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A polarographic method for the determination of the *N*-oxide, *N*-oxide-sulphoxide and sulphoxide metabolites of chlorpromazine

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Cathode ray polarography has been used to measure chlorpromazine-*N*-oxide, *N*-oxide sulphoxide and chlorpromazine sulphoxide in mixtures. The response was linear when the metabolites were present in the range 10^{-5} to 5×10^{-8}M in aqueous solutions. Reductive polarography of the mixed oxides in solution gave an additive wave from which individual oxides were determined by subtraction. Ultraviolet and potentiometric titration methods were used to determine the pKa values of the oxide metabolites of chlorpromazine. The mechanism of the reduction process was investigated using d.c. polarography and preparative micro-electrolysis. Polarographic analysis was applied to the determination of the metabolites after their separation from urine, plasma and microsomal preparations.

Chlorpromazine-*N*-oxide, a major metabolite of chlorpromazine (Beckett & Hewick, 1967), produces maxima in the ultraviolet spectrum at the same wavelength as the parent drug. As the sulphoxide of chlorpromazine-*N*-oxide exhibits maxima at the same wavelengths as the sulphoxide of chlorpromazine, ultraviolet measurement is unsuitable for the determination of chlorpromazine plus its *N*-oxide metabolites in biological fluids.

Gas-liquid chromatography of chlorpromazine-*N*-oxide produces an elimination product, 2-Cl-10-allyl-phenothiazine, in addition to some reduction product, chlorpromazine (Craig, Mary & Roy, 1964); also chlorpromazine-*N*-oxide sulphoxide gives 2-Cl-10-allyl-phenothiazine sulphoxide and a small amount of chlorpromazine sulphoxide (Beckett & Essien, to be published). Direct estimation of the chlorpromazine-*N*-oxides by g.l.c. is therefore impracticable. Chlorpromazine sulphoxide can be chromatographed unchanged but its broad peak and relatively long retention time preclude the use of g.l.c. determination of low levels of this metabolite.

A direct and sensitive method was therefore sought for the analysis of mixtures of these oxide metabolites to facilitate the determination of the importance of *N*-oxidation and sulphoxidation in the metabolism of chlorpromazine and other phenothiazine drugs.

The electrochemically active species can be measured at concentrations as low as 5×10^{-8}M by cathode ray polarography. This has already been used to measure low concentrations (0·5–0·1 μg ml^{-1}) of chlorpromazine sulphoxide in acid solution (Porter & Beresford, 1966; Porter, 1967). Application of the technique to the determination of mixtures of *N*-oxides and sulphoxide was therefore investigated. Because of the non-destructive nature of polarography it is possible for the *N*-oxide and sulphoxide of chlorpromazine to be recovered virtually unchanged after polarographic

analysis of the solution. Samples of the three oxides and the parent drug, chlorpromazine, were subjected to ultraviolet spectral analysis, and d.c. polarographic investigations over a wide pH range and also to potentiometric titrations, to provide information on the acid-base equilibria and reduction processes under the conditions of solvent extraction and polarographic analysis.

MATERIALS AND METHODS

Apparatus

An automatic microtitrator, Radiometer (Copenhagen) Titrator TTT2, was used for acid-base titration. Ultraviolet spectra were recorded using a Unicam SP 800 spectrophotometer with matched 1 cm silica cells. A cathode ray polarograph (Davis A 1660) was used in the single cell mode and was connected to a fast recorder utilizing ultraviolet light sensitive paper.

A pulse polarograph (PAR Model 174) was used in the sampled d.c. mode with a scan speed of 10 mV s^{-1} and a drop time of 0·5 s. Preparative electrolysis was carried out in a vessel designed by Manousek (Manousek, O., private communication).

The polarographic cell (Fig. 1) was designed for use with the standard calomel electrode instead of a reference mercury electrode because the potential of the former does not fluctuate. The cell was immersed at 25° in a water-bath protected from light.

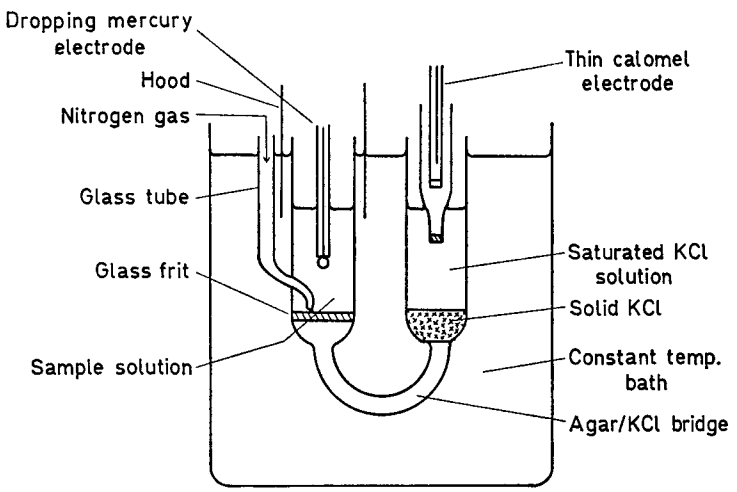

FIG. 1. Polarographic cell.

Reagents and compounds

Britton-Robinson buffers (pH 2 to 12) were prepared by the addition of varying volumes of 0·1N NaOH solution to a stock solution 0·04M in boric, phosphoric and acetic acids (Analar chemicals). 0·05N hydrochloric acid was prepared from the Analar material. n-Heptane and n-butanol were reagent grade. Chlorpromazine-*N*-oxide and the *N*-oxide-sulphoxide were prepared by reacting chlorpromazine base with hydrogen peroxide or *m*-chloroperbenzoic acid and recovering chlorpromazine-*N*-oxide and the *N*-oxide sulphoxide by selective solvent extraction. Chlorpromazine sulphoxide was supplied by Smith Kline and French Ltd. and chlorpromazine hydro-

chloride by May & Baker Ltd.; stock solutions (10^{-3}M) were prepared in fresh double distilled water. Titanous chloride reagent ($TiCl_3$ 30% w/v in HCl 24% w/v) was supplied by BDH.

Methods

(i) *Polarography.* After the sample solution (1–2 ml) had been de-aerated with nitrogen for 3 min, the current-voltage waves were recorded for potentials between 0 and −1·5V, a nitrogen atmosphere being maintained above the sample solution. The peak potential (Ep), peak height (ip) and sensitivity setting were recorded for each polarographic wave using the cathode ray polarograph. Calibration curves (peak height × sensitivity) versus concentration, both expressed on the log scale, were constructed using a stepwise dilution technique, care being taken to flush out undesirable contamination from previous sample solutions. Blank supporting electrolyte solutions were also polarographed.

The variation of $E_{\frac{1}{2}}$ and i_{lim} with pH was studied by recording d.c. polarograms in the cell (Fig. 1) at a concentration of 10^{-4}M and in Britton-Robinson buffers. Preparative microelectrolysis was with 1 ml of 10^{-3}M of the oxide metabolite for a period of 20 h at a potential of −1·33 V. The electrolysis mixture was subjected to solvent extraction at alkaline pH (ammonia 0·88) using n-heptane containing 10% n-butanol and identified by t.l.c. 2 mg of chlorpromazine oxide metabolite in 1 ml aqueous solution was also reacted with 5 drops of titanous chloride reagent for 5 min. The reduction mixture was extracted and the products identified as previously described.

(ii) *Spectrophotometry.* Aliquots (0·2 ml) of 3 × 10^{-3}M solutions of the chlorpromazine metabolites were diluted to 10 ml with Britton Robinson buffer solutions of varying pH (3–12). The ultraviolet absorption of these solutions containing 6 × 10^{-5}M of the metabolites was scanned between 200 and 450 nm using the respective buffer solutions in the reference cell. At the wavelength of maximal change in absorbance, a plot of absorbance against pH was constructed and the pKa value obtained by using the Henderson Hasselbach equation.

(iii) *Acid-base titration.* Aliquots of 0·0025 to 0·005M solutions of the metabolites in water were titrated separately with 0·05N hydrochloric acid and NaOH solutions using the automatic titrator, and the pKa values were estimated from the pH values of half neutralization.

(iv) *Separation and analysis of the* N-*oxides and sulphoxide of chlorpromazine in biological samples.* Chlorpromazine and its metabolites were separated from biological samples by the use of control of pH, ion-pair formation, selective solvent extraction and selective reduction of the *N*-oxide moiety with SO_2 (see Appendix for outline of method; Beckett and Essien, to be published).

(v) *Preparation of organic extracts for polarography.* Organic extracts were evaporated under reduced pressure in the dark, using a rotary film evaporator. The flask containing the residue was then removed under a nitrogen atmosphere, and the residue was dissolved in 1 to 2 ml of supporting electrolyte and polarographed. Where necessary, the residue after evaporation was initially dissolved in 0·2 ml of ethanol before the final solution was made up with the supporting electrolyte.

(vi) *Thin-layer chromatography (t.l.c.).* Chlorpromazine and its oxide metabolites,

and the chemical and electrochemical reduction products of the latter were chromatographed in the dark, on silica gel G (Merck) plates (0·5 mm) using the solvent system, benzene–methanol–diethylamine (75 : 15 : 10 v/v). The plates were sprayed with 50% v/v sulphuric acid.

RESULTS AND DISCUSSION

Cathode ray polarographic behaviour

With the use of a standard calomel electrode as the reference electrode in the cell described (Fig. 1), stable potential readings were maintained throughout the polarographic measurements. Chlorpromazine-*N*-oxide and the sulphoxide showed well defined peaks in acidic media pH 1–6 (Fig. 2) of equal height in the concentration range 10^{-5} to 5×10^{-8}M.

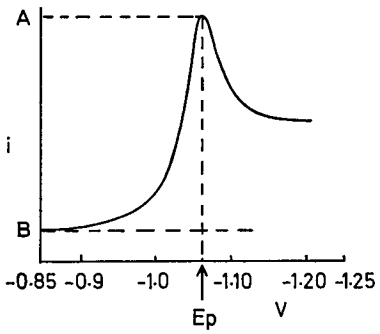

FIG. 2. Cathode ray polarogram of chlorpromazine-*N*-oxide (10^{-5} to 10^{-7}M) in Britton-Robinson buffer pH 5. Ep = peak potential; AB = peak height.

The *N*-oxide sulphoxide gave a wave whose height was twice that of the *N*-oxide or sulphoxide at pH 1–6 and concentration 10^{-5} to 5×10^{-8}M. When polarographed in 0·05N hydrochloric acid (start potential—0·5 V), the three oxide derivatives of chlorpromazine gave reduction peaks (Ep) at the potentials, —0·74 ± 0·01 V. The Ep for the three compounds in Britton Robinson buffer pH 5 was —1·06 ± 0·01 V (start potential —0·85 V).

The above system was therefore adapted (see later) for the analysis of chlorpromazine because chlorpromazine-*N*-oxide is not stable in solutions of low pH, and analytically unsuitable waves are given by the three oxide metabolites of chlorpromazine at alkaline pH values.

Direct current polarography, microelectrolytic and chemical reduction

In the d.c. polarographic investigation, using the sampled d.c. mode of the pulse polarograph, both chlorpromazine-*N*-oxide and the sulphoxide at concentration 10^{-4}M showed no variation of the limiting current (i_{lim}) with pH in the pH range 1–6. The wave heights for both oxides were identical. The *N*-oxide-sulphoxide showed no variation of i_{lim} with pH in the range 1–6 and its wave height was twice that of the *N*-oxide or sulphoxide. The change in half-wave potential with pH ($dE_{\frac{1}{2}}/dpH$) for the three oxides was 95 ± 5 mV/pH unit in this range (i.e. 90 mV for *N*-oxide, 95 mV for sulphoxide and 100 mV for the *N*-oxide sulphoxide were obtained).

The polarographic wave of the *N*-oxide (10^{-3}M, pH 5) was analysed using the equation for irreversible electrode reductions:

$$E = E_{\frac{1}{2}} - \frac{0{\cdot}059}{\alpha n_\alpha} \log i/i_d\text{-}i, \text{ (Meites, 1965),}$$

where E is the potential of the mercury drop; $E_{\frac{1}{2}}$ is the half-wave potential; α is the transfer coefficient for the electrode reaction; n_α is the number of electrons involved in the rate-determining electron-transfer step; i_d is the diffusion current; i is the positive current causing the reduction to proceed, and i is corrected for the residual current at each potential.

A plot of E versus log $i/(i_d\text{-}i)$ gave a slope of 0·112V and intercept on the E axis of −1·07 V which corresponded to the $E_{\frac{1}{2}}$ value (Fig. 3). This gave a value of $\alpha n_\alpha = 0{\cdot}53$. Similar analyses were carried out on the waves of chlorpromazine sulphoxide which agreed with $E_{\frac{1}{2}}$ values of the compounds and an αn_α value of 0·5 in the case of the sulphoxide (Fig. 3). This plot gave two linear portions in the case of the double oxide, chlorpromazine-*N*-oxide sulphoxide, and the implication is discussed later.

The number of protons p involved in the rate-determining steps of the electrochemical reductions was calculated from the equation

$$dE_{\frac{1}{2}}/dpH = \frac{-0{\cdot}059}{\alpha n_\alpha} \cdot p \text{ (Meites, 1965),}$$

and the value of 0·81 was obtained for both chlorpromazine-*N*-oxide and the sulphoxide.

Since αn_α values between 0·3 and 0·6 usually refer to slow reactions involving one electron, and p values are near unity, it is reasonable to assume that the rate-determining step in the polarographic reduction of the *N*-oxide and the sulphoxide is the addition of an electron and a proton followed by a rapid addition of the remaining electron and proton in both cases.

After preparative microelectrolytic reduction of 10^{-3}M solution of chlorpromazine *N*-oxide, the reduction wave height dropped to 35–30% its original height, and the polarographic scan from zero potential showed no appearance of a reduction wave other than that of the remaining chlorpromazine *N*-oxide. The t.l.c. analysis of the extract of the electrolysis mixture using reference chlorpromazine oxide metabolite and chlorpromazine, and the solvent system benzene–methanol–diethylamine (Beckett & Hewick, 1967) showed that the only reduction product was chlorpromazine. Chlorpromazine is the product of chemical reduction of each of the three oxides using $TiCl_3$/HCl reagent (Beckett & Essien, to be published; Brooks & Sternglanz, 1959).

It is therefore deduced that the total electrochemical reaction involved two protons and two electrons with an initial slow step as described above, and the loss of the oxygen atom. The total reaction is represented in Fig. 4 for both *N*-oxide and sulphoxide.

The two linear portions in the plot of E versus log $i/i_d\text{-}i$ (Fig. 3) for the double oxide, chlorpromazine-*N*-oxide sulphoxide, indicate that more than one slow step is involved in the electrochemical reduction. The values of αn_α for the first and second portions of the plot were 0·3 and 0·39, respectively, and the corresponding p values were 0·5 and 0·73. This indicates two slow steps in the reduction of the double oxide, each involving one electron and one proton. The involvement of 4 protons and 4 electrons in the

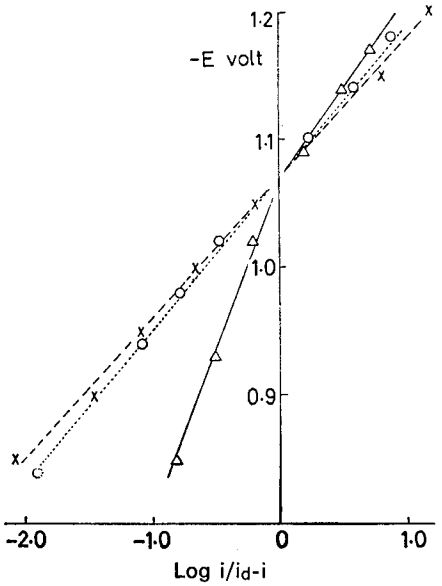

FIG. 3. Graphs of E versus log i/id-i for chlorpromazine-*N*-oxide (×), chlorpromazine sulphoxide (○) and chlorpromazine-*N*-oxide-sulphoxide (△) all at 10^{-4}M. (DC polarography in Britton-Robinson buffer pH 5).

complete reduction of the double oxide is represented in Fig. 4. Its polarographic wave in the pH range 1–6 and at concentrations 10^{-4} to 10^{-3}M appears to have two components which merge into one at lower concentrations, i.e. 10^{-5} to 5×10^{-8}M.

Spectral behaviour

Within the pH range of 1 to 12, chlorpromazine-*N*-oxide showed a constant ultraviolet spectrum identical with that of chlorpromazine between the wavelengths 230 to 350 nm. Similarly, the *N*-oxide sulphoxide exhibited no observable shift of absorption bands in its spectrum with varying pH values (1–12), and its spectrum was virtually that of chlorpromazine sulphoxide (Felmeister & Discher, 1964; Warren, Eisdorfer & others, 1966; Wallace & Biggs, 1971). However, the sulphoxide showed four principal bands in its ultraviolet spectrum over the pH range 1–8 (240, 275, 300 and 342 nm); with further increase in pH, this pattern was replaced by one with principal bands at 240, 276, 303 and 345 nm. Using a wavelength of 305 nm for analysis, we found a pKa of 9·3 which corresponds to that of the basic group of the chlorpromazine molecule previously reported by Sorby, Plein & Benmaman (1966), who used potentiometric titration (see below).

Potentiometric titration

Chlorpromazine-*N*-oxide in water (pH 6·5) was titrated with hydrochloric acid to give an end point which corresponds to a 1 : 1 reaction and a pKa value of 4·7 at the mid-point of the titration step (c.f. pKa's 4·9, 4·7, 4·8 and 4·7 for the *N*-oxides of nicotine, imipramine, chlorcyclizine and trimethylamine, respectively, reviewed by Jenner, 1971). The *N*-oxide sulphoxide of chlorpromazine, titrated from pH 5·8 with hydrochloric acid, gave a similar end point and a pKa value of 4·7 corresponding to the *N*-oxide group, whereas titration with alkali showed an end point corresponding to

1 : 1 reaction and pKa value of 7·0 for the sulphoxide group. When titrated with alkali, chlorpromazine sulphoxide in water (pH 5·5) showed two pKa values, 7·2 and 9·3, for the sulphoxide and basic groups, respectively. The pKa value of 9·2 obtained for chlorpromazine hydrochloride was in good agreement with the values of 9·21 and 9·3 previously reported by Sorby & others (1966) and Marshall (1955), respectively.

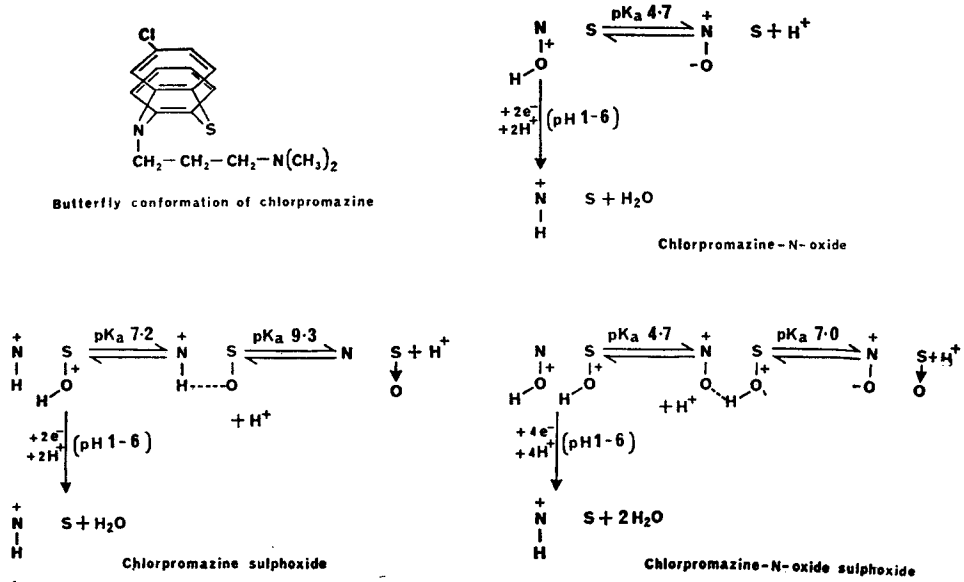

FIG. 4. The acid-base equilibria and polarographic reduction of the oxide metabolites of chlorpromazine. A protonated intermediate of chlorpromazine sulphoxide has been previously suggested by Felmeister & Discher (1964). Only the 'S' and the basic 'N' are shown in the reactions.

Acid-base equilibria existing in aqueous solutions of the oxide metabolites of chlorpromazine

The protonated species of chlorpromazine-*N*-oxide, the sulphoxide and *N*-oxide sulphoxide undergoing polarographic reduction are shown in Fig. 4. The shift in the ultraviolet spectrum of chlorpromazine sulphoxide (pH 8 to higher pH values) indicates the change from ionized to molecular species (neutral molecule) which affects the absorption of the ring system of the molecule. This effect is attributed to hydrogen bonding between the proton on the aliphatic nitrogen and the oxygen on the sulphur atom of the phenothiazine ring system in the ionized species because of the 'butterfly' conformation of the ring system (Gordon & others, 1964) (Fig. 4). The lack of observable change with pH in the spectrum of chlorpromazine-*N*-oxide sulphoxide indicates the absence of, or very weak, hydrogen bonding between the protonated *N*-oxide and sulphoxide groups. The polarographic, spectral and potentiometric steps and the titration results for the above chlorpromazine metabolites are shown in Fig. 4.

Analytical application of polarography to the determination of chlorpromazine oxides

There is a linear relation between the polarographic measurements and concentrations of the chlorpromazine oxide derivatives (Fig. 5) within the range 10^{-5} to

5×10^{-8}M. The calibration factor F is given by—

$$\log \frac{\text{peak height (ip)} \times \text{sensitivity}}{\text{concentration}}$$

Equal concentrations of chlorpromazine-*N*-oxide and chlorpromazine sulphoxide give equal reduction peak heights with the factor F of 8·0, whereas the *N*-oxide sulphoxide (F = 8·3) gives a peak height twice that of the corresponding concentration of the *N*-oxide or the sulphoxide.

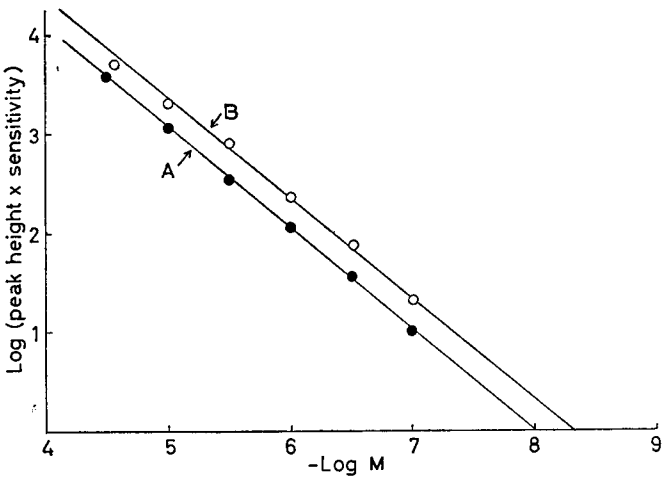

FIG. 5. Polarographic calibration curves for the determination of chlorpromazine oxides in Britton-Robinson buffer pH 5. ● = curve for chlorpromazine-*N*-oxide and chlorpromazine sulphoxide. ○ = curve for chlorpromazine-*N*-oxide-sulphoxide.

Table 1. *Recoveries of the* N-*oxide* (*CPZNO*), N-*oxide sulphoxide* (*CPZNOSO*) *and sulphoxide* (*CPZSO*) *of chlorpromazine* (*CPZ*) *from urine samples containing various concentrations of added compounds* (*analysis by cathode ray polarography*).

Compounds	Quantities added to urine (mol)	Quantities recovered (mol)
CPZNO	1×10^{-7}	$0{\cdot}98 \times 10^{-7}$
CPZNSO	1×10^{-7}	$0{\cdot}96 \times 10^{-7}$
CPZSO	1×10^{-7}	$0{\cdot}97 \times 10^{-7}$
CPZNO	1×10^{-7}	$0{\cdot}98 \times 10^{-7}$
CPZNOSO	1×10^{-8}	$0{\cdot}97 \times 10^{-8}$
CPZSO	1×10^{-8}	$0{\cdot}97 \times 10^{-8}$
CPZNO	1×10^{-8}	$0{\cdot}99 \times 10^{-8}$
CPZNOSO	1×10^{-9}	$0{\cdot}98 \times 10^{-9}$
CPZSO	1×10^{-9}	$0{\cdot}96 \times 10^{-9}$
CPZNO	2×10^{-8}	$1{\cdot}9 \times 10^{-8}$
CPZNOSO	1×10^{-9}	$0{\cdot}97 \times 10^{-9}$
CPZSO	1×10^{-8}	$0{\cdot}98 \times 10^{-8}$

See Appendix showing separation scheme.

When the oxide metabolites of chlorpromazine were added to urine they could be determined quantitatively by the above polarographic method (Table 1). Similar results were obtained for plasma and deactivated liver microsomal preparations.

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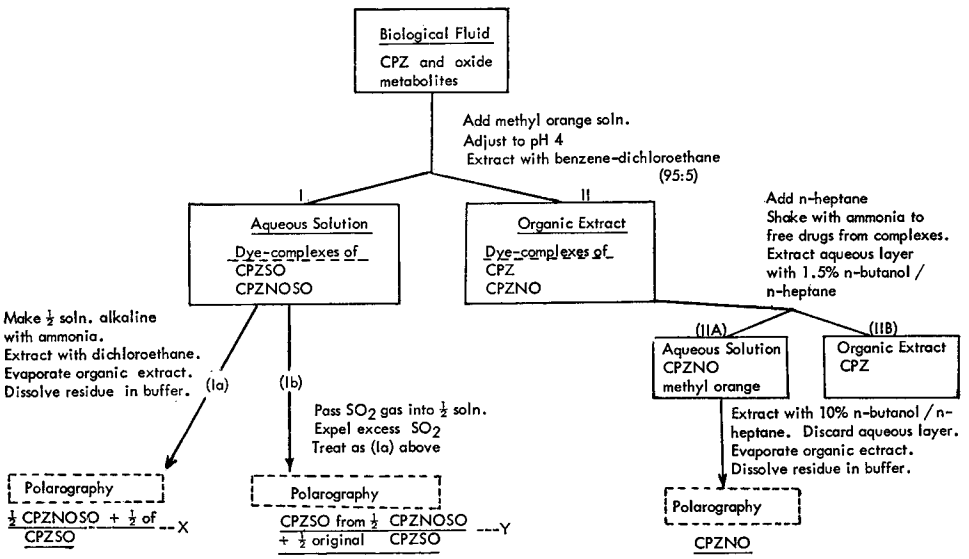
Appendix

Separation and analysis of the *N*-oxides and sulphoxide of chlorpromazine in biological samples by the use of the control of pH and by ion-pair formation, selective solvent extraction, selective reduction and cathode ray polarography.



NOTE: CPZNOSO is reduced by SO_2 to CPZSO. Polarographic reduction peak height of CPZNOSO = 2 × peak height of CPZSO.

X–Y = polarographic value for the NO of ½ CPZNOSO.
= ¼ of total CPZNOSO.
4(X–Y) = total CPZNOSO
2X–4(X–Y) = total sulphoxide.
2(2Y–X) = total sulphoxide.