Determination of Plasma Levels of Tolbutamide (1-Butyl-3-*p*-Tolylsulfonylurea, Orinase*). (23425)

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Tolbutamide (1 - butyl-3-*p*-tolylsulfonylurea, Orinase*) is a potent, orally-active hypoglycemic agent(1). In order to correlate plasma levels of this compound with reduction in blood sugar concentrations, a suitable analytical procedure adaptable to analysis of large numbers of samples is needed. Such a procedure is presented based on the intense absorption by Tolbutamide at 228 mμ.

Materials. A. *Reagents*: (1) M/15 Phosphoric acid: 4.55 ml of concentrated phosphoric acid are diluted to 1000 ml with distilled water. (2) Chloroform: Mallinckrodt A.R. chloroform in sealed glass bottles is used for the extractions. (3) 95% Ethanol: this is redistilled before use. (4) Darco G-60. B. *Apparatus*: (1) Separatory funnels: 60-ml separatory funnels equipped with Teflon stopcock plugs are employed for the extractions. Pledgets of Pyrex wool are inserted into the stems of the funnels and serve to eliminate small droplets of water from the chloroform phase(2). *All* glassware must be free of ultraviolet absorbing substances. Prior to use, the funnels are shaken with 5-10 ml of chloroform which is allowed to filter through the glass wool pledgets in the stems. (2) Filter funnels: 2-inch funnels containing pledgets of Pyrex wool in the stems are used to filter the suspensions of Darco G-60. Prior to use, the funnels and pledgets are rinsed with 95% ethanol and thoroughly dried. (3) Spectrophotometer: a Beckman Model DU Spectrophotometer with 1-cm quartz cells is used for absorbance measurements. Spectra have been obtained with a Cary Model 11 Recording Spectrophotometer. *Procedure.* A. *Extraction*: 1 ml of oxalated plasma is mixed with 1 ml of distilled water and 1 ml of M/15 phosphoric acid in a 60-ml separatory funnel. The acidified plasma is extracted with 10 ml of chloroform by a gentle inversion of the funnel 25-30 times. Occasionally it is necessary to break an emulsion by centrifugation. The chloroform phase is carefully drained through the pledget of glass wool and collected in a vessel suitable for evaporation of the solvent. The extraction is repeated with 2 additional 10-ml portions of chloroform and the extracts combined and concentrated to dryness. In the present study, concentrations have been made in 50-ml round-bottom flasks under reduced pressure below 35°C by means of vacuum evaporating equipment constructed in these laboratories. In other laboratories employing this procedure, evaporation to dryness in a beaker on a steam bath has been satisfactory provided the sample is removed just before going to dryness and remaining chloroform removed by a stream of air at room temperature.† The residue should not be exposed to elevated temperatures but all traces of chloroform must be removed. B. *Charcoal treatment*: the residue is dissolved in exactly 10 ml of 95% ethanol. After sufficient time to insure complete solution, 5-7 mg of Darco G-60 are added and the mixture is swirled several times during 30 minutes. The supernatant solution is then filtered through a 2-inch funnel containing a pledget of Pyrex wool in the stem. C. *Spectrophotometry*: the absorbance of the charcoal filtrate is measured at 228 mμ *vs.* a reagent blank (30 ml of chloroform concentrated to dryness and carried through the remainder of the procedure). A plasma sample obtained from the same individual prior to Tolbutamide administration is also carried through the procedure and similarly measured (plasma blank). The concentration, T, of Tolbutamide (mg/100 ml of plasma) is calculated

* Orinase is the Upjohn trademark for its brand of 1-butyl-3-*p*-tolylsulfonylurea.

† Marble, A., Joslin Clinic, Boston, Mass., personal communication.

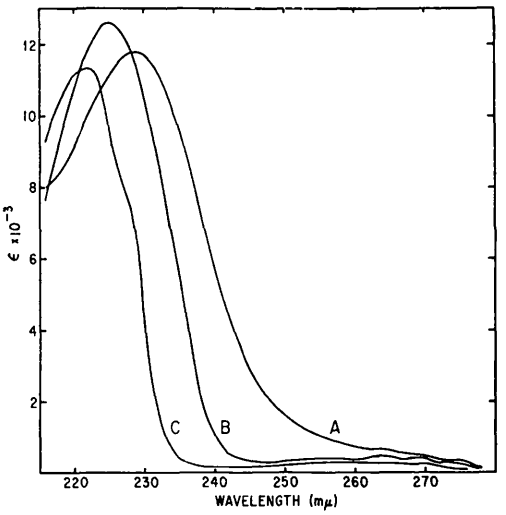

FIG. 1. Absorption spectra in 95% ethanol. A—Tolbutamide (1-butyl-3-*p*-tolylsulfonylurea); B—*p*-Toluenesulfonamide; C—*p*-Toluenesulfonic acid.

from the equation $T = \frac{1000\, A_{228}}{a_{228} \cdot F}$ where A_{228} is the observed absorbance at 228 mμ, a_{228} is the calculated absorbance at 228 mμ for a solution containing 1 mg Tolbutamide/ml of 95% ethanol (about 49), and F is the fraction of added Tolbutamide recovered from plasma by the procedure (about 0.85 for human plasma, 0.91 for dog plasma). This value for plasma Tolbutamide is then corrected for the apparent Tolbutamide concentration obtained for the plasma blank. Both a_{228} and F should be determined for the individual spectrophotometer and laboratory involved.

Results. The absorption spectrum of Tolbutamide in 95% ethanol shows an intense maximum at 228 mμ (Fig. 1). A comparison of this spectrum with those of *p*-toluenesulfonamide and *p*-toluenesulfonic acid (Fig. 1) indicates that the tosyl group is the chromophore responsible for the Tolbutamide absorption at 228 mμ. Absorbance at this wavelength obeys Beer's law over the range 0-24 μg/ml. When Tolbutamide (as its sodium salt) is added to human plasma and carried through the complete procedure the absorbance *vs.* concentration response is linear but is only about 85% of that observed for the pure Tolbutamide standard. A loss of about 15% occurs on extraction and charcoal treatment necessitating use of a correction factor as described above.

An examination of the recovery of Tolbutamide from various activated charcoals has shown recoveries of 92.5% from Darco G-60, 90.0-92.3% from a group of the Nuchars and 74% from Norit A. Since Darco G-60 consistently yields a low plasma blank, it is used routinely. This study indicates that losses of Tolbutamide encountered in this procedure occur, for the most part, during charcoal treatment.

The effect of charcoal treatment on a group of human plasma blanks is shown in Table I. In every case, the apparent Tolbutamide concentration is reduced by Darco G-60 treatment and with the exception of the badly hemolyzed samples, the plasma contribution is equivalent to about 1 mg/100 ml or less.

The recovery of added Tolbutamide from human and dog plasma is shown in Table II. In each case recovery is satisfactory when corrected for inherent losses as described above.

Typical plasma levels of Tolbutamide in the dog following oral administration (as the sodium salt) are presented in Fig. 2. Included for comparison are the corresponding decreases in blood sugar concentrations as determined by the method of Folin and Wu(3). The animals were fasted for 15 hours prior to administration of Tolbutamide and were continued without food during the entire experimental period. Since blood sugar concentrations in fasting controls showed only minor fluctuations, changes due to fasting could be

TABLE I. Effect of Darco G-60 on Human Plasma Blanks.

Plasma condition	Apparent Tolbutamide conc. (mg/100 ml) Before Darco G-60	After Darco G-60
Excellent	2.3	.5
"	1.9	.9
"	1.9	1.1
"	.6	.4
"	1.1	.7
"	1.3	1.0
Slightly hemolyzed	6.9	1.2
Badly "	11.3	4.0
" "	10.6	2.3

TABLE II. Recovery of Added Tolbutamide from Human and Dog Plasma.

Human plasma				Dog plasma			
Sample 1		Sample 2		Sample 1		Sample 2	
Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery	Added (mg/100 ml)	% recovery
2.3	95.7; 82.6	11.4	101.8; 98.2	9.7	99.0	9.7	99.0
4.6	100.0; 84.8	13.7	99.3; 91.2	19.4	100.5	19.4	99.0
6.9	95.7; 95.7	16.0	104.4; 101.9				
9.1	100.0; 100.0	18.3	105.5; 104.4				
		20.6	100.5				
Mean ± stand. dev. = 97.7 ± 6.4%				Mean ± stand. dev. = 99.4 ± .8			

ignored. It can be seen that maximum plasma levels of Tolbutamide correspond well with maximum blood sugar reductions, and that blood sugar concentrations approach control values as the drug slowly clears from the plasma. Disappearance of Tolbutamide from the plasma is typically first-order. Data in Fig. 2 correspond to half-lives in the dog of 13.7 and 17.6 hours after doses of 25 and 100 mg/kg respectively.

Discussion. Tolbutamide is not a "sulfa" because the *para* position is occupied by a methyl rather than an amino group. For this reason the conventional Bratton-Marshall procedure(4) is not applicable to Tolbutamide and the spectrophotometric procedure described above has been devised.

Sensitivity of the procedure depends on the volume of plasma extracted and the volume of the reconstituted alcoholic solution. The procedure described is useful over the range 1-25 mg of Tolbutamide per 100 ml of plasma. Higher concentrations may be accommodated by suitable dilution of the charcoal filtrate.

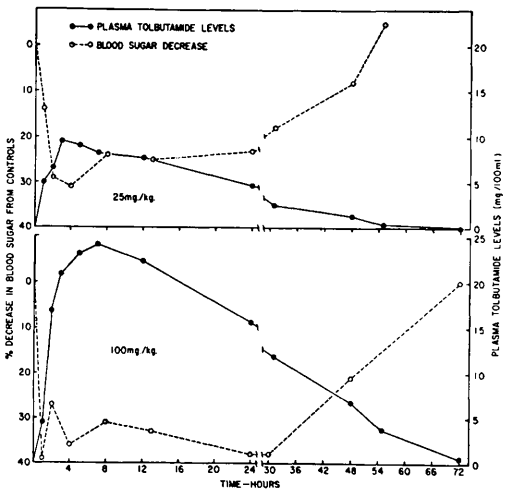

FIG. 2. Relationship of blood sugar decrease to plasma levels of Tolbutamide after a single oral dose to fasting dogs (each point is avg of values obtained from 2 dogs).

Although adequately sensitive, the method lacks specificity. For this reason conditions are presented which result in a minimal apparent Tolbutamide concentration for plasma free of drug (plasma blank). Strong acidification of plasma prior to extraction gives an excellent recovery of Tolbutamide but the plasma blank is higher than obtained on extraction of neutral plasma. Recovery of Tolbutamide from neutral plasma is low due to the fact that Tolbutamide (pKa' 5.43 at 25°C (5)) exists almost completely in the salt form at the natural pH of plasma. Therefore, some reduction in pH is essential in order to convert Tolbutamide into its chloroform-extractable acid form. Dilute phosphoric acid brings plasma to a pH (4.5-4.8) sufficiently low to give 95-100% extraction but does not increase the plasma blank. Heating the residue following removal of chloroform gives highly inconsistent results. For this reason the dry residue should not be heated but *all* chloroform must be removed as it absorbs strongly at 228 mμ.

Application of the procedure as described, except for the charcoal treatment, to the determination of human plasma blanks has given an apparent Tolbutamide concentration of 2.3 ± 0.9 mg/100 ml. However, an occasional sample has run as high as 15 mg/100 ml. Inclusion of the Darco G-60 treatment (Table I) has consistently lowered the human plasma blank to about 1.0 mg/100 ml or less for clear plasma. Reduction has been even more dramatic in the case of hemolyzed plasma and the occasional extremely high

value has been eliminated.

The procedure as described requires no special equipment other than an ultraviolet spectrophotometer. However, the need for scrupulously clean glassware cannot be overemphasized. An analysis may be completed easily within one working day and many may be run simultaneously. The residues following chloroform removal may be stored and this frequently allows a more flexible operation.

After completion of these studies, the method of Spingler and Kaiser(6) appeared, also based on the intense absorption by Tolbutamide at 228 mμ. Utilizing an extraction of lyophilized serum, these investigators compensated for the contribution of the serum by a second absorbance measurement at 280 mμ and treatment of the system as a 2-component mixture (blank + Tolbutamide). Losses of about 30% of the Tolbutamide present were encountered. The procedure presented herein is simpler, losses encountered are considerably less (about 15% for human plasma and 9% for dog plasma), and special apparatus such as lyophilizing equipment is unnecessary. Spingler and Kaiser(6) report a half-life of 8.7 hours for Tolbutamide in the human. The present studies have indicated a half-life of 13.7-17.6 hours in the dog. Species differences in rate of metabolism of the drug appear to be considerable.

Summary. A procedure is presented for determination of Tolbutamide in plasma. The method consists of a chloroform extraction of weakly acidified plasma, concentration of the extract to dryness, dissolution of the residue in a measured volume of ethanol, treatment of the resulting solution with Darco G-60, and measurement of the absorbance of the resulting charcoal filtrate at 228 mμ. Recoveries of Tolbutamide from human and dog plasmas have been satisfactory and plasma levels determined by this procedure have correlated well with the hypoglycemic response elicited by the drug.

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Increased Susceptibility to Herpes Simplex in Mice Subjected to Avoidance-Learning Stress or Restraint.* (23426)

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A variety of factors other than specific immunity modify host response to infectious agents, protecting against repeated exposure to potentially dangerous agents on the one hand, and subtly converting innocuous exposures and inapparent infections into overt disease on the other(1). Psychologic or emotional stress is often proposed as one of the factors predisposing to overt infection(2). Clinical evidence supporting an etiologic role for emotional stress in recurrent herpes simplex is particularly suggestive(3,4), but experimental data are lacking. As the initial step in a study aimed toward elucidation of psychologically induced changes in resistance, an experimental system is described here

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