of stilbamidine fluorescence

fluorescence after a longer period of time is that the fluorescence of the blank does not change, so that proportionately it becomes greater.

A graph of the values for the fluorescence of the standard solutions forms a straight line for the low concentrations measured (Fig. 2). It can be noted that a reading of 60 is obtained when 0.09 mg. of stilbamidine isethionate was present in the original 5 cc. aliquot of urine, the blank on the urine without stilbamidine being, on the average, about 2. By this method the drug content of the urine or other biological fluids can be measured accurately down to 1 γ per cc. of fluid tested.

Recoveries averaging 87 per cent were obtained in experiments in which stilbamidine was added to 5 cc. samples of urine (Table I). The average deviation of a single determination was less than 2 per cent. This is a satisfactory result for a method employing Decalso, the loss being due to an inseparable, permanently adsorbed fraction.

TABLE I

Recovery of Stilbamidine Isethionate

	Stilbamidine added	Per cent recovered*
	mg.	
Urine 1	0.015	96
" 2	0.03	89
" 3	0.06	88
" 4	0.06	87
" 5	0.09	87
" 6	0.09	87
" 7	0.09	84
" 8	0.09	86
" 9	0.09	83
" 10	0.09	87
" 11	0.09	85
" 12	0.12	85
" 13	0.12	87
Plasma 1	0.018	84
" 2	0.018	85
" 3	0.036	88
" 4	0.06	89

* After subtraction of the blank.

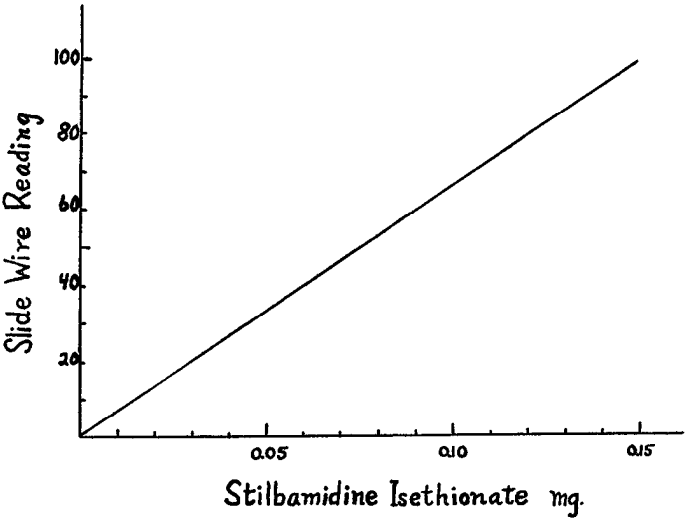

FIG. 2. Fluorescence curve of stilbamidine

Proteins need not be removed before placing the plasma on the Decalso column. A procedure similar to that for urine is applied to 2 cc. samples of plasma. The quinine standard is set at 100 and weaker standard solu-

tions are necessary. Blank values of plasma without stilbamidine are negligible. Recovery of stilbamidine added to plasma was the same as with urine (Table I). Culture media containing stilbamidine can be examined in the same way.

Comment

The pharmacological and clinical studies of stilbamidine and other aromatic amidines have been handicapped by a lack of suitable methods for the estimation of the amounts of these drugs in urine and blood. By making use of the two readily apparent properties of stilbamidine, adsorption and fluorescence, a simple and accurate method for the determination of stilbamidine in biological fluids was evolved. Recently a method has appeared for the fluorometric determination of aromatic amidines based upon the reaction of the latter with glyoxal and benzaldehyde in alkaline aqueous solution with formation of fluorescent glyoxalidone derivatives (6). Although this is a definite improvement over previous methods employing glyoxal, it requires the separation of amidine from plasma proteins with either dialyzed iron or butyl alcohol, and for urine samples there are frequently (6) "extraneous compounds which inhibit the reaction of the aromatic amidines with glyoxal and benzaldehyde." Furthermore, the range to be measured (2 to 10 γ of stilbamidine) is more restricted than with the Decalso method.

SUMMARY

A simple and accurate method of estimation of stilbamidine in urine and blood is given. The stilbamidine is separated from interfering substances by adsorption on a column of Decalso, which is then washed with hot water. Preliminary protein precipitation is not necessary. Elution is carried out with a hydrochloric acid-ethanol mixture, which is further acidified to reduce blank fluorescence. The characteristic blue fluorescence of the stilbamidine is then measured in a fluorophotometer.

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J. Biol. Chem. 1947, 168:699-703.