J. Pharm. Pharmacol. 1986, 38: 795–800
Received March 26, 1986

© 1986 J. Pharm. Pharmacol.

Non-isotopic spectrophotometric determination of the unbound fraction of drugs in serum*

C.-F. LIM, K. N. WYNNE, J. M. BARNED†, D. J. TOPLISS AND J. R. STOCKIGT‡

Ewen Downie Metabolic Unit, Monash University Department of Medicine and †Pharmacy Department, Alfred Hospital, Melbourne, Victoria 3181, Australia

A spectrophotometric method to measure the free fraction of highly-bound drugs in serum has been established for a range of non-steroidal anti-inflammatory drugs (NSAIDs) and for frusemide. Spectrophotometry is used to measure fractional transit of drug from a large volume of dialysate to a small volume of serum during dialysis to equilibrium. The method, which depends on the principle that drug transit from dialysate to serum is proportional to serum binding, requires neither isotopic drug preparations nor specific drug assays, is independent of extraction efficiency from the dialysate and requires no measurements from the serum compartment. Estimates of percent unbound fraction (%UF) for aspirin (6·0 ± 0·9%), phenylbutazone (0·9 ± 0·2%), and frusemide (1·8 ± 0·2%) were comparable with those obtained with ^{14}C drug preparations. Values for %UF were determined for eleven additional NSAIDs. The method was valid for a four-fold change in serum : dialysate ratio. Kinetics of frusemide binding to serum were comparable using [^{14}C]frusemide and the test method. This technique may have general application in establishing the %UF for substances that are extensively bound to serum proteins and for identifying sera that show abnormal binding.

Of the many drugs that are extensively bound to serum proteins, those with anionic properties bind to albumin (Koch-Weser & Sellers 1976; Kwong 1985). It is generally held that the bound fraction is less accessible to tissue receptors and to sites of degradation than is the free or unbound fraction (UF) (Koch-Weser & Sellers 1976). Data on drug binding is usually given by specifying the bound percentage, often only as an approximation. The statement that a drug is 'greater than 95% bound' gives little information about its free concentration because a minor change in percent binding of a highly-bound drug will have a profound effect on the absolute free level (Kwong 1985). The lack of precise information on the percent unbound fraction (%UF) of many drugs became apparent in our studies of the potency of various drugs, including the non-steroidal anti-inflammatory drugs (NSAID) and the diuretic frusemide, as competitors for thyroxine binding sites in serum (Stockigt et al 1985a, b).

Methods for determining the free levels of drugs have recently been reviewed (Kwong 1985). Generally these techniques depend either on the availability of radio-labelled drug or on a specific, sensitive drug assay for the quantitation of the UF. For highly bound drugs, even minor impurities or less tightly bound degradation products in labelled drug preparations can lead to major over-estimates of UF. Where a drug is highly bound to serum, an extremely sensitive assay may be required to measure the minute free concentration in the dialysate, and the extraction efficiency must be monitored from both serum and buffer compartments.

We describe here a general method for measuring the UF of drugs using equilibrium dialysis with spectrophotometric determinations of drug concentration in the dialysate. The method depends on the principle that transit of a freely dialysable substance from buffer to serum compartment will be proportional to its binding by serum. Kwong (1985) and Tozer et al (1983) have emphasized two of the potential artefacts in measurement of free drug concentrations by equilibrium dialysis: (1) transit of drug from serum to buffer compartment, thus lowering the serum concentration, thereby giving a falsely high apparent UF unless the serum is measured at equilibrium, and (2) an osmotic volume shift which results in dilution of the serum compartment. The present method avoids both of these artefacts because no measurement of the drug concentration in serum is required. If the test serum is initially free of the drug under study and of other substances with similar spectrophotometric absorption, the %UF at equilibrium can be established from the ratio between the dialysate drug concentrations (optical density by spectrophotometry after extraction from buffer) in the presence and absence of serum. We have evaluated this technique by comparing values for %UF obtained by isotope

* Presented in part at the 67th meeting of the Endocrine Society, Baltimore, June 1985.

‡ Correspondence.

distribution and the optical density (OD) ratio for several drugs, and have examined the method using various relationships between buffer and serum volumes.

METHODS

Principle of the method

If transit of drug from the buffer to serum compartment during dialysis to equilibrium is directly proportional to the extent of drug binding in serum, removal of a drug from the buffer compartment can be used to quantify serum binding. The UF is derived by comparing the drug concentration in the buffer (dialysate) after dialysis against serum (the equilibrium concentration), with the drug concentration in dialysate in the absence of serum (control concentration). From the known volumes of dialysate and serum, the concentration of drug in the serum compartment can be calculated as follows.

The total mass of drug remains unaltered in the equilibrium dialysis system. Hence, for 20 ml buffer and 0.5 ml serum:

$$20x = 0{\cdot}5b + 20{\cdot}5u \quad (1)$$

where x = control dialysate concentration; u = unbound or final dialysate concentration at equilibrium; b = bound serum concentration.

From (1):

$$b = 40\,x - 41u \quad (2)$$

also

$$\%UF = \frac{u}{b + u} \times 100 \quad (3)$$

Substituting for b:

$$\%UF = \frac{100u}{40x - 40u} \quad (4)$$

For a range of values for x and u in (4), the %UF of a drug can be calculated, giving the relationships shown in Fig. 1. The choice of a small serum volume and large dialysate volume allows precise measurements of changes in the ratio u/x where %UF is very low.

For example, for 0·5 ml serum and 20 ml dialysate, when

x = 10, u = 0; u/x = 0, %UF = 0
x = 10, u = 1; u/x = 0·1, %UF = 0·28
x = 10, u = 3; u/x = 0·3, %UF = 1·07
x = 10, u = 5; u/x = 0·5, %UF = 2·5

Equilibrium dialysis

Equilibrium dialysis was performed in 20 ml glass scintillation vials. The drug was added to 20 ml of 0·04 M Tris buffer, pH 7·4, containing physiological concentrations of K^+, Ca^{2+}, Mg^{2+} and Cl^- (Irvine 1974). An undiluted normal serum pool (0·25–1·0 ml) was dialysed against this buffer in double-knotted dialysis tubing (type 8, Union Carbide, New York). Preliminary studies using [^{14}C] aspirin indicated that equilibrium was reached within 12 h at 37 °C. After dialysis for 18–20 h at 37 °C, the serum was discarded and the drug extracted from control and equilibrium dialysates for determination of %UF from the OD ratio. The test serum was also dialysed against blank buffer to provide a control for circulating drug or any interfering substance which entered the dialysate from the serum compartment. The NSAIDs studied were aspirin, diflunisal, diclofenac, indomethacin, sulindac, naproxen, ketoprofen, fenoprofen, flufenamic acid, mefenamic acid and phenylbutazone, either purchased from Sigma (St Louis, USA) or prepared from standard pharmaceutical preparations. Fenclofenac was a gift from Reckitt and Colman (Hull, UK), meclofenamic acid was a gift from Warner Lambert (Ann Arbor, Michigan, USA) and tolmetin a gift from Ethnor Pty Ltd (Sydney, Australia). Frusemide was the i.v. preparation (10 mg ml^{-1}) supplied by Hoechst (Melbourne, Australia). All NSAIDs were dissolved in 100% ethanol with the exception of meclofenamic acid which was dissolved in 0·01 M NaOH. Addition of NSAID to the dialysate resulted in ethanol concentrations of less than 1%. The test serum used in these studies was a pool from samples obtained for assessment of thyroid function in subjects shown to be euthyroid, who were not taking any of the drugs under study. The albumin concentration of this pool was 41 g $litre^{-1}$ and its total free fatty acid concentration was 0·5 mM.

Isotopic studies

Labelled frusemide (9·8 mCi $mmol^{-1}$) was a gift from Hoechst, Frankfurt, West Germany, [^{14}C]-phenylbutazone (2·98 mCi $mmol^{-1}$) was a gift from Ciba-Geigy, Basle, Switzerland, [^{14}C]aspirin (40 mCi $mmol^{-1}$) was purchased from the NEN Corporation, Boston, MA, [^{14}C]diflunisal (13·1 mCi $mmol^{-1}$) was a gift from Merck Sharp & Dohme, Sydney, Australia and [^{14}C]fenclofenac (13·6 mCi $mmol^{-1}$) was a gift from Reckitt and Colman, Hull, UK. The %UF was determined for these drugs by comparison of tracer concentration in serum and dialysate at equilibrium. Results were comparable whether labelled drug was added to serum or buffer compartment.

Extraction and estimation of drug concentration

At equilibrium, the drug was extracted from the dialysate with C18 SEP PAK cartridges (Water Associates, Milford, Mass, USA). The cartridge was wetted with methanol according to the manufacturer's instruction, followed by application of the 20 ml dialysate which was acidified with 0·1 ml of 4·5 mM HCl. The cartridge was washed with 3·0 ml distilled water followed by elution of drug with 2·0 ml methanol. The optical density of drug in the methanol eluate was determined using a spectrophotometer (Pye Unicam, PU8610, Cambridge, UK), with absorbance between 0·1 and 1·5 units at the wavelength of maximum absorption (Merck Index 1983). The OD ratio was calculated and interpolated in Fig. 1, using the relationship for the appropriate initial serum volume.

The extraction efficiency from control and equilibrium dialysates with C18 SEP PAK cartridges was assessed using labelled fenclofenac and frusemide. Similar extraction efficiencies were obtained both from the control vials (fenclofenac 96·0%; frusemide 98·9%) and from the equilibrium vials (fenclofenac 102·1%; frusemide 97·0%).

The serum drug concentration at equilibrium was calculated from the reduction in buffer concentration of drug, as follows:

$$b = \frac{20\,(x - u)}{\text{equil. serum vol}}$$

The serum volume at equilibrium was established using ^{125}I-human albumin (Amersham International, 0·2 μCi mg^{-1}) which had been pre-dialysed against 2 litres of buffer to remove low molecular weight contaminants. The volume at equilibrium was 0·41, 0·72, and 1·32 ml for initial serum volumes of 0·25, 0·5 and 1 ml, respectively, indicating osmotic dilution similar to that found by Tozer et al (1983).

RESULTS

Table 1 shows the %UF of 14 NSAIDs and frusemide determined by the OD ratio method. The %UF of frusemide, aspirin, and phenylbutazone, determined using the test method, were similar to the values obtained using ^{14}C-preparations (Table 1). The UF value for diflunisal was higher by the OD ratio method than with isotope, while the value for fenclofenac was lower by the OD ratio method. To reach detection limits for spectrophotometric measurement, the binding of some drugs was studied at higher than their therapeutic concentrations. The determinations of %UF by the OD ratio method were done at the final calculated serum concentra-

Table 1. Percent unbound fraction of non-steroidal anti-inflammatory drugs and frusemide measured by optical density ratio using 0·5 ml serum and 20 ml dialysate. The %UF of aspirin, diflunisal, fenclofenac, phenylbutazone and frusemide was also measured using ^{14}C-preparations.

			Spectrophotometric method		
Drug	*Max therap. concn μM	Isotopic method %UF mean ± s.d. (n)	%UF mean ± s.d. (n)	Equil. serum concn μM	Absorption nm
Aspirin	1800	7 ± 0.4 (7)	6·0 ± 0·9 (5)	375	298
Diflunisal	320	0.3 ± 0.08 (3)	0·7, 0·7	530	255
Diclofenac	10		0·5 ± 0·08 (3)	375	275
Fenclofenac	270	1·0 ± 0·08 (5)	0·5 ± 0·09 (7)	375	277
Indomethacin	10		0·7 ± 0·3 (6)	30	318
Sulindac	20		2·2, 2·4	450	330
Naproxen	200		0·6, 0·3	95	262
Ketoprofen	30		0·7, 1·2	105	260
Fenoprofen	90		1·2, 1·6	105	274
Flufenamic acid	70		0·2 ± 0·06 (4)	100	287
Mefenamic acid	80		0·6 ± 0·2 (9)	105	288
Meclofenamic acid	60		0·6 ± 0·2 (4)	210	280
Tolmetin	210		1·2, 1·3	180	260
Phenylbutazone	320	1·4 ± 0·3 (6)	0·9 ± 0·2 (9)	95	240
Frusemide	30	1·6 ± 0·2 (10)	1·8 ± 0·2 (11)	25	275

* American Hospital Formulary Service, Drug Information (1985)
* Martindale: The Extra Pharmacopoeia (1982)

tions ranging from 25 μM (frusemide) to 530 μM (diflunisal). With the ^{14}C-preparations, the estimated drug concentrations in serum were <10 μM for the five drugs so studied.

The %UF of frusemide, aspirin and phenylbutazone were measured by the OD ratio method using different volumes of undiluted serum (0·25, 0·50 and 1·00 ml), with constant dialysate volume (20 ml). Table 2 shows the optical density values for the control and equilibrium dialysate from which the drug concentration ratio is calculated. This ratio varied inversely with the serum volume, so that the %UF, obtained by interpolation from Fig. 1, was independent of changes in serum volume between 0·25 and 1·0 ml (Table 2). Hence, the OD ratio method was valid for various relations between buffer and serum volume.

To assess further the concentration-dependence of drug binding, the binding of [^{14}C]frusemide to serum proteins was studied in the presence of increasing unlabelled frusemide concentrations. [^{14}C]Frusemide was added to 0·5 ml of undiluted serum and dialysed against increasing concentrations of unlabelled frusemide in 20 ml buffer. The %UF of frusemide was 1·4–1·9% over a calculated serum concentration range from 16–300 μM. At 560, 640 and 1400 μM frusemide, the %UF was 2·6, 3·5 and 10·9%, respectively, indicating an abrupt increase in %UF above the molar concentration of albumin (~600 μM). Scatchard analysis of frusemide binding by normal serum was also performed using the OD

Table 2. The percent unbound fraction of frusemide, aspirin and phenylbutazone measured by optical density ratio with 0·25, 0·5 or 1·0 ml derum dialysed against 20 ml buffer (n = 4).

Drug	Serum volume ml	Optical density Control	Optical density Equil.	OD ratio	%UF	Equil. serum concn μM
Frusemide	0·25	0·55 ± 0·02	0·34 ± 0·07	0·62 ± 0·02	2·1 ± 0·2	64
	0·5	0·55 ± 0·02	0·22 ± 0·02	0·40 ± 0·04	1·6 ± 0·2	46
	1·0	1·09 ± 0·05	0·34 ± 0·01	0·31 ± 0·06	2·2 ± 0·6	55
Aspirin	0·25	0·57 ± 0·02	0·49 ± 0·017	0.86 ± 0.01	7·3 ± 0·6	117
	0·5	0·57 ± 0·02	0·42 ± 0·02	0·74 ± 0·04	6·8 ± 1·2	118
	1·0	0·57 ± 0·02	0·33 ± 0·03	0·58 ± 0·05	6·6 ± 1·2	103
Phenylbutazone	0·25	1·54 ± 0·04	0·75 ± 0·065	0·49 ± 0·04	1·2 ± 0·2	267
	0·5	1·54 ± 0·04	0·44 ± 0·058	0·29 ± 0·04	1·0 ± 0·2	198
	1·0	1·54 ± 0·04	0·38 ± 0·031	0·24 ± 0·02	1·5 ± 0·1	125

ratio method at calculated equilibrium drug concentrations from 120 to 3200 μM. The dominant binding site had a K_d of ~4 × 10^{-5} M and a capacity of ~3 × 10^{-3} M, suggesting that frusemide binds to multiple sites per albumin molecule. These results were comparable with those obtained with [^{14}C]frusemide which gave K_d ~2 × 10^{-5} M and capacity ~2 × 10^{-3} M (Stockigt et al 1985a).

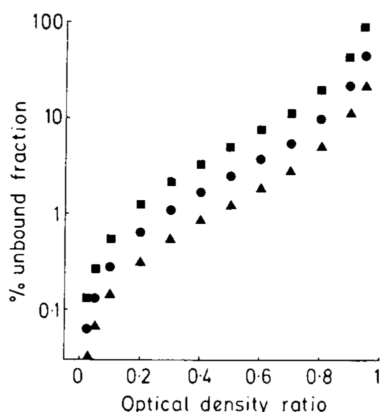

FIG. 1. Calculated relationship between optical density ratio of drug concentrations in the presence and absence of serum and the percent unbound fraction of a drug, using three different volumes of serum with a dialysate volume of 20 ml. This relationship is given for the initial volumes of undiluted serum added as dialysand, rather than the large equilibrium volume after osmotic shift (see Discussion). Key: ■ serum 1·0 ml; ● serum 0·5 ml; ▲ serum 0·25 ml.

DISCUSSION

Recent reviews have emphasized the importance of the free concentration in drug monitoring (Levy & Moreland 1984; Kwong 1985). Methods to measure the UF include the use of radiolabelled drug, gel permeation chromatography, or drug detection by HPLC after equilibrium dialysis or ultrafiltration (Kwong 1985; Melten et al 1985). In general, these methods are at their best where the UF is relatively high. In contrast, the method described here has been designed for substances with very low %UF, particularly where an isotopically labelled preparation is not available.

Because of large variations in the therapeutic concentration and binding of drugs to serum albumin, several factors must be considered when using the OD ratio to establish the UF in serum. Firstly, drug concentration after extraction of the dialysate must be within range for precise quantitation by spectrophotometry. Secondly, the amount of drug added to achieve precise spectrophotometric detection must not be so large as to alter drug binding. As shown here for frusemide, the %UF will generally be independent of total drug concentration until its molar concentration approaches that of albumin (Tozer 1984). For one of the drugs added in relatively high concentration, aspirin, the %UF values obtained by isotope distribution (at very low drug concentration) and by addition of unlabelled drug were comparable, suggesting that the estimate of binding at about 300 μM was little influenced by occupancy of albumin. However, it is well known that the %UF for aspirin may increase markedly at high therapeutic concentrations (Tozer 1984). In contrast, the %UF for diflunisal obtained by the OD ratio method at a concentration of 530 μM was higher than that found with the isotopic preparation, suggestive of concentration-dependent displacement. The opposite discrepancy for fenclofenac, with higher %UF by the isotopic method is so far unexplained, but may relate to less tightly bound

impurities or degradation products in the isotopic preparation.

Numerous methodological details can limit the precision of estimates of the UF of drugs in serum. Equilibrium dialysis methods that use approximately equal volumes of dialysate and dialysand have the disadvantage for highly bound drugs that measurements in the dialysate require great sensitivity, with small errors in the estimate of the minute buffer concentration having a great influence on the apparent UF. For the present method, a large dialysate volume and small serum volume were chosen to allow precise measurement of changes in the equilibrium drug concentration in buffer for small changes in %UF, thereby allowing precise measurements of UF below 2%.

With regard to osmotic effects, our results for %UF have not been corrected for osmotic dilution of the test serum which was found to be 25–40% using ^{125}I-human albumin. In establishing the equations given in Methods, it is important to consider whether the serum volume should be taken as the initial volume added, or the larger volume at equilibrium following osmotic shift. As outlined by Tozer (1984), osmotic shift alters neither the free drug concentration nor the *quantity* bound in the serum compartment. Hence, it is valid to relate the concentration ratio in the dialysate to the *initial* undiluted serum volume in calculating the relationships shown in Fig. 1. It should be noted that Tozer et al (1983) in appendix IV to their paper and Mathor & Wajchenberg (1985) suggested that measurements of UF could be made from the change in concentration of radioactive steroid in the buffer compartment after dialysis against serum, i.e. no measurement of the serum compartment. The concept has been further developed here by using unequal serum and buffer volumes to increase sensitivity and by use of a non-specific detection system after extraction of the dialysate.

The extraction of drug from buffer by SEP PAK cartridges described here for NSAIDs and frusemide is simple and reproducible and can be applied to any drug having lipophilic properties. The estimate of UF does not depend on complete recovery of the drug, or on precise quantitation of absolute drug concentration, because both the control and the equilibrium dialysates are subjected to the same extraction procedures, making the ratio which is used to establish %UF independent of extraction efficiency.

It has been reported that variation in the serum to buffer ratio can be a source of error when measuring the UF using equilibrium dialysis (Smith & Jubiz 1980). We therefore studied the effects of changes in this relationship on the UF of aspirin, phenylbutazone and frusemide and demonstrated that %UF showed little change when the serum : buffer ratio was varied from 1 : 20 to 1 : 80. It should be noted that in the report by Smith & Jubiz (1980) the molar ratio of drug to albumin was often greater than unity, whereas in our studies this ratio did not exceed unity.

The method described here has so far been used mainly with a normal serum pool in order to define the binding characteristics of particular drugs. However, it is also suitable for assessment of individual sera in which abnormal binding is suspected. Where a serum contains the drug in question, a control vial is required to correct for the contribution of the serum concentration to the post-dialysis optical density. A higher UF would be anticipated with subnormal concentrations of albumin, or where another substance acted as a competitor for the same binding site. In this respect, the effects of free fatty acids in impairing binding of drugs such as salicylate, phenylbutazone and phenytoin is important (Spector et al 1973), although such inhibition of drug binding by free fatty acids will usually not occur unless the molar ratio of free fatty acids to albumin exceeds 3·5 (Spector 1975). Hereditary qualitative albumin variants which show abnormal ligand binding may also be identifiable by this method. Studies in familial dysalbuminaemic hyperthyroxinaemia, a condition in which a variant albumin binds thyroxine with about 50 times the normal affinity (Barlow et al 1982), have so far failed to show evidence of abnormal drug binding using frusemide, fenclofenac, aspirin or phenylbutazone (Barlow et al 1986).

There has recently been considerable interest in the possibility that various endogenous substances may inhibit drug and hormone binding in serum, particularly in renal insufficiency (Reidenberg & Drayer 1984). Putative inhibitors are often tested in systems where serum proteins are highly diluted, or absent, thereby overestimating potency if in-vivo activity is limited by binding to serum proteins. The system described here could be used to determine the extent of protein binding for any low molecular weight, dialysable substance, provided that an assay is available to monitor its removal from the dialysis buffer.

Acknowledgement
This work was supported in part by grants from the National Health and Medical Research Council of Australia.

REFERENCES

American Hospital Formulary Service. Drug Information 85 (1985) American Soc. Hospital Pharmacists, pp 705–771

Barlow, J. W., Csicsmann, J. M., White, E. L., Funder, J. W., Stockigt, J. R. (1982) J. Clin. Endocrinol. Metab. 55: 244–250

Barlow, J. W., Csicsmann, J. M., Meinhold, H., Lim, C. F., Stockigt, J. R. (1986) Clin. Endocrinol. (Oxf) 23: in press

Irvine, C. H. G. (1974) J. Clin. Endocrinol. Metab. 38: 655–662

Koch-Weser, J., Sellers, E. M. (1976) N. Engl. J. Med. 294: 311–316

Kwong, T. C. (1985) Clin. Chim. Acta. 151: 193–216

Levy, R. H., Moreland, R. A. (1984) Clin. Pharmacokinet. 9 (suppl 1): 1–9

Martindale: The Extra Pharmacopoeia (1982) 28th edn. Reynolds, J. E. F. (ed.) The Pharmaceutical Press, London, pp 234–282

Mathor, M. B., Wajchenberg, B. L. (1985) Clin. Chim. Acta 145: 213–218

Melten, J. W., Witterbrood, A. J., Willems, H. J. J., Faber, G. H., Wemer, J., Faber, D. B. (1985) J. Pharm. Sci. 74: 692–694

Merck Index, (1983) ed. M. Windholz, 10th edn, Merck & Co Inc, Rahway, NJ, USA

Reidenberg, M. M., Drayer, D. E. (1984) Clin. Pharmacokinet. 9 (suppl 1): 18–26

Smith, J. B., Jubiz, W. (1980) Steroids 36: 393–403

Spector, A. A. (1975) J. Lipid Res. 16: 165–179

Spector, A. A., Santos, E. C., Ashbrook, J. D., Fletcher, J. E. (1973) Ann. N.Y. Acad. Sci. 226:247–258

Stockigt, J. R., Lim, C.-F., Barlow, J. W., Wynne, K. N., Mohr, V. S., Topliss, D. J., Hamblin, P. S., Sabto, J. (1985a) J. Clin. Endocrinol. Metab. 60: 1025–1031

Stockigt, J. R., Lim, C.-F., Wynne, K. N., Barlow, J. W., Barned, J. M. (1985b) The Endocrine Society 67th Annual Meeting, Baltimore, Abstract 1105

Tozer, T. N. (1984) in: Benet, L. Z., Massoud, N., Gambertoglio, J. G. (eds) Pharmacokinetic Basis for Drug Treatment. Raven Press, New York, pp 173–193

Tozer, T. N., Gambertoglio, J. G., Furst, D. E., Avery, D. S., Holford, N. H. G. (1983) J. Pharm. Sci. 72: 1442–1446