Pethidine binding in plasma: effects of methodological variables

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- 1 Several methodological variables potentially influencing the plasma protein binding of [¹⁴C]-pethidine *in vitro* were investigated using equilibrium dialysis and rigorous pH control.
- 2 Ionic strength of buffer, pethidine concentration and 21 days of plasma samples frozen to –8°C did not affect the outcome of the binding experiments.
- 3 Unbound fraction decreased with increasing temperatures between 25°C and 37°C.
- 4 Unbound fraction decreased with increased pH between pH 7.0 and 8.0; the mean unbound fraction at an equilibrium pH of 7.4 and at 37°C was 0.58 (s.d. 0.03, *n* = 58).
- 5 It is likely that previous reports of pethidine unbound fraction as being between 0.2 and 0.4 represent artefacts caused by inadequate pH control during dialysis.

Keywords pethidine binding methodological variables

Introduction

The ease and popularity of equilibrium dialysis for plasma protein binding determinations have led to many variations in the procedures followed by investigators using this method. These variations include such experimental variables as the type and strength of the buffer used, as well as variables associated with the plasma sample itself such as the method of collection, anticoagulation and storage. The importance of detailed methodological reporting by authors has been stressed (Brinkschulte & Breyer-Pfaff, 1979; Kristensen & Gram, 1982). Since methodological variables may influence the results obtained it is appropriate that such variables be evaluated for their influence on drug plasma protein binding in general, but especially in cases where conflicting data exist, if meaningful comparisons are to be made. The purpose of this study was to examine the effect of a number of methodological factors on the plasma protein binding of pethidine, since conflicting data exist for this drug which hamper interpretation of its pharmacokinetic and pharmacodynamic properties (Klotz *et al.*, 1974; Mather *et al.*, 1975; Jackson, 1981; Jackson *et al.*, 1981; Nation, 1981; Pond & Kretschmar, 1981; Holmberg *et al.*, 1982).

Methods

Plasma pool

Aliquots from a pool of plasma that had been obtained from the blood of five healthy male volunteers were used throughout these experiments. The plasma was stored at –8°C for a maximum of 21 days and aliquots were thawed at 4°C overnight before use. Fibrin aggregations were removed by centrifugation and pH was adjusted to 7.4 using small volumes of 0.1M NaOH or 0.1M HCl. In the pH dependence study, plasma pH was adjusted to the pH of the buffer in use for each experiment. Analysis of concentrations of total protein, albumin, α_1 -acid glycoprotein and β -lipoprotein gave values in the normal range observed for adult humans.

Dialysis conditions

Equilibrium dialysis was performed in round dialysis cells made of Delrin® (DuPont de Nemours) and having 2 ml chambers. During dialysis sampling ports were sealed with polyethylene PE160 tubing (Clay Adams, New Jersey).

Cellulose dialysis tubing (type 36, Union

Carbide, Chicago) was washed in several changes of boiling distilled water until the vapour was odour free. The tubing was stored in freshly distilled water at 4°C until used. Binding of [^{14}C]-pethidine to the apparatus was negligible. Two ml plasma and 2 ml buffer (0.067M Sorensen's phosphate pH 7.4, unless otherwise specified) were pipetted into opposite sides of the dialysis cells and (*N*-methyl ^{14}C)-pethidine HCl (1035 d/min/ μl , 0.023 mg/ml base) was added to the buffer to give the desired initial pethidine concentration in buffer (1.2 $\mu\text{g}/\text{ml}$ in most cases). The cells were sealed, gently rotated by hand several times, then placed in an oscillating water bath (100 oscillations/min) at 37°C (unless otherwise specified). Equilibrium was determined to have occurred within a dialysis time of 5 h during preliminary experiments.

Protein leakage was tested with protein reagent strips (Albustix®, Ames, Victoria) and any buffer showing a positive reading was discarded together with its plasma counterpart.

Measurement of pH

All pH measurements were performed at the temperature of the dialysis using an Instrument Laboratories Model 213 digital pH blood-gas analyser. This instrument, which is fitted with a small probe, enabled 20 μl samples to be taken directly from the dialysis cell without removing the cell from the water bath and interrupting the dialysis. The coefficient of variation of this method of pH measurement was less than 0.3% throughout the study.

Analysis of pethidine and calculation of unbound fraction

Radiochemical purity of the [^{14}C]-pethidine was assessed as > 97.5% and 100% in two t.l.c. systems. Its specific activity was determined by liquid scintillation counting and gas chromatography. [^{14}C]-pethidine concentrations were measured in 0.5 ml plasma and buffer aliquots made alkaline with 0.2 ml of 0.1M NaOH and extracted into 2 ml scintillation grade toluene by vortexing for 2 min. The organic layer then was added to 8 ml scintillation cocktail (six parts PCS (Amersham) to three parts scintillation grade toluene (Searle)). Ten min counting was performed in a scintillation counter (Packard model 3255) using the preset radionuclide window for ^{14}C . Quench correction was performed using the channels ratio method. Unbound fraction was calculated from Equation (1).

$$\% \text{ Unbound} =$$

$$\frac{\text{mean of duplicate d min in 0.5 ml buffer aliquots}}{\text{mean of duplicate d min in 0.5 ml plasma aliquots}} \quad (1)$$

Each determination was performed in three or four dialysis cells. Corrections were made for different recoveries of radioactivity from plasma and buffer, respectively 85 and 92%.

Methodological variables

At the same initial concentration of pethidine in buffer (1.2 $\mu\text{g}/\text{ml}$), the following methodological variables were investigated: temperature, buffer ionic strength, plasma pH and storage conditions of plasma samples. The effect of increasing the equilibrium pethidine concentrations up to 3.5 $\mu\text{g}/\text{ml}$ also was investigated.

Dialyses were performed at three temperatures; 25, 30 and 37°C. $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffers of the desired pH and ionic strength were prepared according to Perrin & Dempsey (1974).

Freshly obtained plasma from healthy volunteers was subjected to the same dialysis procedure as aliquots from the plasma pool that had been stored at -8°C for 21 days. In order to determine whether a more convenient substitute to plasma could be used for binding studies of pethidine, another series of studies were performed using commercially available stable plasma protein solution (SPPS, Commonwealth Serum Laboratories, Melbourne, Australia) in comparison to fresh human plasma.

Statistical analyses

Differences between groups of data were evaluated with Student's *t*-test or analysis of variance while correlations between variables were evaluated by linear regression analysis. A probability of less than 5% was considered significant in two-tailed tests.

Results

There was an inverse relationship between the unbound fraction of pethidine and temperature over the range studied (Table 1) ($r = -0.81$, $P < 0.005$). Buffer ionic strength did not influence pethidine binding under the conditions of the study (Table 2) ($r = 0.24$, $0.1 < P < 0.2$).

The degree of binding was markedly influenced

Table 1 Effect of temperature on the plasma protein binding of [^{14}C]-pethidine ($n = 4$ at each temperature)

Temperature (°C)	Total plasma concentration of pethidine at equilibrium ($\mu\text{g/ml}$) Mean (s.d.)	% unbound in plasma mean (s.d.)	pH at equilibrium*
25	0.70 (0.03)	60 (3)	7.44
30	0.71 (0.02)	57 (2)	7.40
37	0.74 (0.02)	54 (1)	7.43

* pH was measured in all cells (plasma and buffer) at the temperature of the dialysis and had a coefficient of variation of less than 0.3% at each temperature.

by pH (Figure 1) such that there was a negative correlation between unbound fraction and plasma pH at equilibrium ($r = -0.98$, $P < 0.0005$).

Binding in plasma that had been stored at -8°C for 21 days was not different ($0.05 < P < 0.1$) from that in freshly collected plasma where the final plasma pH was 7.40 ± 0.05 in each case. The mean unbound fraction in fresh plasma was 0.61 (s.d. 0.04, $n = 6$) while that in frozen plasma was 0.57 (s.d. 0.03, $n = 12$). The unbound fraction in SPPS was 0.96 (s.d. 0.06, $n = 4$) which was significantly different to both fresh and frozen plasma.

Binding was not influenced by pethidine concentrations up to $3.5 \mu\text{g/ml}$ (Table 3) ($r = 0.25$, $0.1 < P < 0.2$).

Discussion

The results of this investigation have provided background information that not only describes the plasma protein binding characteristics of pethidine, but assists in the interpretation of

previous studies of this drug and possibly assists in providing guidelines for the study of other basic drugs as well.

Although most investigators now perform dialysis experiments at 37°C , many earlier studies were performed at or below room temperature. In the present study, different temperatures resulted in different unbound fractions, although there remains the possibility that this may have arisen due to longer equilibration times being required at lower temperatures.

Phosphate buffers are commonly used in equilibrium dialysis studies. Although 0.067M and 0.1M phosphate buffers have been used frequently in studying pethidine (e.g. Jackson, 1981; Jackson *et al.*, 1981; Pond & Kretschmar, 1981) these strengths would seem to be chosen by tradition rather than by systematic study. In the present study however, it was shown that pethidine plasma protein binding at pH 7.4 was not dependent on buffer ionic strength over a wider range of ionic strengths ($I = 0.4$ to 0.086).

The present study has demonstrated marked pH dependent binding for pethidine. At pH 7.03 the unbound fraction was 0.70 whilst at pH 7.91

Table 2 Effect of ionic strength of buffer on plasma protein binding of [^{14}C]-pethidine ($n = 4$ at each strength)

Ionic strength	Total plasma concentration of pethidine at equilibrium ($\mu\text{g/ml}$) mean (s.d.)	% unbound in plasma mean (s.d.)	pH*
0.4	0.70 (0.04)	56 (3)	7.38
0.3	0.68 (0.03)	57 (1)	7.39
0.2	0.67 (0.02)	59 (3)	7.39
0.175 (0.067 M)	0.69 (0.05)	58 (2)	7.35
0.1	0.65 (0.02)	57 (2)	7.39
0.086 (0.033 M)	0.65 (0.02)	57 (2)	7.37

* pH was measured in all cells (plasma and buffer pH) at 37°C and had a coefficient of variation of less than 0.4% at each ionic strength.

Figure 1 Relationship between percent unbound pethidine in plasma and plasma pH ($r = 0.98$, $P < 0.0005$). Initial pethidine concentration in buffer in each case was $1.2 \mu\text{g/ml}$.

this value decreased to 0.36. The mean value at pH 7.43 was 0.54. Similarly, an increase in pH has been reported to result in the reduction in the unbound fraction for other basic drugs such as lignocaine (Burnley *et al.*, 1978), propranolol (Rochester *et al.*, 1982), fentanyl (Bower, 1981) and for acidic drugs such as warfarin (Wilting *et al.*, 1981) and digoxin (Brock, 1974). The results of binding determinations for many drugs thus are critically dependent on the plasma pH at the end of the experiment, and data from experi-

ments without adequate pH control can result in misleading conclusions. In the light of this knowledge, the importance of adequate pH control during the course of an equilibrium dialysis cannot be emphasised too strongly. Due to removal of the erythrocytes and evaporation of CO_2 , plasma pH often will be nearly 8.0. Since buffer capacity is not sufficient to restore a physiological pH value by re-equilibrating with CO_2 alone, the pH must be adjusted prior to dialysis.

Thus, it seems likely that the reason pethidine unbound fraction resulting from this study (0.58) differs from those previously reported may be because others failed to control the plasma pH. These reports (Klotz *et al.*, 1974; Jackson, 1981; Jackson *et al.*, 1981; Nation, 1981; Pond & Kretschmar, 1981) each stated the initial buffer pH only, and reported a pethidine unbound fraction of around 0.4—a value which was obtained in the present study at a pH of approximately 7.8 and a value not unusual in experiments where initial pH adjustments are not made (Kristensen & Gram, 1982; Morgan *et al.*, 1982). Similarly, results obtained for pethidine binding from ultrafiltration experiments (Mather *et al.*, 1975) indicated an unbound fraction of between 0.2 and 0.4, despite the use of 5% CO_2 in the driving gas. It would seem that pH dependent binding is a common attribute of basic drugs, hence strict attention must be directed to controlling pH as a dominating variable in the study of their binding.

It is often necessary to store plasma samples prior to analysis, yet the effects of storage on the plasma protein binding determination rarely have been investigated. In the present study, the binding of [^{14}C]-pethidine in fresh plasma was not significantly different from that in plasma that had been stored at -8°C for 21 days even though the unbound fraction tended to be lower in the frozen samples. Similar findings have been reported for impiramine (Kristensen & Gram, 1982) and for etidocaine (Morgan *et al.*, 1982). The effects of long term storage await investigation.

Concentration-dependent plasma protein binding throughout the concentration range of interest for the drug under study must be investigated in order to facilitate correct interpretation of pharmacokinetic and pharmacodynamic information. Failure to appreciate concentration-dependent binding may lead to erroneous conclusions in subsequent comparisons between drugs and between recipients of drugs. Concentration-dependence of pethidine plasma binding has been suggested previously (Mather *et al.*, 1975; Jackson, 1981; Jackson *et al.*, 1981). The present study indicated conclusively that the binding of ^{14}C -pethidine at final

Table 3 Effect of pethidine concentration on plasma protein binding of [^{14}C]-pethidine ($n = 4$ at each concentration)

Total plasma concentration of pethidine at equilibrium ($\mu\text{g/ml}$) mean (s.d.)	% unbound in plasma mean (s.d.)	pH*
3.4 (0.2)	59 (3)	7.37
1.4 (0.2)	59 (1)	7.36
0.71 (0.02)	59 (2)	7.31
0.36 (0.01)	58 (1)	7.33
0.19 (0.01)	57 (3)	7.39
0.074 (0.003)	56 (1)	7.38

* pH was measured in all cells (plasma and buffer pH) at 37°C and had a coefficient of variation of less than 0.4% at each concentration.

plasma pethidine concentrations between 0.07 and 3.5 $\mu\text{g/ml}$ was not concentration dependent at controlled pH over the range of clinical interest (Mather & Meffin, 1978).

It is our conclusion that certain methodological variables can affect the extent of pethidine

plasma protein binding. Of these, pH is critical. The evidence obtained suggests that artefactually low unbound fractions for pethidine may have been reported previously and that these have arisen from inadequate pH control.

References

- Bower, S. (1981). Plasma protein binding of fentanyl. *J. Pharm. Pharmac.*, **33**, 507–514.
- Brinkschulte, M. & Breyer-Pfaff, U. (1979). Binding of tricyclic antidepressants and perazine to human plasma. *Naunyn Schmiedebergs Arch. Pharmac.*, **308**, 1–7.
- Brock, A. (1974). Binding of digoxin to human serum proteins: influence of pH on the binding of digoxin to human albumin. *Acta Pharmac. Tox.*, **34**, 260–266.
- Burney, R. G., DiFazio, C. A. & Foster, J. A. (1978). Effects of pH on protein binding of lidocaine. *Anesth. Analg. (Cleve.)*, **57**, 478–480.
- Holmberg, L., Odar-Cederlof, I., Nilsson, J. L. G., Ehrnebo, M. & Boreus, L. (1982). Pethidine binding to blood cells and plasma proteins in old and young subjects. *Eur. J. clin. Pharmac.*, **23**, 457–461.
- Jackson, A. J. (1981). Determination of meperidine blood to plasma concentration ratio (λ)—effect of temperature and pH. *Res. Comm. Chem. Path. Pharmac.*, **32**, 365–368.
- Jackson, A. J., Miller, A. K. & Narang, P. K. (1981). Human blood preservation: effect on *in vitro* protein binding. *J. pharm. Sci.*, **70**, 1168–1169.
- Klotz, U., McHorse, T. S., Wilkinson, G. R. & Schenker, S. (1974). The effect of cirrhosis on the disposition and elimination of meperidine in man. *Clin. Pharmac. Ther.*, **16**, 667–675.
- Kristensen, C. B. & Gram, L. F. (1982). Equilibrium dialysis for determination of protein binding of imipramine—evaluation of a method. *Acta Pharmac. Tox.*, **50**, 130–136.
- Mather, L. E. & Meffin, P. J. (1978). Clinical pharmacokinetics of pethidine. *Clin. Pharmacokin.*, **3**, 359–365.
- Mather, L. E., Tucker, G. T., Pflug, A. E., Lindop, M. J. & Wilkerson, C. (1975). Meperidine kinetics in man. *Clin. Pharmac. Ther.*, **17**, 21–30.
- Morgan, D. J., Koay, B. B. & Paull, J. D. (1982). Plasma protein binding of etidocaine during pregnancy and labour. *Eur. J. clin. Pharmac.*, **22**, 451–457.
- Nation, R. L. (1981). Meperidine binding in maternal and fetal plasma. *Clin. Pharmac. Ther.*, **29**, 472–479.
- Perrin, D. D. & Dempsey, B. (1974). *Buffers for pH and Metal Ion Control*, pp 62–71. New York: John Wiley and Sons.
- Pond, S. M. & Kretschmar, K. M. (1981). Effect of phenytoin on meperidine clearance and normeperidine formation. *Clin. Pharmac. Ther.*, **30**, 680–686.
- Rochester, C. L., Gammon, D. E., Shane, E. & Bilezikian, J. P. (1980). A radioreceptor assay for propranolol and 4-hydroxypropranolol. *Clin. Pharmac. Ther.*, **28**, 32–39.
- Wilting, J., Van Der Giesen, W. F. & Janssen, L. H. M. (1981). The effect of chloride on the binding of warfarin to albumin as a function of pH. *Biochem. Pharmac.*, **30**, 1025–1031.

(Received July 19, 1983,
accepted December 1, 1983)