Determination of tolbutamide and chlorpropamide in biological fluids

Assays exist for the determination of the oral hypoglycemic sulphonylureas, tolbutamide and chlorpropamide, in biological fluids. The most common involve modification of Spingler's colorimetric assay (Spingler, 1957; Carmichael, 1959; Nelson, O'Reilly & Chylski, 1960). However, methods utilizing these reactions lack specificity, so too do methods employing the ultraviolet spectrum of the intact molecule and they are generally unsuitable below 10 μg ml^{-1} (Forist, Miller & others, 1957). The gas chromatographic assay of Sabih & Sabih (1970) is more sensitive, but in our hands both *N*-methyl *p*-toluenesulphonamide and tolbutamide gave a peak with an identical retention time. Also it is rather time consuming.

A procedure for tolbutamide and chlorpropamide that overcomes some of the difficulties of existing assays is now described.

A Varian 1200 model gas chromatograph was used equipped with a nickel-63 detector, containing a 6 ft. 1/8 inch o.d. glass column packed with 3% OV–17 on 100–120 mesh, acid washed DMCS Chromosorb W. Injection port, column and detector temperatures were 225, 210 and 310° respectively. Carrier gas (5% methane in argon) flow was maintained at 30 ml min^{-1}. Colorimetric measurements were made on either a Cary 15 or a Beckman DBG instrument.

Sodium phosphate buffer, 2·55 M was prepared by dissolving 0·255 mol sodium hydrogen phosphate in 60 ml water and adjusting the volume to 100 ml. Dinitrofluorobenzene (0·05%) in pentyl acetate (Reagent grade) was prepared weekly from a 0·5% stock solution and stored at +4° in a dark bottle. *N*-Propyl and *N*-butyl 2,4-dinitroanilines were prepared according to Walle (1968).

Colorimetric procedure. To plasma or blood (0·5 ml) add phosphate buffer (1 ml) and extract with 0·5% isoamyl alcohol in hexane (10 ml). Centrifuge or allow to separate, pipette 7 ml of hexane extract into another tube containing 1 ml N sodium hydroxide, shake and after phase separation remove the hexane layer. Add 1 ml of 2N hydrochloric acid and 3 ml pentyl acetate to the basic layer, shake for 2 min, and centrifuge. Pipette the pentyl acetate extract (2 ml) into another tube containing 1 ml 0·05% dinitrofluorobenzene solution, lightly stopper and heat for 1 h at 125° on a constant temperature oil bath. After cooling, the absorbance is read at 420 nm against pentyl acetate. The concentration of tolbutamide or chlorpropamide is calculated by reference to a calibration curve, obtained by taking 0–50 μg sulphonylurea in 0·5 ml plasma through the assay procedure.

Gas chromatographic procedure. The extraction and reaction procedure is the same as that in the colorimetric assay with the following additions and modifications. To plasma or blood (0·5 ml) and the phosphate buffer, add aqueous internal standard solution (0·1 ml). For the determination of tolbutamide use chlorpropamide and vice versa. The amount of internal standard can be adjusted to the anticipated concentrations of the respective sulphonylurea. Also, 2 ml instead of 3 ml of pentyl acetate is used and only 1 ml pentyl acetate extract is pipetted off and reacted with 0·1 ml 0·5% dinitrofluorobenzene solution. After cooling 1–5 μl is injected into the gas chromatograph. The peak height ratio of the sulphonylurea to the internal standard is measured and the concentration of sulphonylurea is calculated by reference to a calibration curve of peak height ratio versus concentrations obtained by taking 0–35 μg sulphonylurea in 0·5 ml plasma or blood through the assay procedure.

Occasionally, samples assayed colorimetrically give too low absorbance readings. Under these circumstances an appropriate amount of internal standard, *N*-propyl-2, 4-dinitroaniline for tolbutamide, and *N*-butyl-2,4-dinitroaniline for chlorpropamide, in 0·1 ml pentyl acetate can be added to the pentyl acetate solution to be read

colorimetrically. The sample can now be assayed by gas chromatography and the concentration of sulphonylurea in plasma ascertained by reference to an appropriate calibration curve, obtained by taking known amounts of sulphonylurea through this modified procedure.

The differential absorbance between either *N*-propyl or *N*-butyl-2,4-dinitroaniline and the excess 2,4-dinitrofluorobenzene is maximal at 380 nm (Carmichael, 1959). However, at this wavelength, this excess of reagent still has an appreciable absorbance and if the absorbance of the sample is measured at 420 nm, instead there is little loss in sensitivity and the absorbance of the reagent is negligible.

Above 25 μg sulfonylurea ml^{-1} biological sample, the colorimetric assay is sensitive and satisfactory but below 10 μg it is not. Thus, the coefficient of variation at 5, 10, 15, 20, 25, 30, 40 and 50 μg ml^{-1} was respectively 19, 36, 16, 14, 6, 6, 9, 7%. To improve the sensitivity and specificity of this assay, the electron capturing properties of the resultant substituted anilines, formed in the colorimetric assay, were utilized (Walle, 1968). Propyl and butyl 2,4-dinitroaniline, formed from chlorpropamide and tolbutamide respectively, are well resolved by gas chromatography. The similarity in the physico-chemical properties of these sulphonylureas allows one to be used as an internal standard for the other drug. No detectable peaks at the same retention time as the substituted anilines were observed when blood or plasma, from subjects not receiving sulphonylureas, were taken through the procedure. Also, the chromatographic assay is more reliable than the colorimetric procedure, the coefficient of variation at 5, 10, 15, 20 and 30 μg ml^{-1} being 0·7, 0·7, 1·2, 2·9, 6·4 % respectively. The greater variance at higher concentrations resulted from non-linearity of the electron capture detector response when large quantities are injected. One can readily determine 1 μg sulphonylurea ml^{-1} sample with this procedure and if desired the sensitivity can be increased by concentrating the pentyl acetate layer before gas chromatographic assay thereby allowing flexibility. The two methods can be overlapped, although the more rapid colorimetric assay is more used for high concentration and the chromatographic assay for low concentrations of drug. Even if the colorimetric assay is used but the absorbance readings are too low, by adding a known amount of substituted aniline, e.g., propyl 2,4-dinitroaniline for tolbutamide, the pentyl acetate solution can still be analysed by gas chromatography.

The hexane extraction separates tolbutamide from its two major metabolites, hydroxytolbutamide and carboxytolbutamide. When these metabolites are either known to be absent, or are shown to be insignificant, the assay may be much simplified. The sample pH is reduced to below 2 with hydrochloric acid and extracted with 3 ml pentyl acetate. (Less than 1% of either metabolite is extracted into the hexane layer.) The 2,4-dinitrofluorobenzene is added to this extract and assayed colorimetrically or gas chromatographically depending upon the anticipated concentration of sulphonylurea.

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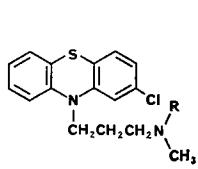
Chlorpromazine 'hydroxylamines' in red blood cells as major metabolites of chlorpromazine in man

In man, 6–10 h after a single dose of 32 mg of chlorpromazine (Ia), about 10% of the dose is found in the red blood cells as a mixture of 'free' *N*-hydroxynorchlorpromazine (Ib) and its sulphoxide (IIb) in roughly equal amounts, while about 2% of norchlorpromazine (Ic) but only traces of the parent drug (Ia) and its *N*-oxide are present in these cells. At the same time, the plasma contains only about 0·5% of the dose as a mixture of the hydroxylamines (Ib) and (IIb) and less than a total of 0·3% of the parent drug and its *N*-demethylated derivatives.



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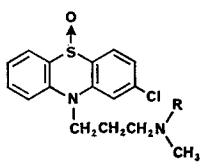
b = OH
c R = H



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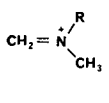

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After 24 h, there is little change in the concentration of the hydroxylamines (Ib) and (IIb) in the cells but about 5% of norchlorpromazine (Ic) and negligible amounts of unchanged drug (Ia) are present; there is about 0·2% of (Ic) and negligible amounts of (Ia) in the plasma. After 13 days, the concentration in the cells of the hydroxylamines (Ib) and (IIb) is only slightly reduced and there is 0·5 to 1% of (Ib) in the plasma but neither parent drug (Ia) nor the demethylated compound (Ic) could be detected in cells or plasma.

Patients being treated with 300 to 600 mg of chlorpromazine (Ia) daily had concentrations of (Ib) and (IIb) in the cells that indicated a total amount of 20 to 40 mg in the cells and 5 to 10 mg of (Ic) but negligible amounts of the parent drug.

In addition to the above concentrations of "free" hydroxylamines (Ib) and (IIb), additional concentrations of conjugated forms, sometimes almost equal to those of the free forms, are present.

The free compounds were isolated by separately diluting red blood cells and plasma with water, complexing with methyl orange and selective extraction of these complexes by the control of pH and the use of different organic solvents. The organic extracts were concentrated under reduced pressure in the dark, in a nitrogen atmosphere, and then examined by t.l.c., g.l.c., polarography, mass spectrometry and for chemical reactions.

The metabolically produced hydroxylamines (Ib) and (IIb) gave results identical to those obtained using authentic synthetic material produced by careful oxidation of norchlorpromazine (Ic) by hydrogen peroxide or *m*-chlorperbenzoic acid.

Using t.l.c. silica gel, (Ib) and (IIb) gave R_F values 0·8 and 0·6 respectively [(Ia)