Prediction of *In Vivo* Tissue Distribution from *In Vitro* Data 1. Experiments with Markers of Aqueous Spaces

Peter Ballard,[1,4] David E. Leahy,[2] and Malcolm Rowland[3]

Received January 7, 2000; accepted March 8, 2000

Purpose. The aim of this study was to evaluate the ability of an *in vitro* method of tissue distribution to accurately predict total water and extracellular aqueous spaces using marker compounds urea and inulin.
Methods. Slices (50–200 mg) of all the major tissues in the rat were incubated with Hanks/HEPES pH7.4 buffer containing ^{14}C-urea and ^{3}H-inulin for 2 h at 37°C. Tissue weight was noted before and after incubation and the tissue-to-buffer ratios determined.
Results. ^{14}C-Urea *Kp* estimates were generally greater than total tissue water due to tissue swelling, which varied widely among the tissues, up to 41% in muscle. In most cases, *Kp* values were much closer to *in vivo* values after correcting for the ^{14}C-urea in the imbibed media (Kp_{corr}). The method was able to distinguish between ^{14}C-urea and ^{3}H-inulin *Kp* values and indicated that inulin occupied a smaller space than urea, which for the majority of tissues corresponded to the extracellular space.
Conclusions. The Kp_{corr} values for ^{14}C-urea and *Kp* for ^{3}H-inulin were consistent with total tissue water and extracellular space for the majority of tissues studied, indicating their suitability as marker compounds for checking the viability of this *in vitro* method for estimating tissue distribution.

KEY WORDS: tissue distribution; swelling; *in vitro*; inulin; urea; rat.

INTRODUCTION

Drug discovery has been revolutionised in recent years by advances in chemistry and molecular biology that have resulted in a host of new therapeutic targets and chemical leads. Secondary screening to characterise drug like molecules requires a variety of rapid and reliable physiocochemical (e.g., solubility and lipophilicity) and biological tests (e.g., binding affinity, metabolism, Caco-2), to find compound series suitable for lead optimisation projects (1). This data can be encompassed within physiologically based pharmacokinetic (PBPK) models, which can describe or predict the disposition of chemicals in animal and man (2,3). However, additional data for these models are required that describe the drug distribution in tissues (tissue-to-plasma partition coefficients, Kp). While *Kp* values can be determined from *in vivo* studies, a more attractive alternative is to develop an *in vitro* methodology that can accurately predict the *in vivo* tissue distribution for compounds with a wide range of physicochemical properties.

Many *in vitro* tissue distribution methods have been reported for volatile compounds using tissue homogenates (4,5) with relatively few reports available for non-volatile compounds (6,7). While these estimates are valuable, there is the concern that the use of tissue homogenates can result in an overestimate for drugs that are not able to penetrate cellular membranes. Tissue slices have been used to estimate tissue distribution for drug molecules (8) and marker compounds for total tissue water and extracellular spaces (9,10) although these rarely equate to the corresponding *in vivo* values. Other workers have predicted *Kp* values for drugs and volatile organics from tissue volumes, composition and lipophilicity (11,12).

This paper describes the use of a physiologically relevant and rapid *in vitro* technique for estimating the tissue distribution of non-volatile compounds and examines the behaviour of markers of specific tissue spaces.

MATERIALS AND METHODS

Reagents

Hanks' Balanced Salt Solution containing D-glucose (HBSS) was supplied by Life Technologies Limited (Paisley, UK); ^{3}H-inulin and ^{14}C-urea by Amersham Life Science, Little Chalfont, Buckinhamshire, UK; Optisorb '1' and Optisorb 'S' by Fisons Chemicals, Loughborough, UK and Monophase S by Packard Instrument Company, Meriden, USA. All other chemicals were obtained from Sigma Chemical Company, Poole, Dorset, UK and used as received. Hanks/HEPES buffer was prepared by adding 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid sodium salt (HEPES) to HBSS at 10mM. Tissues (see Table I) were obtained from three male Wistar rats (200–300 g) sacrificed by cervical dislocation and stored on ice for up to 2 h prior to use. Intestine and stomach samples were washed with sodium chloride solution (0.15%) to remove luminal contents prior to incubation.

Methodology for *In Vitro* Tissue Incubations

Prior to incubation, fresh tissues were cut into pieces (50–200 mg) freehand, using scissors, accurately weighed, and incubated with Hanks/HEPES buffer (2 ml) containing ^{14}C-urea (0.25 μCi/ml) and ^{3}H-inulin (0.25 μCi/ml). After incubation at 37°C in a shaking waterbath for 2 h, the tissues were removed, blot dried on filter paper and reweighed. The water content and the ^{14}C-urea and ^{3}H-inulin concentrations in tissue and medium were determined by drying the samples overnight in a vacuum oven at 40°C, reweighing, oxidising using a Packard Instruments sample oxidiser and measuring the radioactivity by liquid scintillation counting, using Optisorb '1' (12 ml) and Optisorb 'S' (9 ml) for ^{14}C and Monophase 'S' (15 ml) for ^{3}H, respectively. The recoveries of ^{3}H-inulin and ^{14}C-urea from the oxidiser were approximately 90 and 100% respectively, with carryover between oxidations less than 1%.

[1] Discovery-DMPK, AstraZeneca, Alderley Park, Cheshire, SK10 4TG, United Kingdom.
[2] Cyprotex, Skelton House, Science Park, Manchester, M15 4SH, United Kingdom.
[3] School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PL, United Kingdom.
[4] To whom correspondence should be addressed. (e-mail: peter.ballard@astrazeneca.com)

ABBREVIATIONS: Kp, tissue-to-medium partition coefficient; Kp_{corr}, tissue-to-medium partition coefficient corrected for media imbibed during the *in vitro* incubation.

0724-8741/00/0600-0660$18.00/0

Table I. Mean Values (± Standard Error, n = 10) of Tissue Swelling, Tissue Water Content, ^{14}C-urea Partition Coefficient, with and Without Swelling (Kp and Kp_{corr}, Respectively), After a 2 h Incubation in Hanks/HEPES Buffer at 37°C and ^{14}C-urea K_p Following i.v. Bolus Dosing in the Rat[a]

Tissue	Swelling (%)	Fractional Water Content	^{14}C-Urea Kp	^{14}C-Urea Kp_{corr}	^{14}C-Urea Kp *in vivo*[a]
Adipose	12 (1.0)	0.047 (0.002)	0.23 (0.008)	0.099 (0.011)	0.131
Bone	3.5 (1.7)	0.16 (0.0044)	0.37 (0.042)	0.33 (0.030)	0.559
Brain	34 (2.1)	0.62 (0.014)	1.2 (0.026)	0.61 (0.013)	0.137
Heart	8.5 (1.2)	0.79 (0.006)	0.92 (0.016)	0.77 (0.010)	0.837
Intestine	−5.0 (2.8)	0.76 (0.002)	0.68 (0.026)	0.79 (0.064)	1.06
Kidney	39 (1.4)	0.73 (0.004)	1.0 (0.033)	0.46 (0.014)	2.27
Liver	13 (0.9)	0.65 (0.005)	0.80 (0.011)	0.59 (0.014)	0.69
Lung	19 (1.2)	0.70 (0.035)	1.0 (0.022)	0.70 (0.008)	0.625
Muscle, thigh	41 (3.8)	0.73 (0.005)	1.2 (0.043)	0.55 (0.017)	0.735
Skin	18 (1.1)	0.54 (0.008)	0.88 (0.011)	0.59 (0.011)	0.617
Spleen	6.7 (0.6)	0.74 (0.003)	0.75 (0.021)	0.64 (0.013)	0.735
Stomach	−0.4 (2.4)	0.73 (0.023)	0.88 (0.022)	0.91 (0.037)	0.496
Testes	−34 (2.8)	0.83 (0.024)	0.65 (0.023)	0.55 (0.028)[b]	0.551
Thymus	3.9 (4.0)	0.74 (0.017)	0.98 (0.039)	0.93 (0.065)	ND

[a] (23).
[b] Kp calculated using the tissue weight at end of incubation.
ND - not determined.

The water content of tissue pieces was calculated as:

$$\text{Fractional water content} = \frac{W - W_{dry}}{W} \quad (1)$$

where W and W_{dry} are the wet and dry tissue weights.

The percentage swelling during the incubation was calculated as:

$$\text{Swelling (\%)} = \frac{W_{inc} - W}{W} \times 100 \quad (2)$$

where W_{inc} is the weight of tissue after incubation

The distribution of a marker between tissue and medium (Kp) was determined experimentally as:

$$Kp = \frac{cpm_T/W}{cpm_M/V_M} \quad (3)$$

where cpm_T and cpm_M are the counts per min of the radioisotope in the tissue and medium respectively and V_M the volume of medium.

The tissue-to-medium partition coefficient correcting for the imbibed media (Kp_{corr}) was calculated as:

$$Kp_{corr} = \frac{(cpm_T - cpm_{imb})/W}{cpm_M/V_M} \quad (4)$$

where cpm_{imb} is the additional amount of radioactivity in the tissue at the end of the incubation resulting from imbibed medium, which was calculated by assuming that the concentration of radioisotope in imbibed medium was the same as in the bulk medium at the end of the incubation.

RESULTS

Tissue Swelling

The mean extent of swelling (n = 6) observed following a 2 h incubation at 37°C varied widely among the tissues, with the most swelling observed in muscle (41%) and least in stomach (−0.4%) and intestine (−5%), Table I. This slight loss in stomach and intestine weight was probably a result of the luminal washing procedure when the tissue was excised, effectively meaning that the tissue receives a pre-incubation with subsequent unrecorded swelling and an unspecified amount of adhered buffer. A reduction in testes mass (34%) was recorded due to disintegration of the tissue during incubation and consequent incomplete recovery from the vial at the end of the incubation.

Partitioning of ^{14}C-Urea in Rat Tissues

Preliminary experiments with muscle, liver and adipose tissue indicated that ^{14}C-urea and ^{3}H-inulin attained equilibrium within 0.5 h of incubation. The partition of ^{14}C-urea between 14 separate fresh tissues and Hanks/HEPES buffer (n = 10), based on the original weight of tissue (Kp) varied from as low 0.23 for adipose tissue to 1.2 for brain and muscle, Table I. The majority were also greater than the fractional tissue water content as determined by desiccation. In the worst cases, the overestimation was 86% for brain and 400% for adipose tissue. When corrected for the extent of swelling, the agreement between ^{14}C-urea Kp_{corr} and fractional water content was substantially improved, with the majority of tissues showing a difference of less than 20%. However, Kp_{corr} was still insufficient to totally account for the difference in adipose tissue (110%).

Kp_{corr} could not be applied to testes due to the disintegration of tissue during the incubation. Consequently for testes, the best estimate of partitioning is obtained through Kp, using the tissue weight at the end of the incubation, accepting that this will be an overestimate due to an unknown amount of imbibed buffer. For tissues that demonstrated a large amount of swelling during the incubation, notably muscle and kidney, there was a tendency for Kp_{corr} to underestimate the fractional

water content. Overall though, Kp_{corr} results in a more accurate estimate of the total water space of the tissue than *Kp*.

Partitioning of ^{3}H-inulin into Rat Tissues

The mean ^{3}H-inulin *Kp* values (n = 10) based on both initial and final tissue weights are listed in Table II, in comparison with literature values for the fractional extracellular spaces. Values based on the preincubation tissue weight were generally higher than the extracellular tissue fraction, particularly for muscle (0.605 vs 0.146) and stomach (0.441 vs 0.130). As expected, the *Kp* values were somewhat reduced when based on the tissue weight after 2 h incubation, due to swelling. Overall there was good agreement between extracellular fraction and *Kp* values based on the incubated tissue weight, although for some tissues there were significant discrepancies. Also, the *Kp* values for ^{3}H-inulin were smaller than the corresponding values for ^{14}C-urea, indicating that inulin is accessing a smaller tissue space.

DISCUSSION

Preliminary investigations were conducted on several physiological buffers, including Hanks/HEPES, Leibovitz and Krebs-Henseleit bicarbonate. ^{14}C-Urea *Kp* values were equivalent in muscle incubated in all three buffers although the pH of the Krebs buffer had increased substantially by the end of the incubation and consequently it was not investigated further. The buffers used were intentionally protein free, so that the system would permit an estimate of *Kpu*, the tissue-to-unbound plasma partition coefficient. This is of particular relevance for highly protein bound drugs. ^{14}C-Urea and ^{3}H-inulin attained equilibrium after 0.5 h incubation. However, the incubation was extended to 2 h to mirror the conditions likely to be needed to ensure equilibrium for drugs with *Kp* values greater than that of the fractional tissue water content.

In agreement with the literature (9,10,13,14) tissue swelling was observed to occur in two phases, with an initial rapid uptake lasting approximately 20 min, followed by a slower phase lasting until the end of the incubation. The swelling is presumably driven by osmotic pressure to equilibrate the protein containing tissue with the protein-free medium. Other factors that can effect tissue swelling are the temperature at which the slice is held prior to incubation (10), the length of the incubation as well as the ionic buffer strength, particularly for potassium and chloride (13,15).

^{14}C-Urea and ^{3}H-inulin were chosen as markers of total tissue water and extracellular space respectively because they have been investigated extensively with *in vitro* (16,17), *in situ* (18,19) and *in vivo* (20,21) studies and because neither binds to tissue components. Tissue swelling during *in vitro* incubation resulted in ^{14}C-urea *Kp* values that were generally greater than the fractional tissue water content. However, correcting for imbibed medium under the reasonable assumption that the marker concentration of imbibed fluid was the same as that in the bulk medium at the end of the incubation (9,22) resulted in good agreement between Kp_{corr} and the fractional tissue water content. For drugs that are freely able to penetrate cellular membranes, failure to correct for tissue swelling will lead to the greatest overestimation of *Kp* for compounds with low *Kp* values. For compounds exhibiting high *Kp* values (generally greater than 10), the majority of compound is partitioned or bound within tissue cells or membranes and consequently the overestimation of *Kp* from imbibed medium is comparatively small and effectively can be ignored. However, the experiments presented here only indicate the applicability of the technique for hydrophilic substrates.

The ^{14}C-urea Kp_{corr} values generally agreed closely with *Kp* values obtained following i.v. bolus dosing in the rat (Table I) and an *in situ* rat hind limb preparation (19) in muscle (0.55 vs 0.52 respectively), skin (0.59 vs 0.58), bone (0.33 vs 0.42) and adipose tissue (0.10 vs 0.37). However, there are some notable differences between the *in vitro* and *in vivo* results in brain and kidney, presumably due to the blood-brain barrier and an overestimation *in vivo* of ^{14}C-urea in the kidney tubules, respectively (23). Additional validation of the *in vitro* methodology is observed as the volume of distribution calculated from the sum of individual *in vitro* Kp_{corr} values and tissue volumes

Table II. Reported Extracellular Fraction and Observed Mean ^{3}H-Inulin Estimates (±SE, n = 10) Based on the Initial ([b]) and Final ([c]) Weight of Tissue Following a 2 h *In Vitro* Incubation in Hanks/HEPES buffer at 37°C in Comparison to Literature Values for *In Vitro* Incubations[d]

Tissue	Extracellular fraction[a]	Kp[(b)]	Kp[(c)]	Kp[d]
Adipose tissue	0.145	0.158 (0.010)	0.142 (0.009)	
Bone	0.141	0.201 (0.047)	0.192 (0.038)	
Brain	0.038	0.334 (0.017)	0.248 (0.011)	0.36, 0.42, 0.43, 0.30
Heart	0.358	0.471 (0.017)	0.433 (0.013)	
Intestine	0.118	0.267 (0.014)	0.285 (0.019)	
Kidney	0.305	0.384 (0.022)	0.276 (0.014)	0.37
Liver	0.278	0.253 (0.009)	0.223 (0.009)	0.27
Lung	0.448	0.457 (0.020)	0.384 (0.014)	
Muscle	0.146	0.605 (0.043)	0.426 (0.021)	0.45
Skin	0.321	0.427 (0.014)	0.360 (0.009)	
Spleen	0.438	0.163 (0.012)	0.152 (0.010)	
Stomach	0.130	0.441 (0.017)	0.444 (0.016)	
Testes	No Result	0.218 (0.011)	0.332 (0.019)	
Thymus	No Result	0.352 (0.033)	0.335 (0.020)	

[a] (23, 24).
[d] (9, 13, 25, 26).

(0.64 l/kg) accurately predicts that from the plasma pharmacokinetics following an i.v. bolus dose (23) in the rat (0.66 l/kg).

The *Kp* estimates for ^{3}H-inulin were in good agreement with literature values for brain, kidney, liver and muscle, Table II. In all tissues, ^{3}H-inulin accessed a smaller space than ^{14}C-urea which corresponded to the extracellular fraction determined *in vivo* (f_e) for the majority of tissues. However, in brain, intestine, muscle and stomach, the inulin space was larger than f_e and smaller in spleen. A volume of distribution of 0.32 l/kg is predicted from the ^{3}H-inulin *Kp* values based on the final tissue weight, which compares favourably with the fractional extracellular tissue volume in the rat (0.19).

It would be useful to correct for media entering the extracellular space on swelling, but only the total amount of media imbibed is known and not its distribution between intra and extracellular spaces. An attempt was made to calculate this distribution, knowing f_e and tissue swelling *in vitro*, but unfortunately this proved inconsistent due to the variability in the data and the small values involved. Therefore, where a test compound demonstrates an *in vitro Kp* value equivalent to that of inulin, for the purposes of *in vitro/in vivo* scaling, through a physiologically based pharmacokinetic model for example, it is recommended that the *in vivo* value for f_e replaces *in vitro Kp*.

In conclusion, this study has demonstrated that accurate estimates of total tissue water and extracellular spaces can be achieved through an *in vitro* system with marker compounds. For total tissue water, and consequently for drugs having no barrier to permeability, the effect of tissue swelling should be taken into account when calculating *Kp*. With a limited number of tissues inulin only provides an indication of the extracellular space and in this case, the *in vivo* value should be used for scaling purposes. It is recommended that both urea and inulin are suitable compounds to check on the viability of the *in vitro* technique prior to the investigation of test compounds.

ACKNOWLEDGMENTS

The authors acknowledge the technical help of Brian Shuker. Peter Ballard would like to thank Zeneca Pharmaceuticals for financial support.

REFERENCES

1. D. E. Leahy, R. Duncan, H. J. Ahr, M. K. Bayliss, A. G. de Boer, F. Darvas, J. H. Fentem, J. R. Fry, R. Hopkins, J. B. Houston, J. Karlsson, G. L. Kedderis, M. K. Pratten, P. Prieto, D. A. Smith, and D. W. Straughan. Pharmacokinetics in early drug research. The report and recommendations of ECVAM workshop 22. *ATLA* **25**:17–31 (1997).
2. T. Iwatsubo, N. Hirota, T. Ooie, H. Suzuki, and Y. Sugiyama. Prediction of *in vivo* drug disposition from *in vitro* data based on physiological pharmacokinetics. *Biopharm. Drug Disp.* **17**:273–310 (1996).
3. M. Rowland. Physiologic pharmacokinetic models and interanimal species scaling. *Pharmacol. Ther.* **29**:49–68 (1985).
4. V. Fiserova-Bergerova and M. L. Diaz. Determination and prediction of tissue-gas partition coefficients. *Int. Arch. Occ. Env. Health* **58**:75–87 (1986).
5. M. L. Gargas, R. J. Burgess, D. E. Voisard, G. H. Cason, and M. E. Andersen. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharm.* **98**:87–99 (1989).
6. J. E. Murphy, D. B. Janszen, and M. L. Gargas. An *in vitro* method for determination of tissue partition coefficients on non-volatile chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and estradiol. *J. Appl. Toxicol.* **15**:147–152 (1995).
7. J. M. Gearhart, G. W. Jepson, H. J. Clewell, III, M. E. Andersen, and R. B. Conolly. Physiologically based pharmacokinetic and pharmacodynamic model for the inhibition of acetylcholinesterase by diisopropylfluorophosphate. *Toxicol. Appl. Pharmacol.* **106**:295–310 (1990).
8. R. N. Upton, L. E. Mather, and W. B. Runciman. The *in vitro* uptake and metabolism of lignocaine, procainamide and pethidine by tissues of the hindquarters of sheep. *Xenobiot.* **21**:1–12 (1991).
9. H. M. Pappius and K. A. C. Elliott. Water distribution in incubated slices of brain and other tissues. *Can. J. Biochem.* **34**:1007–1022 (1956).
10. S. Varon and H. McIlwain. Fluid content and compartments in isolated cerebral tissues. *J. Neurochem.* **8**:262–275 (1961).
11. P. Poulin and K. Krishnan. Molecular structure-based prediction of the partition coefficients of organic chemicals for physiological pharmacokinetic models. *Toxicol. Meth.* **6**:117–137 (1996).
12. P. Poulin and F.-P. Theil. A Priori prediction of tissue:plasma partition coefficients of drugs to facilitate the use of physiologically-based pharmacokinetic models in drug discovery. *J. Pharm. Sci.* **89**:16–35 (2000).
13. R. S. Bourke and D. B. Tower. Fluid compartmentation and electrolytes of cat cerebral cortex *in vitro*—I. Swelling and solute distribution in mature cerebral cortex. *J. Neurochem.* **13**:1071–1097 (1966).
14. W. M. Sperry and F. C. Brand. Absorption of water by liver slices from "physiological" saline solutions. *Proc. Soc. Exp. Biol. (New York)* **42**:147–150 (1939).
15. R. S. Bourke. Studies of the development and subsequent reduction of swelling of mammalian cerebral cortex under isosmotic conditions *in vitro*. *Exp. Brain Res.* **8**:232–248 (1969).
16. K. Okamoto and J. H. Quastel. Water uptake and energy metabolism in brain slices from the rat. *Biochem. J.* **120**:25–36 (1970).
17. S. R. Cohen. The dependence of water content and "extracellular" marker spaces of incubated mouse brain slices on thickness. Alterations produced by slicing, and fluid spaces in intact and altered tissue. *Exp. Brain Res.* **20**:435–457 (1974).
18. W. Herms, and G. Arnold. Diuresis, inulin clearance, sodium reabsorption and oxygen consumption of the isolated erythrocyte–free perfused rabbit kidney. *Bibl. Haem.* **33**:404–407 (1969).
19. A. C. Heatherington and M. Rowland. Discrepancies in pharmacokinetic parameter estimation between bolus and infusion studies in the perfused rat hindlimb. *J. Pharmaco. Biopharm.* **23**:441–462 (1995).
20. S. M. Gad and R. L. Preston. *In vivo* prediction of extracellular and intracellular water in cattle. *J. Animal Sci.* **68**:3649–3653 (1990).
21. K. F. Rothe. Fractional extracellular space and fractional water content of various rat tissues at different extracellular pH values and in uremia. *Lab. Animals* **13**:171–174 (1979).
22. J. R. Stern, L. V. Eggleston, R. Hems, and H. A. Krebs. Accumulation of glutamic acid in isolate brain tissue. *Biochem. J.* **44**:410–418 (1949).
23. G. E. Blakey. Tissue Kinetics for a Series of Barbiturates, in *Department of Pharmacy.* University of Manchester, Manchester (1995).
24. S. P. Khor and M. Mayersohn. Potential error in the measurement of tissue to blood distribution coefficients in physiological pharmacokinetic-modeling. Residual tissue blood 1. Theoretical considerations. *Drug Metab.Disp.* **19**:478–485 (1991).
25. J. N. Allen. Extracellular space in the central nervous system. *Arch. Neurol. Psych.* **73**:241–248 (1955).
26. H. S. Bachelard, W. J. Campbell, and H. McIlwain. The sodium and other ions of mammalian cerebral tissues, maintained and electrically stimulated *in vitro*. *Biochem. J.* **84**:225–232 (1962).