ATYPICAL CONDITIONS FOR QUANTITATIVE RECOVERY OF ACEPROMAZINE AND CHLORPROMAZINE FROM PLASMA

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Recoveries of acepromazine and chlorpromazine from equine plasma were examined. Recoveries of both drugs from plasma were poor under theoretically optimal conditions for basic drugs. When a wide range of extraction pH was examined, it was found that more complete recoveries of these drugs from plasma were achieved at pH 5-6. Use of [³H]chlorpromazine showed that the rate of migration of the drug from an aqueous to a nonpolar environment was much faster at pH 6.0 than at pH 9.2 from both plasma and buffer solutions. Times required for equilibration with agitation were 15 min at pH 6.0, 1 h at pH 9.2, and 2 h at pH 11.0. With these agitation times and pH values, recoveries were more than 95% complete.

INTRODUCTION

Acepromazine, a phenothiazone derivative, is a widely used tranquilizer in equine medicine. Like most phenothiazines, it blocks a range of central effects including locomotor activity, respiratory response, and control of body temperature (Tobin and Ballard, 1979). Since horses retain much of their coordination and alertness while becoming easier to handle with acepromazine, the drug is frequently used in the transport of valuable or unruly animals. It may be used in competition in small doses to calm an otherwise excitable animal and allow it to perform in a more relaxed manner. It is also used in veterinary medicine as a preanesthetic during surgical procedures, sometimes in conjunction with other agents such as potent analgesics. Because acepromazine is widely used and is prohibited in most racing situations, sensitive and specific methods of detection are needed to test for its presence in performance horses (Booth, 1978).

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We found that standard procedures for the detection of basic drugs in biological fluids yielded surprisingly poor recoveries of phenothiazines. Methods described by Curry (1968) and by Bailey and Guba (1979) were helpful in the detection of chlorpromazine in human serum, but required complex extraction procedures, which could affect yields. Since there have been few studies of the extraction and recovery of nanogram quantities of acepromazine in biological fluids, we developed a simple, specific method utilizing a gas-liquid chromatograph equipped with a nitrogen/phosphorus detector. We report here some unexpected behavior of acepromazine and chlorpromazine in plasma and buffer samples and show how this behavior may lead to anomalously low recoveries of these drugs from biological systems.

MATERIALS AND METHODS

For the initial extraction experiments (Fig. 1), we used 3-ml samples containing various concentrations of plasma diluted with 50 mM phosphate buffer (pH 7.4). Samples were spiked with 0.5 ml of a 100 μg/ml solution of the indicated drug and mixed on a vortex mixer. This was followed by addition of 2 ml saturated tetraborate solution and 2 ml dichloromethane (DCM). The tubes were then agitated for 5 min. The samples were centrifuged for 1 h at 0°C to break up the thick emulsion that was formed. The aqueous layer was discarded, and 1 ml DCM was removed and evaporated to dryness under a stream of N_2 at 24°C. The dried residue was reconstituted with 50 μl cyclohexane, and 5 μl was injected onto the gas chromatograph for analysis.
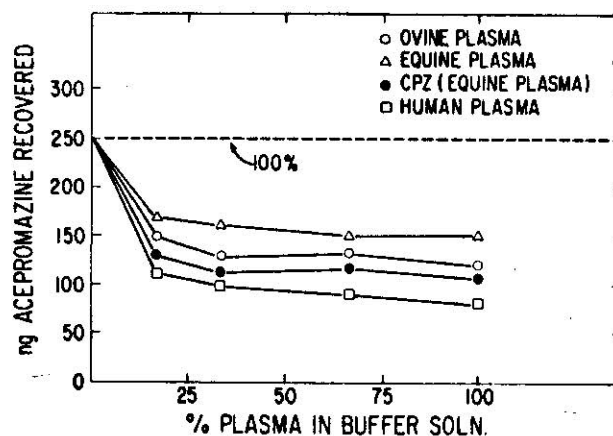

FIGURE 1. Partial recovery of acepromazine from plasma. Acepromazine (250 ng) was added to 3 ml plasma and sufficient 50 mM phosphate buffer (pH 7.4) was added to yield the indicated dilutions of plasma. Samples were then adjusted to pH 9.2 as described in the text and extracted into DCM. The points show recovery of acepromazine from different dilutions of ovine (o), equine (Δ), and human (□) plasmas and also recovery of chlorpromazine (CPZ) from equine plasma (●).
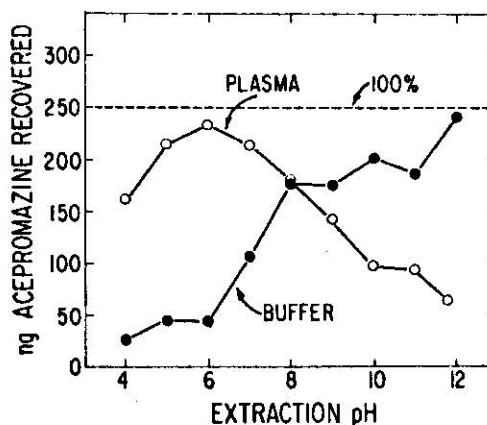
FIGURE 2. Recovery of acepromazine from spiked plasma and buffer samples at different pH values. Acepromazine (250 ng) was added to 3 ml equine plasma or 50 mM phosphate buffer, adjusted to the indicated pH with 0.1 N HCl or 0.1 N NaOH, and then extracted into DCM as described in the text. (○) Recovery from plasma; (●) recovery from buffer.

To measure recovery as a function of pH (Figs. 2 and 3), samples were again spiked with 0.5 ml of a 100 μg/ml solution of the indicated drug. The plasma and phosphate buffer solutions were adjusted to the desired pH by addition of 0.1 N HCl and 0.1 N NaOH. To each sample 2 ml DCM was added and the tubes were agitated for 5 min. The samples were centrifuged for 1 h at 0°C to break up any emulsion that may have formed. The aqueous layer was then discarded, and 1 ml DCM was removed and evaporated to dryness. Cyclohexane (0.05 ml) was added to dissolve the extract residue, and 5 μl was injected for analysis.
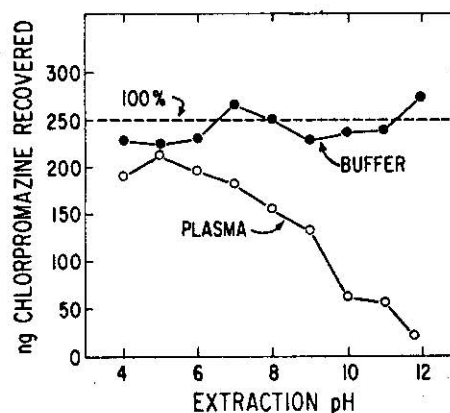

FIGURE 3. Recovery of chlorpromazine from spiked plasma and buffer samples at different pH values. Chlorpromazine (250 ng) was added to 3 ml equine plasma or 50 mM phosphate buffer, adjusted to the indicated pH with 0.1 N HCl or 0.1 N NaOH, and then extracted into DCM as described in the text. (○) Recovery of chlorpromazine from plasma; (●) recovery from buffer.
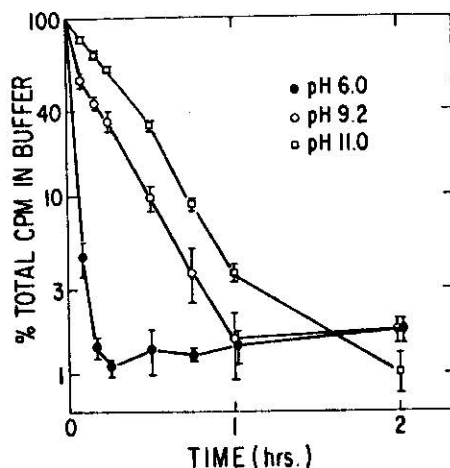
FIGURE 4. Rate of extraction of chlorpromazine from buffer at three pH values. [³H]-Chlorpromazine (50 μCi) and unlabeled chlorpromazine (50 μg) were added to 5 ml 50 m*M* phosphate buffer at the indicated pH values and extracted into DCM as described in the text. The points show the time course of disappearance of [³H]chlorpromazine from the buffer solutions during agitation at pH 6.0, 9.2, and 11.0.

To measure the migration of chlorpromazine from the polar to the nonpolar phase (Figs. 4 and 5), [³H]chlorpromazine was utilized. The specific activity of the labeled compound was 38 Ci/mmol and its purity was calculated as 91.3%. Phosphate buffer and plasma (5 ml each) were added to 15-ml screw-top tubes. The samples were adjusted to the desired
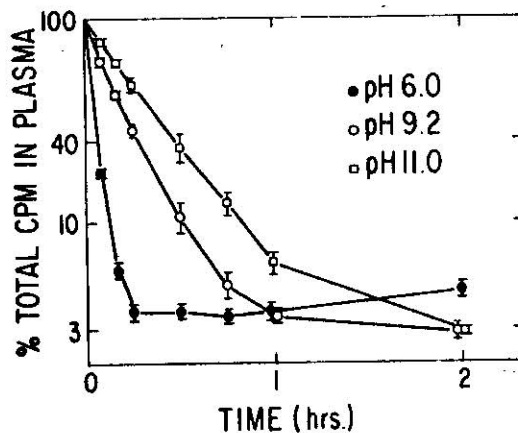
FIGURE 5. Rate of extraction of chlorpromazine from buffer at three pH values. [³H]-Chlorpromazine (50 μCi) and unlabeled chlorpromazine (50 μg) were added to 5 ml equine plasma at the pH indicated and extracted into DCM as described in the text. The points show the time course of disappearance of [³H]chlorpromazine from the plasma solutions during agitation at pH 6.0, 9.2, and 11.0.

pH with 0.1 N HCl and 0.1 N NaOH, after which 50 μg unlabeled chlorpromazine and 50 μCi [^{3}H]chlorpromazine in methanol were added. At this point the solutions were mixed on a vortex mixer and the initial sample was taken. DCM (5.0 ml) was added and the tubes were capped and agitated on a Rotorack (Fisher Scientific, Pittsburgh, Pa.) at low speed (8 rpm). At regular intervals the Rotorack was stopped, and 100-μl samples of the aqueous layer were removed, transferred to 15-ml scintillation vials, and counted. The scintillation fluid was 3A20 (2 parts) plus Triton X-100 (1 part), and the counting was done on a Beckman LS-3150T scintillation counter. Counts were corrected for loss of volume and counts of the 100-μl samples that were taken during the course of the experiment.

Except for the radioisotope experiments, samples were analyzed with a Perkin-Elmer 3920B gas-liquid chromatograph equipped with a nitrogen/phosphorus detector. Column packing was 3% OV-101 on 100/120 Gas Chrom Q; operating temperatures were 260°C for injector ports, 250°C for the column oven, and 285°C for the interface. The carrier gas flow rate was 29 ml/min. A 50 mM phosphate buffer was used in all experiments and was found to be sufficient to allow accurate titration of the buffer solutions to the required pH. DCM and cyclohexane were nanograde and obtained from Mallinckrodt Inc. (St. Louis, Mo.). Chlorpromazine HCl was obtained from Sigma Chemical Co. (St. Louis, Mo.), and acepromazine maleate was graciously donated by Ayerst Laboratories (New York). [^{3}H]Chlorpromazine was purchased from Research Products International Corp. (Elk Grove Village, Ill.).

RESULTS

When extractions for acepromazine and chlorpromazine from equine plasma were carried out by a standard procedure for basic drugs (Sunshine, 1969), relatively poor, fractional recoveries of these agents were obtained. Figure 1 shows results of an experiment in which acepromazine and chlorpromazine were added to various dilutions of ovine, equine, and human plasma and the standard recovery method was applied. Even when the plasma was diluted to 16.7% of the normal concentration, less than 50% recovery of these drugs was obtained, compared to recovery from buffer. The data suggested that binding action of the plasma interfered with the recovery of phenothiazine tranquilizers under standard recovery conditions for basic drugs.

Because of these poor recoveries from plasma at basic pH values, we studied the recovery of acepromazine from buffer solutions and plasma over a wide pH range. Figure 2 shows that optimal recovery of acepromazine from plasma occurred at a pH of about 6.0, while recovery from aqueous solution was optimal at a pH of about 12.0. Similarly, experiments with chlorpromazine (Fig. 3) showed optimal recovery of this drug from plasma at pH 5.0, while recovery from buffer was essentially independent of pH.

Because of these anomalous results, we investigated the effects of pH on the migration of radiolabeled chlorpromazine from polar to nonpolar environments. As shown in Fig. 4, [^{3}H]chlorpromazine was lost from the aqueous environment rapidly at pH 6.0, at about one-fifth of this rate when the pH was 9.2, and at about one-tenth of this rate when the pH was 11.0. Figure 5 shows roughly the same effects in plasma, with chlorpromazine again migrating more rapidly from the aqueous environment at pH 6.0.

DISCUSSION

From these experiments it is clear that acepromazine and chlorpromazine exhibit unusual extraction properties for basic drugs. Since both drugs have a pK_a of 9.3, one might expect most rapid and complete recoveries under basic conditions. Although recoveries from aqueous solutions can be complete at basic values, our results suggest that the movement of chlorpromazine and acepromazine from polar to nonpolar solvents is more rapid at acidic pH values.

It has been suggested that the slow movement of chlorpromazine, and the proposed similar behavior of acepromazine, at basic pH may be explained by protein binding. While some researchers concluded that the binding of phenothiazines to serum albumin is essentially hydrophobic (Perrin and Hulshoff, 1976), Keeler and Sharma (1974) found that promazine was more highly bound at basic pH. They attributed this effect to partial ionic binding of promazine to the carboxyl groups of the tryptophan residues in the albumin molecules. At basic pH, they concluded, more of the carboxyl groups would be ionized, and this would permit higher binding affinities. This, however, does not explain the similar, more rapid extractions observed for chlorpromazine from phosphate buffer solutions (Fig. 4). While chlorpromazine is basic, Schubert (1967) observed that it can be extracted from aqueous solvents at an acidic pH. In fact, Schubert suggested that the chloride of this drug is soluble in some chlorinated solvents, such as chloroform and DCM; therefore one would expect recovery to be efficient over a wide range of pH, as the graph in Fig. 3 indicates. The more rapid recovery of chlorpromazine at acidic pH from the buffer solutions, however, remains unexplained at this time.

For our own purposes in the laboratory and for routine analysis of acepromazine and chlorpromazine, we use an extraction pH of 9.0 and an agitation time of 1 h at 24°C. We find this to be the simplest method of extraction because it allows us to screen for acepromazine and chlorpromazine during our routine testing for basic drugs. Therefore, the only variable between the analysis of these phenothiazines and other basic drugs would be the increased time of agitation. This is important because the use of basic buffer systems greatly simplifies extraction procedures when a large number of samples must be processed.

These studies indicate that two factors must be considered by researchers when performing analytical studies with acepromazine and chlorpromazine. First, it must be clear that recoveries from plasma solution are much more rapid under acidic conditions (pH 6.0) than under the more standard basic conditions (pH 9-11). Second, the time of agitation must be sufficient to allow complete recovery at the pH to be used. These times can be determined from the graphs in Figs. 4 and 5.

These data should be valuable to clinicians for accurate analysis of phenothiazines in biological fluids. In the past, there has been a pressing need for sensitive assays of these drugs. With this information and the utilization of sensitive and specific instruments such as the nitrogen detector, more accurate quantitative studies should be possible.

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