Journal of Medicinal and Pharmaceutical Chemistry

VOL. 1, No. 3 (1959)

Spectrophotometric Method for the Determination of Chlorpromazine and Chlorpromazine Sulphoxide in Biological Fluids

T. L. FLANAGAN, T. H. LIN, W. J. NOVICK, I. M. RONDISH, C. A. BOCHER and E. J. VAN LOON

Research and Development Division, Smith Kline and French Laboratories, Philadelphia 1, Pa.

Introduction

A number of investigators have developed colorimetric methods for the determination of the *N*-substituted phenothiazines.[1-6] Essentially, all of these methods involve a solvent extraction of an alkaline biological medium followed by a salt isolation and subsequent colour development with a concentrated mineral acid or a dilute mineral acid plus a ferric salt or other oxidizing agent.

In 1956 Berti and Cima[7] published a method by which chlorpromazine* was removed from biological media by treatment with Amberlite IRC-50. The resin in turn was treated with concentrated sulphuric acid, producing an amaranth-red colour which was proportional to the phenothiazine content of the resin. In 1957 Forrest and Forrest,[8] and Cavanaugh and Ervin[9] developed simple colorimetric tests for the detection of chlorpromazine in body fluids with the elimination of the solvent extraction step. However, these colorimetric procedures do not differentiate between chlorpromazine and some of the metabolites of chlorpromazine.

Salzman and Brodie[10] published a method in which they quantitatively determined chlorpromazine and chlorpromazine sulphoxide† in biological material by extracting the two components from an alkaline medium into an organic solvent. The sulphoxide was isolated in an acetate buffer at pH 5·6, while the chlorpromazine was isolated in 0·1 N hydrochloric acid. Both compounds were determined spectrophotometrically.

* 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine.

† 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine-5-oxide.

A quantitative spectrophotometric procedure has been developed in our laboratory for the determination of free and bound chlorpromazine and chlorpromazine sulphoxide in biological fluids.

Method

The biological specimen is made alkaline (pH 12) and chlorpromazine and its sulphoxide in the form of free bases are extracted with ether. The ether is washed to remove impurities. The two compounds are isolated from the ether by extraction with sulphuric acid and their concentration determined spectrophotometrically. These are designated 'free' chlorpromazine and 'free' chlorpromazine sulphoxide.

It was found in biliary and urinary specimens that additional amounts of chlorpromazine and chlorpromazine sulphoxide remained in the aqueous residue after exhaustive ether extraction.

In 1954 Schmalz and Burger[11] reported on the reduction of phenothiazine sulphoxides by refluxing with concentrated hydrochloric acid. Acid hydrolysis of the biological specimens with hydrochloric acid was tried, and this led to increased recoveries of chlorpromazine, but in this process variable quantities of the sulphoxide were converted to chlorpromazine, depending upon the acid concentration and the duration of the hydrolysis. Therefore alkaline hydrolysis of the biological specimens was used, and increased quantities of chlorpromazine and its sulphoxide were obtained.

The aqueous residue from the free extraction is subjected to an alkaline hydrolytic treatment. Following hydrolysis and subsequent cooling, the extraction and spectrophotometric procedures are repeated. The additional chlorpromazine and chlorpromazine sulphoxide which are obtained from the second extraction technique are termed 'bound' chlorpromazine and 'bound' chlorpromazine sulphoxide.

Apparatus and Reagents

(1) Recording spectrophotometer, Process and Instrument Co., Model R.S., No. 3.
(2) Centrifuge, International Equipment Co., Model No. B.E.
(3) Distilled water and reagent grade chemicals were used in the preparation of all solutions.

Experimental

Free Determination—Urine Specimen

Place 5 ml of urine in a 125-ml separating funnel; add 5 ml of water and a few drops of 10 N NaOH to bring the pH of the solution to 12. Add 25 ml of ether and shake vigorously for 2 min. Allow the phases to separate, and draw off the aqueous phase into a second 125-ml separatory funnel. Decant the organic phase into a 250-ml separatory funnel. If the sample should emulsify, transfer the contents to a 40-ml centrifuge tube, centrifuge for a few minutes at 3,000 rev/min, and then transfer the clear ether layer to a 250-ml separatory funnel by means of a plasma lifter or a dropping pipette. Repeat the ether extractions four more times. Mechanical shaking may be substituted for manual shaking, particularly when the number of samples is large.

Combine the ether extracts in a 250-ml separatory funnel. Add 1 ml of 0·1 N NaOH and shake vigorously for 1 min. Allow the layers to separate and draw off the alkali into the urine residue. Repeat the alkali washing procedure until this layer becomes clear, always adding the alkali layer to the residue. Save the residue for the determination of bound chlorpromazine and bound chlorpromazine sulphoxide. Add 2 ml of water to the ether layer and once again shake vigorously for 1 min. Allow the layers to separate, and after 5 min draw off the water layer and discard.

Add 5 ml of 1 N H_2SO_4 to the ether layer in the separatory funnel and shake vigorously for 3 min. Allow the layers to separate for 10 min. Draw off the acid layer into a beaker. If the acid layer is not clear, centrifuge for 3 min. Additional acid extractions are advisable to insure removal of the drug from the ether phase when the drug concentration in the sample is high.

Transfer the clear acid solution to a 1-cm cuvette and determine the absorption spectrum of the sample over the range of 220 to 320 mμ on a recording (or manual) spectrophotometer, using 1 N H_2SO_4 in the blank cell. If the sample is too concentrated, dilute it accordingly, using 1 N H_2SO_4 as the diluent.

The ultraviolet spectra of chlorpromazine and chlorpromazine sulphoxide are such that they can be determined individually from a single spectrum curve.

A typical mixture (chlorpromazine and chlorpromazine sulphoxide) type curve is indicated by absorption at 240 and/or 255 mμ, 274 (secondary peak for chlorpromazine sulphoxide) and 305 mμ (common peak for chlorpromazine and chlorpromazine sulphoxide). The concentration of chlorpromazine, in the presence of its sulphoxide, is calculated using a background cancellation method. A diagonal line is drawn connecting the points on the spectrum at 236 and 265 mμ. The absorbance is measured from the point on the spectrum at 255 mμ (maximum absorption for chlorpromazine) down to the corresponding point on this diagonal line. If a spectrum is obtained which indicates the presence of only chlorpromazine, the same method of calculation is employed. The concentration of chlorpromazine sulphoxide is calculated by subtracting the absorbance obtained at 315 mμ (trough) from the absorbance at 270 mμ. The concentrations of chlorpromazine and chlorpromazine sulphoxide are read from a standard curve prepared for each compound by obtaining the ultraviolet spectra of standard solutions in 1 N H_2SO_4 and plotting the absorbances determined, as described above, against concentration.

The chlorpromazine sulphoxide results may be expressed in terms of chlorpromazine by multiplying by a factor of 0·953.

$$\frac{\text{(M.W. of chlorpromazine)}}{\text{(M.W. of chlorpromazine sulphoxide)}} = 0 \cdot 953$$

Bound Determination—Urine Specimen

For the determination of bound chlorpromazine and chlorpromazine sulphoxide, take the aqueous residue after the extraction of the free drug and remove the ether remaining in the residue by warming in hot water with constant shaking. Add NaOH pellets to the residue to bring the concentration of NaOH in this medium to approximately 3 per cent. After the pellets dissolve, place the medium in a boiling water bath for one hour. Cool to room temperature and extract the medium with five 40-ml portions of ether, using 125-ml separatory funnels for the extractions. Pool the ether extracts in a 250-ml separatory funnel and then continue the analysis as outlined in the previous section. The quantities

obtained may be added to the amounts of free drug and expressed as total drug per ml of urine.

If, when undergoing the same procedure, either in the determination of the free or bound drug, the biological material itself gives rise to ultraviolet absorption that interferes with the determination of chlorpromazine and/or chlorpromazine sulphoxide, a blank should be obtained and allowance be made in calculation of chlorpromazine and chlorpromazine sulphoxide.

In case only the total drug content in the urine is desired, the determinations of the free and the bound drug may be combined. Add NaOH pellets to the urine sample to bring the concentration of NaOH to approximately 3 per cent, and subject it to an alkaline hydrolysis initially by heating the alkaline sample in a boiling water bath for 1 h. Cool, and extract the hydrolysed residue with five 40-ml portions of ether and determine the amount of chlorpromazine and chlorpromazine sulphoxide as outlined for the determination of the free drug. It appears that the same method can be applied to the determination of total drug content in bile samples, though its applicability to other biological samples has yet to be established experimentally.

Specificity of the Method

Bile and urine specimens from dogs that received chlorpromazine were analysed for free and bound chlorpromazine and chlorpromazine sulphoxide by this procedure. The ultraviolet absorption spectra of the acid extracts in the free determination were the same as those of authentic mixtures of the two components. Additional proof of the specificity of the method was obtained by chromatography.

The acid extract from the free determination was made alkaline, and extracted with ether. The ether extract was reduced in volume under vacuum and then applied to Whatman 3 MM filter paper. Ascending paper chromatography of this material and authentic samples of chlorpromazine and chlorpromazine sulphoxide was carried out in a freshly prepared solvent system consisting of isoamyl alcohol-ethyl alcohol-formic acid-water in a ratio of 100:15:10:100, the upper layer being used as the mobile phase. After drying in air the paper was sprayed with

nitrite reagent which consisted of a few crystals of sodium nitrite dissolved in 10 c.c. of concentrated hydrochloric acid.[12] Chlorpromazine and chlorpromazine sulphoxide are stained pink immediately upon contact with this reagent. Two spots were observed on the chromatograms; one at *Rf* 0·70 was identical with authentic chlorpromazine, while the second at *Rf* 0·50 was identical with authentic chlorpromazine sulphoxide.

When a portion of the aqueous phase following the free extraction was chromatographed in this system, a single large spot was obtained in the *Rf* region of 0·05–0·12. No spots were observed in the region of the authentic compounds.

Following the alkaline hydrolysis step and subsequent ether and acid extractions, the ultraviolet spectrum of the acid was the same as authentic mixtures of chlorpromazine and chlorpromazine sulphoxide. The acid extract was made alkaline and the chromatography was carried out as described previously. Two spots were again obtained which corresponded to authentic samples of chlorpromazine and chlorpromazine sulphoxide.

The aqueous residue following the alkaline hydrolysis did not show any spots corresponding to the authentic samples.

To further check the authenticity of the method, extracts from biological specimens which had been analysed by the free and bound procedures were chromatographed in the solvent system of Salzman and Brodie,[10] and in every case spots were obtained which corresponded to authentic samples of chlorpromazine and chlorpromazine sulphoxide.

Discussion and Results

In the described procedure, free and bound chlorpromazine and its sulphoxide are determined spectrophotometrically in biological specimens. To obtain the bound components, the biological specimens are subjected to an alkaline hydrolysis. Recoveries of known quantities of chlorpromazine and chlorpromazine sulphoxide from bile and urine specimens ranged from 91 to 101 per cent. However, when aqueous solutions of chlorpromazine and chlorpromazine sulphoxide were subjected to the same treatment as the biological specimens (i.e. the alkaline hydrolysis step), the recovery of chlorpromazine was low. Fels, *et al.*[13]

observed that in N sodium hydroxide the absorption peak characteristic of chlorpromazine decreased with time. It appears that in biological media some protection is afforded the chlorpromazine, as shown by the recovery data in Table I.

A comparison was made of the chlorpromazine and chlorpromazine sulphoxide content of the same biological fluids using the Salzman–Brodie and the present procedures. The results (Table II) show that larger quantities of the drug and its metabolite were found in the specimens by our procedure. In this comparison the spectrophotometric results obtained by the Salzman-Brodie method were calculated as they had outlined and by the background cancellation technique. The results obtained by the two calculation procedures were similar.

An additional heptane-isoamyl alcohol extraction did not increase the amount of free drug obtained by the Salzman and Brodie technique, and since this procedure stops at the determination of the free components, it cannot account for the components present in the bound form. However, for purposes of comparison, the aqueous residues from the S and B procedure were subjected to an alkaline hydrolysis and the hydrolysate extracted with heptane–isoamyl alcohol. The results indicate that this procedure is not as efficient as the present one for the determination of the bound drug in alkaline hydrolysed samples.

The described procedure has also been applied to the determination of free and bound chlorpromazine and chlorpromazine sulphoxide in bile, plasma, intestinal fluid, gastric juice, spinal fluid, and milk, and could be used conceivably for the analysis of saliva. The volume of ether used for extraction may be adjusted according to the volume of the sample and the drug content expected in the sample. Each sample should contain at least 5μg of chlorpromazine or chlorpromazine sulphoxide.

Milk samples from bitches primed with chlorpromazine have been analysed and shown to contain free and bound chlorpromazine and its sulphoxide. During the process of ether extraction, milk has a strong tendency to form emulsions from which it is difficult to recover the ether. The best results have been obtained by adding approximately equal volumes of 20 per cent NaOH to the milk sample and heating the mixture in a boiling water bath for 1 h, followed by the procedure for the spectrophotometric

Table I. Recoveries of chlorpromazine and chlorpromazine sulphoxide from various media

Media	Treatment (hydrolysis)	Chlorpromazine			Chlorpromazine sulphoxide		
		μg added	μg found	% recovery	μg added	μg found	% recovery
Water	None	100	92	92	—	—	—
Water	None	2,000	1,941	97	—	—	—
Water	None	—	—	—	100	96	96
Water	None	—	—	—	2,000	1,955	98
Water	Alkaline	2,000	1,414	71	—	—	—
Water	Alkaline	—	—	—	2,000	2,034	102
Water	Alkaline	1,000	653	65	1,000	965	97
Bile	None	100	91	91	—	—	—
Bile	None	—	—	—	100	93	93
Bile	Alkaline	2,000	1,951	98	—	—	—
Bile	None	1,000	925	93	1,000	1,012	101
Bile	Alkaline	1,000	951	95	1,000	957	96
Urine	None	2,000	1,947	97	—	—	—
Urine	None	—	—	—	2,000	1,944	97
Urine	Alkaline	2,000	1,834	92	—	—	—
Urine	None	1,000	909	91	1,000	909	91
Urine	Alkaline	1,000	922	92	1,000	913	91

Table II. Comparison between Salzman–Brodie method and the present procedure

Specimen	Method of analysis	Method of calculation	Results in terms of μg chlorpromazine hydrochloride						Total chlorpromazine (free and bound)
			free			after alkaline hydrolysis			
			chlor-promazine	chlor-promazine sulphoxide	total as chlor-promazine	chlor-promazine	chlor-promazine sulphoxide	total as chlor-promazine	
Bile	Present	B.C.M.*	72	28	100	116	234	350	450
	S–B	S–B	66	18	84	75	170	245	331
	S–B	B.C.M.	73	19	92	74	188	262	354
Urine	Present	B.C.M.	13	20	33	6	67	73	106
	Total	B.C.M.	—	—	—	15	85	100	100
	S–B	S–B	3	23	26	0	21	21	47
	S–B (Additional solvent extraction)	S–B	0	0	0	—	—	—	—
	S–B	B.C.M.	2	21	23	0	22	22	45

* B.C.M. = Background cancellation method.

determination of chlorpromazine and chlorpromazine sulphoxide previously described.

In the absorption spectrum for chlorpromazine sulphoxide the characteristic secondary peak occurs at 274 mμ. However, in our procedure the absorbance for chlorpromazine sulphoxide is obtained by subtracting the absorbance at 315 mμ (trough) from the absorbance at 270 mμ. This latter wavelength was selected because chlorpromazine has the same absorbance at 270 mμ as

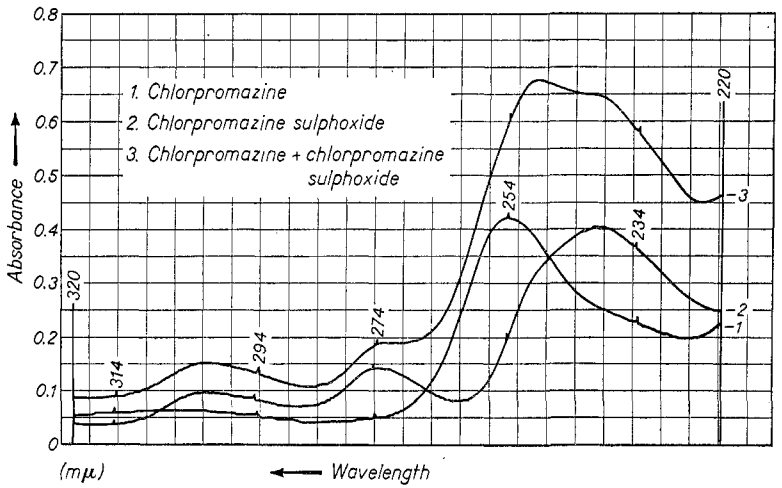

Fig. 1. Ultra-violet spectra of chlorpromazine, chlorpromazine sulphoxide, and a mixture of chlorpromazine and chlorpromazine sulphoxide.

at 315 mμ. Hence, any increase in absorbance at 270 mμ, when specimens containing mixtures of the two components are analysed, is attributable to chlorpromazine sulphoxide.

Using the above procedure, the ether extractable chlorpromazine metabolites are determined as either chlorpromazine or its sulphoxide. If demethylation of the side chains occurs,[14] the demethylated components would be determined as chlorpromazine and/or chlorpromazine sulphoxide, since these components have absorption spectra similar to the parent compounds.

Fig. 1 shows the ultraviolet spectra of chlorpromazine, chlorpromazine sulphoxide, and a mixture of chlorpromazine and chlorpromazine sulphoxide.

Although it has been shown by this procedure that chlorpromazine and its sulphoxide are bound in biological media, we have not identified the substance or substances which bind these compounds.

At the present time we are adapting this method to the determination of chlorpromazine and chlorpromazine sulphoxide in various body tissues.

Summary. A method has been developed for the determination of free and bound chlorpromazine and free and bound chlorpromazine sulphoxide in biological fluids. The free compounds are determined by extracting with ether an alkaline aliquot of the biological fluid to remove the two components. They are re-extracted into acid and both compounds are determined spectrophotometrically.

For the determination of the bound chlorpromazine and bound chlorpromazine sulphoxide, the aqueous residue from the free extraction is made strongly alkaline and hydrolysed. After cooling, the extraction and spectrophotometric procedures are repeated.

The procedure has been applied to the determination of these components in bile, urine, plasma, intestinal fluid, gastric juice, spinal fluid, and milk specimens. Recoveries of known quantities of chlorpromazine and chlorpromazine sulphoxide from biological fluids range from 91 to 101 per cent.

(*Received 27 April, 1959.*)

References

[1] Berti, T. and Cima, L. *Arch. int. pharmacodyn.*, **98,** 452 (1954)
[2] Brignon, J. J. *Bull. soc. pharm. Nancy*, No. 25, June (1955)
[3] Dubost, P. and Pascal, S. *Ann. pharm. franç.*, **11,** 615 (1953)
[4] Dubost, P. and Pascal, S. *Ann. pharm. franç.*, **13,** 56 (1955)
[5] Kok, K. *Acta physiol. pharmacol. néerl.*, **4,** 388 (1955)
[6] Leach, H. and Crimmin, W. R. *J. Clin. Path.*, **9,** 164 (1956)
[7] Berti, T. and Cima, L. *Farmaco*, **11,** 451 (1956)
[8] Forrest, F. M. and Forrest, I. S. *Amer. J. Psychiat.*, **113,** 931 (1957)
[9] Cavanaugh, D. J. and Ervin, F. R. *Amer. J. Psychiat.*, **114,** 171 (1957)
[10] Salzman, N. P. and Brodie, B. B. *J. Pharmacol.*, **118,** 46 (1956)
[11] Schmalz, A. C. and Burger, A. *J. Amer. chem. Soc.*, **76,** 5455 (1954)
[12] Sprogis, G. R., Lezdins, V., White, S. D., Ming, C., Lanning, M., Drake, M. E. and Wyckoff, G. *Amer. J. ment. Defic.*, **61,** 737 (1957)
[13] Fels, I. G., Kaufman, M. and Karczmar, A. G. *Nature, Lond.*, **181,** 1266 (1958)
[14] Ross, Jr., J. J., Young, R. L. and Maass, A. R. *Science*, **128,** 1279 (1958)