ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 175–180

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Short communication

Differences in pre- and post-prandial plasma lipid profiles affect the extraction efficiency of a model highly lipophilic drug from beagle dog plasma

Christopher J.H. Porter *, Suzanne M. Caliph, William N. Charman

The Department of Pharmaceutics, Victorian College of Pharmacy, Monash University, Parkville Campus, 381 Royal Pde., Parkville, Victoria 3052, Australia

Received 26 August 1996; accepted 24 October 1996

Keywords: Halofantrine; HPLC; Extraction efficiency; Post-prandial

1. Introduction

The validation of assays for the determination of drug concentration in plasma is well defined in terms of reproducibility, accuracy, precision, sensitivity and specificity [1]. The data to support these validation-parameters are characteristically generated by replicate analyses of known quantities of drug when 'spiked' into blank (drug-free) plasma. Typically, pooled pre-prandial (fasted) plasma is used as a representative plasma matrix.

Under some circumstances, the nature of the plasma matrix may change between- and within-dosing schedules (e.g. during fed/fasted bioavailability studies). After ingestion of a meal, dietary fat is digested and resynthesised by the enterocyte into chylomicrons. These intestinally-derived lipoproteins gain access to the systemic circulation resulting in post-prandial plasma lipid concentrations which can be elevated 5-fold compared with pre-prandial plasma [2]. Post-prandial plasma collected after ingestion of a fatty meal is visually turbid due to the increased levels of plasma lipoproteins such as chylomicrons, very low density lipoproteins and low density lipoproteins. Lipophilic drugs may associate with the various plasma lipoproteins and the extent of association changes as a function of plasma lipid concentration [3–6]. Variations in drug binding to lipoproteins as a function of food ingestion may also alter drug distribution and clearance [3,7].

Changes in plasma lipid levels, and the known association of lipophilic drugs with the hydrophobic core lipids of plasma lipoproteins [4], stimulated our interest into the effect of changing plasma lipid profiles (representative of that encountered during a fed/fasted bioavailability study) on the extraction efficiency of a model highly lipophilic drug (Halofantrine HCl, calc.

* Corresponding author. Tel.: + 61 3 99039649; fax: + 61 3 99039583; e-mail: chris.porter@vcp.monash.edu.au

PII S0731-7085(97)00008-3

log P 8.5). The effect of extraction solvent volume on extraction efficiency from pre- and post-prandial plasma, and the role of an internal standard to correct for these differences, was examined. Extraction efficiency was also assessed from spiked blank plasma and an authentic plasma sample obtained after drug administration.

2. Experimental

2.1. Chemicals

Halofantrine HCl (Hf), the internal standard (IS) 2,4-Dichloro-6-trifluoromethyl-9-{1-[2-(dibutyl-amino)ethyl]}phenanthrene methanol HCl and Halfan® tablets containing 250 mg Hf HCl were obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). Acetonitrile (Mallinckrodt, Paris, KY, USA) and tert-butyl methyl ether (Fluka, Buchs, Switzerland) were HPLC grade. AR grade hydrochloric acid and glacial acetic acid (Ajax Chemicals, Sydney, Australia) and electrophoresis grade sodium dodecyl sulphate (SDS, Eastman Kodak, Rochester, NY, USA) were used as received. All other chemicals were AR grade, and water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system.

2.2. Equipment

The plasma assay was based on a published method [8]. The HPLC system consisted of a Waters 610 pump, a Waters 717 plus autosampler, a Waters 486 UV detector (257 nm) (Waters, Milford, MA, USA), an Ultrasphere C8 bonded-phase column (5 μm particle size, 25 cm × 4.6 mm i.d., Beckman Instruments, CA, USA) and a Newguard RP-8 cartridge guard column (Aquapore 7 μm, 3.2 × 15 mm, Perkin Elmer, CA, USA). Data were analyzed on a Shimadzu C-R5A integrator (Shimadzu Corp, Japan). The mobile phase consisted of 75:25 (v/v) acetonitrile:water with 0.2% (w/v) SDS and 0.2% (v/v) glacial acetic acid. The flow rate was 1.5 ml min^{-1} and separations were performed at ambient temperature.

2.3. Standards

Stock solutions of Hf and the IS were prepared at 100 μg ml^{-1} (reported as mass of base ml^{-1}) by dissolving the corresponding HCl salt in acetonitrile, and the solutions were stored in glass at −20°C. Standard solutions were prepared in acetonitrile on each day of analysis at concentrations in the range of 0.05–20 μg ml^{-1} for Hf and at 2 μg ml^{-1} for the IS.

2.3.1. Blank (drug-free) plasma

Fasted blank plasma was obtained from blood samples taken from the cephalic vein of beagles which had been fasted for 12 h with water available ad libitum. Blank fed plasma was obtained from blood samples taken 2 h after ingestion of 600 g of standard dog food (typical composition of 2.5% protein and 7.5% fat). Blood samples were collected into sterile tubes containing dipotassium EDTA as an anti-coagulant, and plasma was separated by centrifugation at 1000 × *g* for 10 min and stored at −70°C until required.

2.3.2. Spiked fed and fasted plasma standards

Spiked Hf plasma standards were prepared by spiking 500 μl blank fed or blank fasted plasma samples with 50 μl of Hf stock solution to produce final drug concentrations of 20, 200, 2000 and 8000 ng ml^{-1}. The spiked standards were mixed by vortexing for 1 min.

2.3.3. Authentic plasma standards with varying lipid content

Blood samples were taken from fed beagles 2 h after oral administration of a 250 mg Hf HCl tablet (dogs were fed 600 g standard dog food 1 h prior to drug administration). The plasma was isolated and diluted with equal volumes of either blank fed plasma, blank fasted plasma or a combination of blank fed and fasted plasma to produce five different plasma standards with the same nominal Hf concentration (200 ng ml^{-1}) but different plasma lipid concentrations. The concentration of plasma triglyceride lipid (mmol l^{-1} of C_{18} triglyceride) was determined using an enzymatic colourimetric test kit (Triglycerides GPO-PAP, Boehringer Mannheim, GmBH, Mannheim, Ger-

many) in a Cobas Bio clinical chemistry analyser (Roche Diagnostics, Melbourne, Australia).

2.4. Extraction procedure

A 200-μl aliquot of IS solution (2 μg ml^{-1}) was added to either 500 μl of a spiked plasma standard (containing either 20, 200, 2000 or 8000 ng Hf per ml) or 500 μl of an authentic plasma standard (containing a nominal concentration of 200 ng ml^{-1}) in a 12 ml polypropylene centrifuge tube. A 950 μl aliquot of acetonitrile was added to precipitate plasma proteins, after which the samples were vortexed for 1 min and centrifuged at $700 \times g$ for 2 min. Extraction of Hf was accomplished by addition of either 2, 4, 6 or 8 ml tert-butyl methyl ether (TBME), after which the samples were mixed by vortexing for 2 min and centrifuged for 5 min at $700 \times g$. All the upper organic phase was carefully removed using a glass pipette and transferred to a second polypropylene centrifuge tube containing 100 μl of 0.005 M HCl prepared in acetonitrile. The contents of the centrifuge tube were then evaporated under a stream of high purity nitrogen at 35°C using a N-EVAP 112 evaporator (Organomation, MA). The residue was reconstituted with 200 μl of acetonitrile, vortexed for 1 min, and centrifuged at $700 \times g$ for 2 min prior to injection of a 25 μl aliquot onto the HPLC.

2.5. Assay recovery

Extraction recovery was calculated by comparison of the peak heights of Hf recovered from spiked plasma standards with the peak heights of injected standard solutions prepared in acetonitrile. The concentration of the authentic plasma standards was calculated by comparison of the peak heights of Hf recovered from the plasma standards with the peak heights of the injected standard solutions. The extraction ratio of Hf–IS was calculated by comparing the peak heights of Hf and IS recovered from the authentic plasma standards.

3. Results and discussion

The validation of assay and extraction procedures for the quantitation of plasma drug concentrations is typically performed using samples of pooled fasted plasma spiked with known quantities of drug. An underlying assumption is that the spiked fasted plasma is representative of the authentic plasma samples to be analysed. However, in fed/fasted bioavailability studies the nature and composition of the plasma matrix changes due to food ingestion. In this communication, the effect of a change in the plasma lipid concentration on the efficiency of extraction of halofantrine, a model lipophilic drug, was examined.

Blank plasma samples from fasted and fed beagle dogs were spiked with a range of Hf concentrations (20, 200, 2000 and 8000 ng ml^{-1}) and extracted with either 2 or 8 ml of TBME. As depicted in Fig. 1, the extraction efficiency of Hf from both spiked fasted and spiked fed plasma

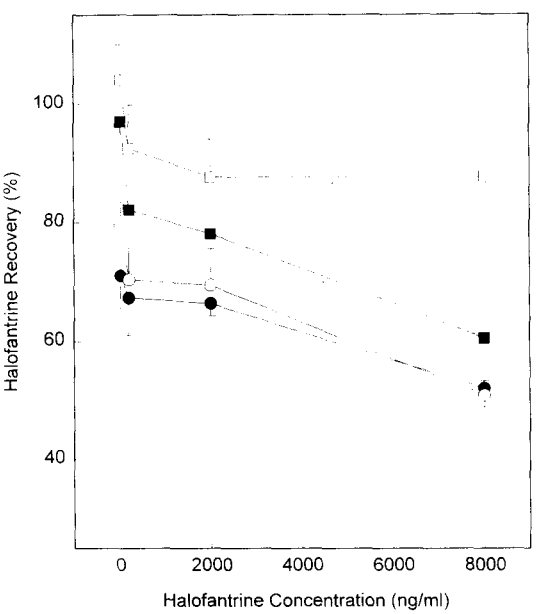

Fig. 1. The percent recovery of Halofantrine, as a function of concentration, from spiked fasted plasma (open symbols) and spiked fed plasma (closed symbols) when using either 2 ml TBME (○ or ●) or 8 ml TBME (□ or ■) as the organic extractant. Data are presented as mean ± S.D., $n = 4$. In some cases the error bars are contained within the symbol.

samples decreased as the spiked drug concentration increased, or the volume of extractant decreased. The recovery of Hf from spiked fed plasma was less than from spiked fasted plasma (when employing 8 ml of TBME as extractant and the same Hf concentration) suggesting that increased plasma lipid levels were responsible for the decreased drug extraction. A similar trend was seen using 2 ml TBME although the differences were not statistically significant. Subsequent studies which examined the extraction efficiency of Hf, when spiked at 200 ng ml^{-1}, into plasma of varying lipid loads confirmed the decreased extraction efficiency with increasing lipid load (data not shown). The increase in triglyceride load of the plasma did not affect the chromatographic separation of Hf and the chromatograms obtained after extraction of Hf from fed plasma were not substantially different to those reported previously after extraction of Hf from fasted plasma [8].

The hydrophobic triglyceride and cholesterol ester core lipids of plasma lipoproteins [9] (when present at post-prandial concentrations) are not necessarily fully miscible with organic solvents commonly used in plasma extractions. Therefore, the presence of any residual non-solubilized hydrophobic lipid in the subnatant fraction of the plasma extraction could effectively sequester lipophilic drugs thereby reducing extraction of drug into the supernatant organic phase.

The extent and character of association of lipophilic drugs with plasma lipoproteins is complex and likely to be affected by the volume and choice of organic solvent used to spike plasma samples. Therefore, the integrity and drug binding characteristics of plasma lipoproteins in samples prepared by spiking blank plasma with drug dissolved in a small volume of an organic solvent may be altered when compared to authentic plasma samples.

In an attempt to exclude the influence of the spiking organic solvent, the recovery of Hf after extraction from authentic plasma samples (obtained after oral administration of Hf HCl to a beagle) was assessed. Non-spiked plasma standards were obtained by dilution of a plasma sample (taken 2 h post dosing) with equal volumes of blank plasma (with varying lipid loads) to produce five plasma standards with the same nominal Hf concentration but different lipid concentrations. A direct measure of the extraction efficiency of Hf from these samples could not be determined as the exact concentration of drug could not be independently determined. Therefore, the concentration of Hf was calculated by comparison of the peak heights of Hf recovered from the authentic plasma samples with the peak heights of injected standard solutions.

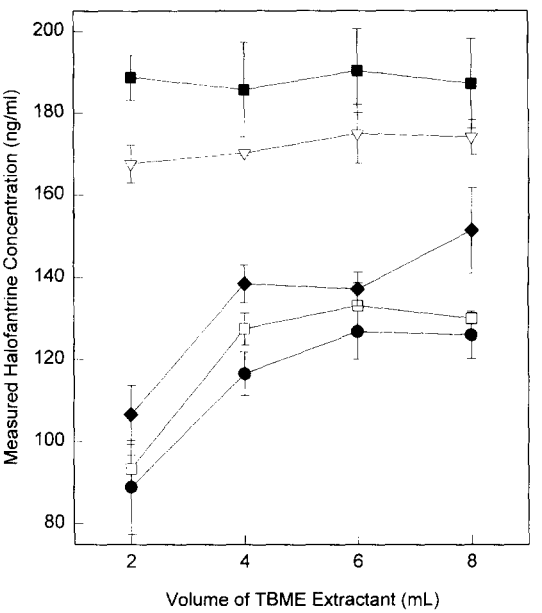

Fig. 2. Measured Halofantrine concentration from authentic plasma samples (of different lipid concentrations with a constant Hf concentration of 200 ng ml^{-1}) as a function of volume of organic extractant (TBME). The plasma triglyceride concentrations were 0.31 mmol l^{-1} (■), 0.39 mmol l^{-1} (▽), 0.66 mmol l^{-1} (◆), 0.88 mmol l^{-1} (□) and 1.10 mmol l^{-1} (●). Data are presented as mean ± S.D., $n = 4$.

Fig. 2 describes the Hf concentration recovered from these plasma samples as a function of plasma lipid concentration and volume of organic extractant. The measured concentration of Hf decreased as the lipid load increased consistent with the results presented in Fig. 1 where spiked plasma was employed. Furthermore, an increase in the volume of the extractant above 4 ml did not further increase drug recovery from a plasma sample of a particular lipid concentration. When

Table 1
Peak height ratios ($\times 10^2$) of Halofantrine (Hf) to internal standard (IS)[a]

Volume of extractant	Plasma triglyceride concentration				
	0.31 mmol/l	0.39 mmol/l	0.66 mmol/l	0.88 mmol/l	1.10 mmol/l
2 ml	9.25 ± 0.65[b]	7.71 ± 0.26[b]	8.17 ± 0.33	7.67 ± 0.24[b]	7.66 ± 0.25[b]
4 ml	8.75 ± 0.10	8.45 ± 0.57	8.64 ± 0.21	8.21 ± 0.23	8.19 ± 0.23
6 ml	8.72 ± 0.31	8.65 ± 0.67	8.49 ± 0.25	8.48 ± 0.18	8.38 ± 0.18
8 ml	8.31 ± 0.57	8.93 ± 1.01	8.74 ± 0.19	8.25 ± 0.11	8.24 ± 0.11

The concentration of Hf in each plasma sample was 200 ng/ml. Data are presented as mean $\pm$ S.D., $n = 4$.
[a] When extracted with different volumes of organic extractant (TBME) from authentic plasma samples containing different amounts of triglyceride lipid.
[b]Significantly different (1 way ANOVA, $P < 0.05$).

employing extractant volumes of 4 ml or greater, the maximum decrease in measured drug concentration over the 3-fold range of plasma triglyceride concentrations studied was approximately 30%.

The extraction procedure and HPLC assay utilised in these studies was developed using 8 ml of TBME as the extractant and fasted plasma as previously described [8]. A closely related structural analogue of Hf was intentionally chosen as the internal standard (IS) for the assay. Various other methodologies have been published describing the quantitation of Hf in blood or plasma [10–13], using this and other internal standards, however non of these studies have specifically addressed the issue of changes in extraction efficiency with changes in biological matrix.

The role of the IS in correcting for changes in the absolute extraction efficiency of Hf, as a function of different plasma lipid concentrations, was assessed by examining the Hf/IS peak height ratios after extraction of the same plasma samples as described in Fig. 2. The Hf/IS peak height ratios as a function of the volume of extractant and the plasma lipid concentration are presented in Table 1. With the exception of the data obtained using the 2 ml of extractant (which showed significant variation), changes in the absolute extraction of Hf as a function of plasma lipid load and extractant volume were correspondingly mirrored by changes in the extraction efficiency of the IS as demonstrated by the consistent peak height ratios reported in Table 1. Therefore, use of this IS and comparison of Hf/IS peak height ratios for unknown samples with plasma standards effectively corrects for the changes which occur in the absolute extraction efficiency between pre- and post-prandial plasma samples of variable lipid composition.

It should be noted that whilst inclusion of an IS may correct for relative changes in extraction efficiency, the absolute extraction efficiency of a lipophilic drug from post-prandial plasma is likely to be significantly lower than from fasted plasma. It would be expected that a decrease in the efficiency of drug extraction would lead to an increased variability which may not otherwise be evident if the assay validation procedure is only performed using pre-prandial plasma. Therefore, the choice of the plasma matrix with which to conduct the validation aspects of assay development should reflect the possible range of plasma matrices for which assays will be conducted (eg. fed/fasted bioavailability studies, differences between healthy volunteers and patients).

These preliminary data have been obtained using beagle dog plasma, however, post prandial increases in human plasma triglyceride levels are typically as large, or larger, than that in beagle dogs (human plasma triglyceride concentrations range from < 1 mmol l^{-1} (fasted) to 4 mmol l^{-1} (fed) [9]) and a similar decrease in the extraction efficiency of lipophilic drugs from post prandial human plasma is likely.

The data presented here suggest that plasma assays supporting fed/fasted bioavailability stud-

ies of lipophilic drugs (or clinical studies where significant variation in the plasma lipid load are expected) should be conducted using an appropriate internal standard. Furthermore, the suitability of the IS should be evaluated by determination of the extraction efficiency of both the drug and IS from pre- and post-prandial plasma. Similarity of elution characteristics of drug and IS from a reverse phase LC column may not be sufficient to guarantee similar partitioning profiles between pre- and post-prandial plasma samples.

Acknowledgements

This work was supported in part by SmithKline Beecham (UK).

References

[1] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C. Edgar Cock, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 81 (1992) 309–312.
[2] P. Tso, in L.R. Johnson (Ed.), Physiology of the Gastrointestinal Tract, Raven Press, New York, 1994, pp. 1867–1908.
[3] A.J. Humberstone, C.J.H. Porter and W.N. Charman, Pharm. Res., 12 (1995) S356.
[4] S. Glasson, R. Zini and J.P. Tillement, Biochem. Pharmacol., 31 (1982) 831–835.
[5] B. Cenni and B. Betschart, Chemotherapy, 41 (1995) 153–158.
[6] B. Cenni, J. Meyer, R. Brandt and B. Betschart, Br. J. Clin. Pharmacol., 39 (1995) 519–526.
[7] S.K. Gupta and L.Z. Benet, Pharm. Res., 7 (1990) 46–48.
[8] A.J. Humberstone, G.J. Currie, C.J.H. Porter, M.J. Scanlon and W.N. Charman, J. Pharm. Biomed. Anal., 13 (1995) 265–272.
[9] J.S. Cohn, J.R. McNamara, S.D. Cohn, J.M. Ordovas and E.J. Schaefer, J. Lipid Res., 29 (1988) 469–479.
[10] J.W. Hines, P.D. Elkins, C.E. Cook and C.M. Sparacino, J. Pharm. Sci., 74 (1985) 433–437.
[11] K.A. Milton, S.A. Ward and G. Edwards, J. Chromatogr., 433 (1988) 339–344.
[12] Y. Gaillard, J.M. Prevosto, V. Cheminel, O. Soares and J.F. Chaulet, J. Chromatogr., 668 (1995) 315–321.
[13] D.R. Brocks, M.J. Dennis and W.H. Schaefer, J. Pharm. Biomed. Anal, 13 (1995) 911–918.