ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1999) 927–938

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Development of an assay for the extraction and quantification of nine 5-*n*-alkyl-5-ethyl barbituric acids in various rat tissues

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Received 2 October 1997; received in revised form 12 February 1998; accepted 23 February 1998

Abstract

Methods were developed to quantify a series of nine homologous 5-*n*-alkyl-5-ethyl barbituric acids in 15 rat tissues. Tissue homogenates were spiked with one of four multicomponent mixtures (methyl to *n*-propyl, *n*-propyl to *n*-pentyl, *n*-pentyl to *n*-heptyl and *n*-pentyl to *n*-nonyl). Liquid–liquid extraction was used to extract the homologues from the rat tissues. Reverse phase HPLC with UV detection at 214 nm was used to separate and quantify the individual barbiturates. The limit of detection for each respective homologue was 1 μg g^{-1} except skin and bone (2 μg g^{-1}). The methodology developed reduced a potential 135 individual assays to a more manageable 16. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Barbiturates; HPLC; Rat; Tissues; Extraction; Homologous series; Validation

1. Introduction

Overall distribution and elimination of a drug is often estimated from the study of plasma pharmacokinetics. This analysis provides little information on how individual tissues interact to produce the observed plasma drug concentration–time profile. Determination of drug concentrations in tissues at various times post dose helps to solve this problem. In humans, tissue sampling presents practical difficulties, thus animals are commonly used to investigate the kinetics of drug distribution in tissues. From such studies an estimate of the tissue-to-plasma partition coefficient (K_p) can be determined. K_p describes the relative affinity of a particular tissue for a drug and is an important parameter in the development of physiologically-based pharmacokinetic (PBPK) models [1]. By using a homologous series of compounds, which, although similar structurally, have widely different physicochemical properties, quantitative structure PBPK models can be developed. The aim of the present study was to develop a reproducible technique capable of extracting and analysing a

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0731-7085/99/$ - see front matter
PII S0731-7085(98)00098-3

congeneric series of nine 5-*n*-alkyl-5-ethyl barbituric acids (Fig. 1) (log $P = 0.02–4.13$, $pK_a = 7.8–8.11$) [2,3] from the following rat tissues: adipose, brain, bone, gut, heart, kidney, liver, lung, muscle, pancreas, skin, spleen, stomach, testes and plasma.

The design of the proposed in vivo experiments included administration of a multicomponent mixture of three different barbiturates to each rat. Previous work has demonstrated that there is no evidence of a pharmacokinetic interaction when the barbiturates are administered as a multicomponent mixture [2,4]. This regimen improves the probability of detecting real differences in pharmacokinetics between congeners. The associated number of sample assays required per investigation is also reduced. The complexity of the assay is increased, however, as three homologues need to be extracted and separated from each tissue sample.

2. Experimental

2.1. Reagents and equipment

All reagents were of analytical grade unless otherwise stated. Trisodium phosphate, sulphuric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, hexane, ethyl acetate, tertiary-butyl methyl ether (HPLC grade) and acetonitrile (HPLC grade) were obtained from BDH, Poole, Dorset, UK. The nine 5-alkyl-5-ethyl barbituric acids (congeneric consecutive straight chain series from methyl to *n*-nonyl) were synthesised by condensing urea with the appropriately substituted diethylmalonate, in the School of Pharmacy and Pharmaceutical Sciences, University of Manchester (purity greater than 95% by ^{1}H NMR and C, H and N analysis). The nine compounds of the homologous series were divided into four groups based on their similar lipophilicity. The composition of each group is listed below:

Group A, 5-methyl-; 5-ethyl-; 5-*n*-propyl-; 5-ethyl barbituric acid.

Group B, 5-*n*-propyl-; 5-*n*-butyl-; 5-*n*-pentyl-; 5-ethyl barbituric acid.

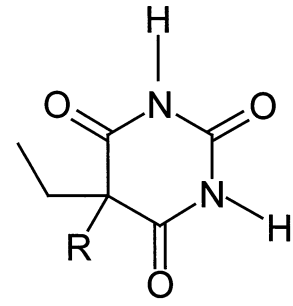

Fig. 1. The generic structure of 5-*n*-alkyl-5-ethyl barbituric acid.

Group C, 5-*n*-pentyl-; 5-*n*-hexyl-; 5-*n*-heptyl-; 5-ethyl barbituric acid.

Group D, 5-*n*-heptyl-; 5-*n*-octyl-; 5-*n*-nonyl-; 5-ethyl barbituric acid.

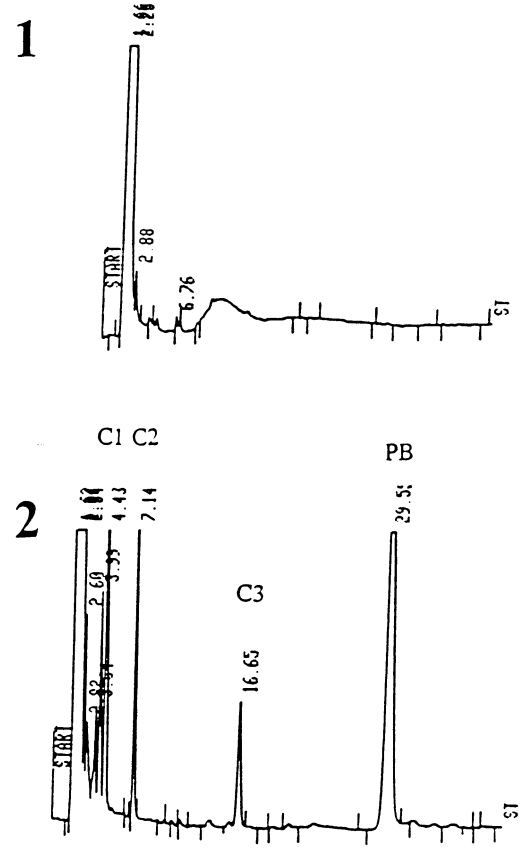

Fig. 2. Chromatograms of liver homogenate extracts containing group A barbituric acids at (1) 0 μg g^{-1} and (2) 15 μg g^{-1}: C1, methyl (4.43 min); C2, ethyl (7.14 min); C3, *n*-propyl (16.65 min); PB, phenobarbitone (29.58 min).

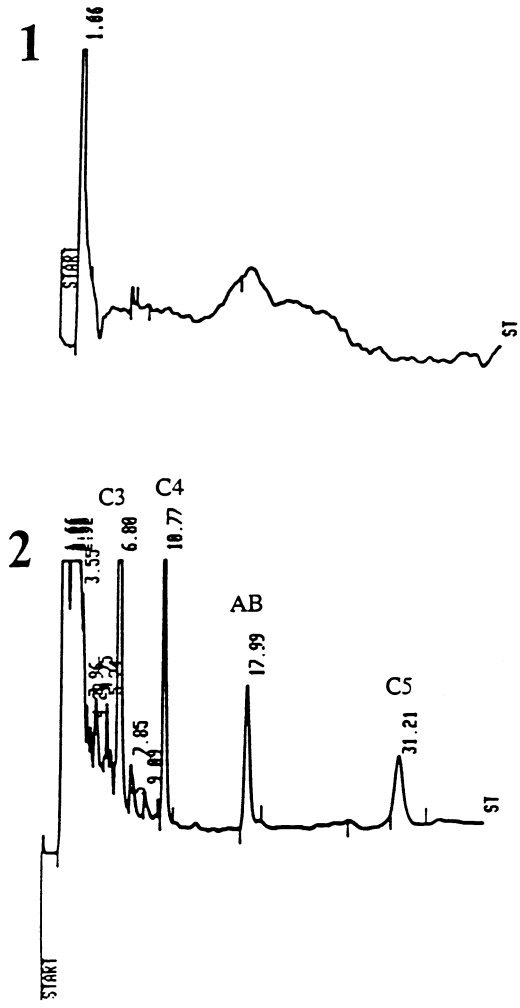

Fig. 3. Chromatograms of liver homogenate extracts containing group B barbituric acids at (1) 0 μg g^{-1} and (2) 15 μg g^{-1}: C3, *n*-propyl (6.88 min); C4, *n*-butyl (10.77 min); C5, *n*-pentyl (31.21 min); AB, amylobarbitone (17.99 min).

The extraction methods used the following apparatus: a Dremel Motoflex drill, Dremel, UK; a homogenisation vessel (15 ml), Jencons, UK; a Teflon pestle end, Jencons, UK; an ultrasonicator, Scientific and Medical Products, UK; a centrifuge, Mistral 300I MSE, Fisons, UK; a reciprocating shaker, MR Supplies, UK; and a Tecam Dribloc DB-3.

Chromatographic separations were achieved in the analytical method using a Hypersil ODS 5 micron 25 cm × 4.6 mm i.d. column (Hichrom, UK). Additionally a Pellicular ODS 60 micron, 30 mm × 2 mm i.d. guard column (Anachem, UK) was used. The chromatographic system used consisted of a Waters 712 Wisp autoinjector, a Waters model 510 solvent delivery system, a Hewlett-Packard 10813 liquid chromatographic oven, an LDC spectromonitor 3 UV HPLC monitor and a Hewlett-Packard integrator model 3390A.

2.2. Mobile phases and buffers

The mobile phase was a mixture of acetonitrile and Sorensen's [5] phosphate (0.01 M, pH 5.4) buffer. The acetonitrile content was dependent on

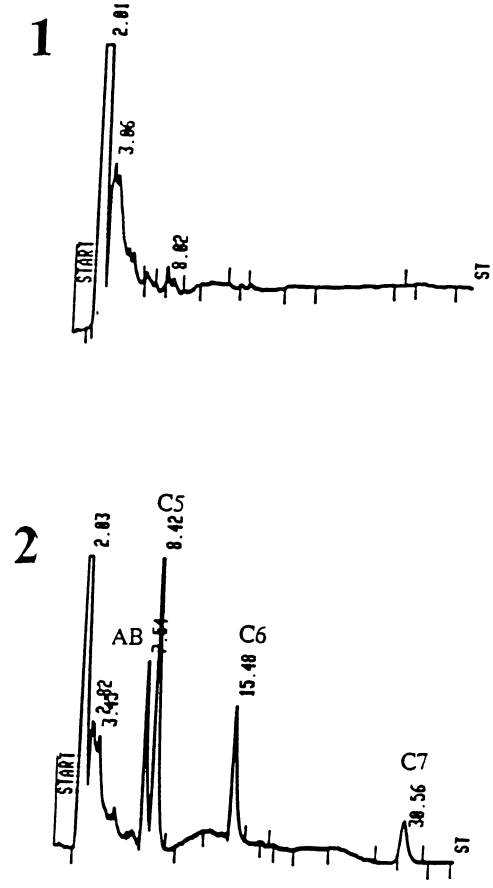

Fig. 4. Chromatograms of adipose homogenate extracts containing group C barbituric acids at (1) 0 μg g^{-1} and (2) 15 μg g^{-1}: C5, *n*-pentyl (8.42 min); C6, *n*-hexyl (15.48 min); C7, *n*-heptyl (30.56 min); AB, amylobarbitone (7.54 min).

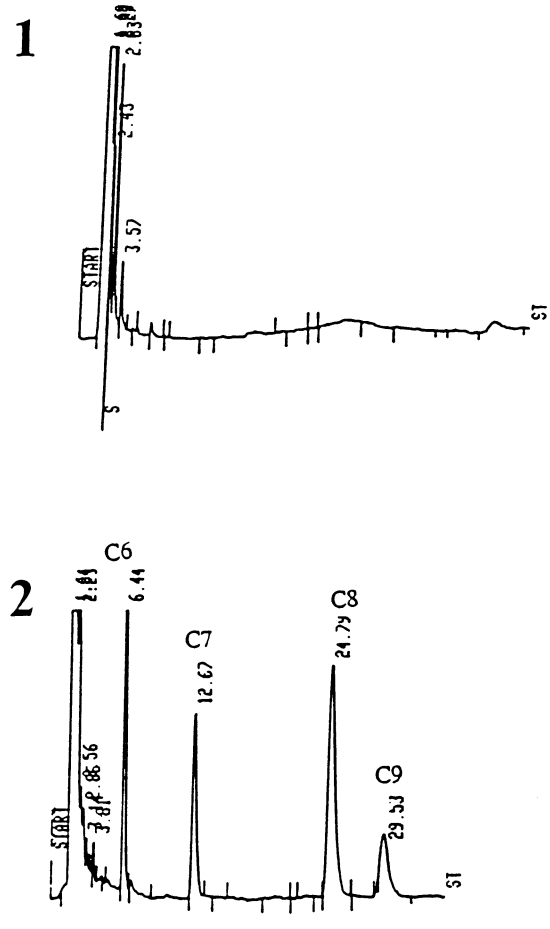

Fig. 5. Chromatograms of skin homogenate extracts containing group D barbituric acids at (1) 0 μg g^{-1} and (2) 15 μg g^{-1}: C6, *n*-hexyl (6.44 min); C7, heptyl (12.67 min); C8, *n*-octyl (24.79 min); C9, *n*-nonyl (29.53).

the barbiturate group being analysed, group A: acetonitrile–Sorensen's buffer (12.5:87.5 v/v), group B: acetonitrile–Sorensen's buffer (22.5:77.5 v/v), group C: acetonitrile–Sorensen's buffer (34.0:66.0 v/v), group D: acetonitrile–Sorensen's buffer (42.0:58.0 v/v). The buffers used for the tissue homogenates were trisodium phosphate (0.1 M, pH 11) and Sorensen's phosphate buffer (pH 7.4).

2.3. Tissue source

The tissues were obtained from freshly killed male Sprague-Dawley rats. The animals were sacrificed using cervical dislocation and the excised tissues were stored at −20°C for up to 2 months.

2.4. Extraction methods

The following procedures were employed to extract the barbiturates from the rat tissues. A number of assay characteristics were common to each barbiturate group. A sample size of 200 mg was sufficient for tissues containing the groups A and B homologues, but 600 mg was required for the groups C and D. Ethyl acetate (5 ml) was the extraction solvent employed for the groups A and B assay procedures. Tertiary-butyl methyl ether (t-BME, 10 ml) was used for the group C and D mixtures. All extractions and clean-up steps were achieved using a reciprocating shaker. The maximum peak heights were achieved when the following solvents were used for reconstitution of the residues for analysis: groups A and B, distilled water, acetonitrile–distilled water (30:70 v/v); groups C and D, acetonitrile–distilled water (40:60 v/v). Prior to analysis all reconstituted samples were ultrasonicated for 1 min.

2.4.1. Procedure 1: extraction of groups A, B and C barbiturates from brain, gut, heart, kidney, liver, lung, muscle, pancreas, spleen, stomach and testes tissues

The tissue samples were homogenised in Sorensen's phosphate buffer (pH 7.4) to yield a

Table 1
Capacity ratios of the barbiturate homologues and internal standards at the mobile phase strengths (v/v%) of acetonitrile used

Compound	12.5	22.5	34	42
Methyl	1.59			
Ethyl	3.16			
n-Propyl	8.70	2.67		
n-Butyl		6.17		
n-Pentyl		15.45	2.38	
n-Hexyl			5.02	3.45
n-Heptyl			10.54	5.95
n-Octyl				10.65
n-Nonyl				19.18
Phenobarbitone	16.29			
Amylobarbitone		12.73	2.03	

Table 2
Percentage recovery and coefficients of variation for group A homologues and internal standard phenobarbitone from spiked tissue homogenates

Tissue	Procedure No.	Methyl	Ethyl	*n*-Propyl	Phenobarbitone
Adipose	2	57 ± 23	64 ± 19	67 ± 18	70 ± 9
Bone	3	95 ± 10	75 ± 12	70 ± 12	70 ± 5
Brain	1	75 ± 4	83 ± 5	83 ± 1	98 ± 17
Gut	1	68 ± 12	74 ± 14	77 ± 13	93 ± 12
Heart	1	73 ± 4	79 ± 5	81 ± 3	89 ± 9
Kidney	1	74 ± 5	81 ± 7	83 ± 7	85 ± 8
Liver	1	72 ± 3	78 ± 4	79 ± 3	82 ± 5
Lung	1	71 ± 6	77 ± 4	80 ± 3	87 ± 8
Muscle	1	73 ± 6	78 ± 4	81 ± 5	88 ± 5
Pancreas	1	71 ± 1	77 ± 4	76 ± 4	86 ± 7
Plasma	4	90 ± 4	86 ± 4	92 ± 4	95 ± 4
Skin	3	94 ± 7	61 ± 7	63 ± 12	72 ± 6
Spleen	1	57 ± 23	64 ± 5	67 ± 9	74 ± 15
Stomach	1	68 ± 4	74 ± 4	80 ± 5	92 ± 2
Testes	1	74 ± 7	79 ± 4	82 ± 2	93 ± 4

10% homogenate (w/v). After addition of sulphuric acid (1.0 M, 1 ml) the homogenate was centrifuged for 20 min at 3000 rpm. The supernatant was aspirated and rotary mixed with solvent for 20 min, centrifuged at 2000 rpm for 3 min and the aqueous layer frozen at −20°C. The decanted organic phase was evaporated to dryness at 60°C under a constant nitrogen stream. The residues were reconstituted in 400 μl of a suitable medium, see Section 2.4.

2.4.2. Procedure 2: extraction of groups A, B, C and D from adipose and group D homologues from all tissues except skin, bone and plasma

An appropriate size of tissue sample was homogenised in trisodium phosphate (0.1 M, pH 11) to produce a 10% suspension (w/v). Followed by rotary mixing with hexane (4 ml) for 20 min. After decanting the hexane, the aqueous phase was then extracted as in procedure 1, from the addition of sulphuric acid (1.0 M, 1 ml).

2.4.3. Procedure 3: extraction of groups A to D from skin and bone tissue

An appropriate size of tissues was digested with sulphuric acid (3.5 M, 2–6 ml depending on the tissue size) at 60°C overnight. After the addition of the extraction solvent, the aspirated digest was rotary mixed for 20 min, centrifuged for 2 min at 2000 rpm and the aspirated solvent evaporated to dryness at 60°C under nitrogen. The residues were reconstituted in trisodium phosphate (0.1 M, 2 ml) and the sample extracted in a similar manner to procedure 2 from the addition of hexane (4 ml) followed by rotary mixing for 20 min.

2.4.4. Procedure 4: extraction of group A–D from plasma

Sorensen's buffer (pH 7.4, 1 ml) was added to the plasma sample volume followed by the addition of sulphuric acid (1 M, 0.5 ml). After the addition of the extraction solvent (3 ml) and rotary mixing for 20 min, the sample was centrifuged at 2000 rpm for 3 min. The aqueous phase was frozen (−20°C), the organic phase was decanted and evaporated to dryness under a constant N_2 stream at 60°C.

2.5. Typical chromatographic conditions

The column oven was set at 37°C, the mobile phase of acetonitrile–phosphate buffer pH 5.4

Table 3
Percentage recovery and coefficients of variation for group B homolgues and internal standard, amylobarbitone, from spiked tissue homogenates

Tissue	Procedure No.	*n*-Propyl	*n*-Butyl	*n*-Pentyl	Amylobarbitone
Adipose	2	95 ± 4	88 ± 4	71 ± 17	83 ± 6
Bone	3	75 ± 17	80 ± 18	37 ± 19	70 ± 19
Brain	1	93 ± 6	88 ± 3	79 ± 3	89 ± 2
Gut	1	74 ± 5	74 ± 10	69 ± 12	78 ± 4
Heart	1	70 ± 13	59 ± 10	94 ± 5	70 ± 6
Kidney	1	70 ± 7	70 ± 3	70 ± 17	70 ± 6
Liver	1	78 ± 4	75 ± 1	65 ± 2	71 ± 3
Lung	1	75 ± 4	73 ± 7	63 ± 3	77 ± 7
Muscle	1	80 ± 1	78 ± 5	68 ± 3	73 ± 4
Pancreas	1	84 ± 6	72 ± 7	68 ± 4	95 ± 6
Plasma	4	91 ± 3	89 ± 3	85 ± 8	87 ± 5
Skin	3	83 ± 3	86 ± 4	75 ± 10	77 ± 4
Spleen	1	38 ± 16	52 ± 12	45 ± 36	52 ± 14
Stomach	1	65 ± 9	60 ± 17	60 ± 12	62 ± 2
Testes	1	71 ± 6	76 ± 8	76 ± 9	72 ± 6

(relative content dependent on the particular group of analytes), the flow rate was 1.5 ml min^{-1} and the UV detection was at 214 nm.

2.6. Assay validation

2.6.1. Preparation of calibration standards

Stock solutions (1 mg ml^{-1} total barbiturate) of the multicomponent mixtures, groups A–D, were prepared in acetonitrile. The standards were prepared by spiking 2 ml aliquots of the blank tissue homogenate, tissue–buffer (10:90, w/v) and 200 μl of the blank plasma with stock spiking solutions over the range 1–60 μg g^{-1} tissue. The spiked samples were left to equilibrate for 10 min before use in any assay procedure. The standards were spiked with internal standards, in acetonitrile, as listed below:

Group A, 10 μl (1 μg ml^{-1}) phenobarbitone,
Group B, 10 μl (1 μg ml^{-1}) amylobarbitone,
Group C, 6 μl (1 μg ml^{-1}) amylobarbitone,
Group D, 20 μl (1 μg ml^{-1}) 5-*n*-hexyl-5-ethyl barbituric acid.

Calibration graphs of the peak height ratio of each compound to the internal standard against the drug concentration were constructed using linear regression analysis. For all the tissues, other than skin, bone and adipose, liver was used to prepare the calibration curve. Procedures 2, 3 and 4 used spiked adipose, skin and plasma tissue, respectively.

2.6.2. Recovery

The recovery of each homologue from the homogenates was assessed at a concentration corresponding to 15 μg g^{-1} tissue. For each tissue, three homogenate samples and three blank tubes were spiked with one of the four barbiturate mixtures, followed by extraction using the appropriate procedure described above. Both the extracted homogenate and the spiked blank tube were reconstituted using the same volume of solvent and a fixed aliquot was injected into the HPLC. The assay recovery was calculated as the percent ratio of the extracted to the unextracted peak height.

2.6.3. Assay precision

The inter- and intra-assay precision were assessed at barbiturate concentrations of 2, 4, 15 and 30 μg g^{-1} tissue. The spiked samples were analysed as three replicates per concentration on three occasions. Precision was calculated by 1-way ANOVA.

Table 4
Percentage recovery and coefficients of variation of group C homologues and internal standard, amylobarbitone, from spiked tissue homogenates

Tissue	Procedure No.	*n*-Pentyl	*n*-Hexyl	*n*-Heptyl	Amylobarbitone
Adipose	2	55 ± 7	38 ± 3	25 ± 16	46 ± 4
Bone	3	52 ± 12	39 ± 3	34 ± 6	102 ± 3
Brain	1	67 ± 6	57 ± 4	51 ± 12	83 ± 5
Gut	1	84 ± 14	80 ± 10	64 ± 17	38 ± 32
Heart	1	82 ± 5	70 ± 2	73 ± 2	105 ± 16
Kidney	1	70 ± 7	59 ± 7	58 ± 5	70 ± 9
Liver	1	79 ± 10	63 ± 17	60 ± 28	75 ± 8
Lung	1	77 ± 3	64 ± 2	61 ± 3	81 ± 4
Muscle	1	76 ± 3	62 ± 1	55 ± 6	80 ± 19
Pancreas	1	51 ± 30	43 ± 32	42 ± 33	67 ± 16
Plasma	4	87 ± 4	90 ± 4	80 ± 1	84 ± 5
Skin	3	54 ± 13	43 ± 21	36 ± 36	61 ± 15
Spleen	1	86 ± 4	64 ± 6	57 ± 5	81 ± 1
Stomach	1	63 ± 21	76 ± 32	40 ± 35	71 ± 18
Testes	1	83 ± 8	72 ± 11	74 ± 22	84 ± 6

2.6.4. Limit of detection

The limit of detection (LOD) was determined by spiking several calibration series and then quantifying the %CV at each concentration. The LOD was taken to be the concentration below which the %CV value increased above 20%.

3. Results

Procedures 1, 2, 3 and 4 proved successful in the analysis of the multicomponent mixtures A–D. The barbiturates and internal standards were sufficiently resolved from each other and not compromised by interfering peaks, see Figs. 2–5 for representative chromatograms. The capacity ratios are displayed in Table 1.

3.1. Recovery

The optimum extraction solvent for barbiturates in groups A and B was ethyl acetate and for groups C and D t-BME was used. The recovery of the barbiturates in groups A, B, C and D from the tissues investigated are recorded in Tables 2–5, respectively.

3.1.1. Procedure 1

For groups A and B procedure 1 resulted in a greater than 65% recovery from most tissues. The spleen tissue was the exception with a lower recovery being observed; although the internal standards (phenobarbitone and amylobarbitone) were of a comparable recovery. Extraction of *n*-pentyl from the spleen with ethyl acetate gave a recovery of 45%, compared to 86% when t-BME was used. Variability in recovery, calculated as %CV, was greatest for the spleen and gut. The use of procedure 1 gave a reasonable recovery of *n*-pentyl, *n*-hexyl and amylobarbitone, whilst the recovery of *n*-heptyl was generally lower. The lowest recovery was observed from the pancreas.

3.1.2. Procedure 2

The recovery from adipose was optimal for group B congeners (mean recovery 85%). The corresponding values for groups A, C and D were 67.7, 40 and 51%, respectively. Procedure 2 was also used to extract group D from brain, gut, heart, kidney, liver, lung, muscle, pancreas, spleen, stomach and testes tissues. Recovery from these tissues was generally poor compared with the percentage drug recovered using procedure 1; only muscle having a percentage recovery similar to that achieved for barbiturates in groups A, B and C.

Table 5
Percentage recovery and coefficients of variation of group D homologues and internal standard, 5-*n*-hexyl-5-ethyl barbituric acid, from spiked tissue homogenates

Tissue	Procedure No.	*n*-Hexyl	*n*-Heptyl	*n*-Octyl	*n*-Nonyl
Adipose	2	60 ± 8	55 ± 7	49 ± 10	44 ± 7
Bone	3	63 ± 18	105 ± 10	80 ± 6	61 ± 7
Brain	2	45 ± 7	31 ± 6	22 ± 9	19 ± 16
Gut	2	45 ± 4	62 ± 6	68 ± 12	75 ± 10
Heart	2	70 ± 6	65 ± 12	51 ± 22	58 ± 9
Kidney	2	57 ± 5	50 ± 2	61 ± 10	42 ± 7
Liver	2	64 ± 5	43 ± 7	45 ± 11	48 ± 15
Lung	2	34 ± 18	32 ± 19	36 ± 11	39 ± 5
Muscle	2	86 ± 5	74 ± 10	73 ± 15	78 ± 4
Pancreas	2	19 ± 11	12 ± 33	14 ± 29	17 ± 18
Plasma	4	82 ± 5	75 ± 4	76 ± 9	74 ± 7
Skin	3	57 ± 5	67 ± 6	64 ± 11	62 ± 10
Spleen	2	47 ± 6	31 ± 13	52 ± 4	27 ± 15
Stomach	2	73 ± 7	64 ± 9	58 ± 14	55 ± 7
Testes	2	49 ± 10	41 ± 7	47 ± 13	44 ± 16

3.1.3. Procedure 3

For both skin and bone recovery decreased with increasing lipophilicity of the congeneric series. The average recovery for group A homologues from the skin was 73% compared to 64% for group D members. For the bone the mean barbiturate recovery from group A was 80% compared with 82% from group D. The recovery of individual barbiturates from the skin and bone in groups A–C were closely matched within a group except for *n*-pentyl from bone in group B, 37% compared with 75% in skin, and amylobarbitone in group C, 102% in bone compared to 61% in skin.

3.1.4. Procedure 4

Recovery using this method was generally good ($>73\%$) although it did decrease as the lipophilicity of the homologues increased (mean recovery for group A, 89%, mean recovery for group D, 75%). Ethyl acetate and t-BME were equally effective in extracting *n*-pentyl (85 and 87%) and amylobarbitone (87 and 84%).

3.2. Calibration curves

The peak height ratio was proportional to barbiturate concentration, for all nine homologues, over the range 1–60 μg g^{-1} tissue ($r^2 > 0.95$). The origin always lay within the 95% confidence interval of the intercept, indicating no endogenous interference (see Table 6 for typical calibration curve statistics).

3.3. Assay precision

The inter- and intra-assay precision associated with the simplest and most complex methodology (procedures 1 and 3) are shown in Table 7. The variability associated with procedure 3 is considerably higher than that obtained with procedure 1.

3.4. LOD

The LOD for the homologues in all tissue except skin and bone was 1 μg g^{-1} tissue (Fig. 6). The LOD in skin and bone was found to be 2 μg g^{-1} tissue; which is consistent with the greater variability observed with a more involved extraction.

4. Discussion

4.1. Chromatography

The assay of barbiturates has been well docu-

Table 6
Typical statistical characteristics (mean and S.E.) of the calibration curves

Procedure No.	Barbiturate group	Slope (S.E.)	Intercept (S.E.)
1	*n*-Heptyl	2.1×10^{-2} (3.52×10^{-4})	1.9×10^{-2} (5.0×10^{-3})
	n-Octyl	1.84×10^{-2} (1.33×10^{-4})	-4.97×10^{-3} (1.89×10^{-3})
	n-Nonyl	9.58×10^{-3} (2.89×10^{-4})	7.41×10^{-3} (4.11×10^{-8})
2	*n*-Pentyl	1.09×10^{-1} (1.11×10^{-3})	9.36×10^{-2} (1.7×10^{-3})
	n-Hexyl	4.64×10^{-2} (3.75×10^{-3})	5.15×10^{-2} (1.0×10^{-7})
	n-Heptyl	1.64×10^{-2} (2.67×10^{-4})	1.26×10^{-2} (3.79×10^{-3})
3	*n*-Propyl	2.56×10^{-2} (2.52×10^{-4})	2.74×10^{-2} (7.12×10^{-3})
	n-Butyl	2.1×10^{-2} (7.3×10^{-5})	5.09×10^{-2} (2.01×10^{-3})
	n-Pentyl	2.62×10^{-2} (1.43×10^{-7})	-5.9×10^{-3} (6.82×10^{-3})
4	Methyl	4.55×10^{-2} (1.23×10^{-3})	1.03×10^{-1} (3.23×10^{-2})
	Ethyl	4.35×10^{-2} (2.76×10^{-4})	2.2×10^{-2} (3.91×10^{-3})
	n-Propyl	1.92×10^{-2} (1.67×10^{-4})	-1.4×10^{-2} (2.36×10^{-3})

mented [3,6–10]. The majority of techniques employ HPLC, invariably coupled with the UV detection of barbiturate. The UV absorbance maximum observed for barbiturates is dependent on the state of ionisation of the molecule. The 5-*n*-alkyl-5-ethyl barbituric acids are weak diprotic acids with pK_as of approximately 7.8 and 11 [3]. A pH rise to 12 results in the formation of the dianionic barbiturate species. The UV absorbance maximum for the monoanion occurs at 245 nm and for the dianionic species it is between 260 and 270 nm. When ionised the barbiturates can be easily detected within the normal UV range, but are poorly separated by HPLC. Randall-Clark and Chan [3] resolved this problem by using post column ionisation of the separated unionised barbiturates. Many workers have subsequently adopted this approach to detect barbiturates extracted from adipose, liver and muscle [11] and plasma [2]. Although, this method was initially tried the post column solvent mixing caused an increase in detector noise which reduced the signal to noise ratio substantially. The problem could not be circumvented and was attributed jointly to the use of two HPLC pumps in parallel and the turbulent effects of the mixing T-piece [10].

Table 7
Mean percentage inter- and intra-assay precision of the methyl–*n*-heptyl barbiturate homologues for procedures 1 and 3

Homologue	Procedure 1		Procedure 3	
	Inter	Intra	Inter	Intra
Methyl	5.3	4.1	15.3	23.3
Ethyl	8.0	5.3	12.9	24.0
n-Propyl	9.7	0.7	18.3	18.2
n-Butyl	5.3	0.9		9.4
n-Pentyl	3.0	1.4		17.2
n-Hexyl		5.4		28.3
n-Heptyl		7.7		10.3
n-Octyl		7.9		25.0
n-Nonyl		14.7		27.0

Although the methodology developed reduced a potential 135 individual assays to a more manageable 16, the number of assay could potentially be reduced even further by using more sophisticated chromatographic techniques. Isocratic LC-MS would allow the simultaneous analysis of the multicomponent mixtures in a relatively short retention time (baseline resolution would not be an issue due to the specificity of the detector). Furthermore, gradient LC-UV has the potential to allow the quantification of all nine homologues in a single assay.

4.2. Extraction

Most tissue could be directly homogenised in a suitable buffer using an electric drill fitted with a pestle end. Although the structure of skin and

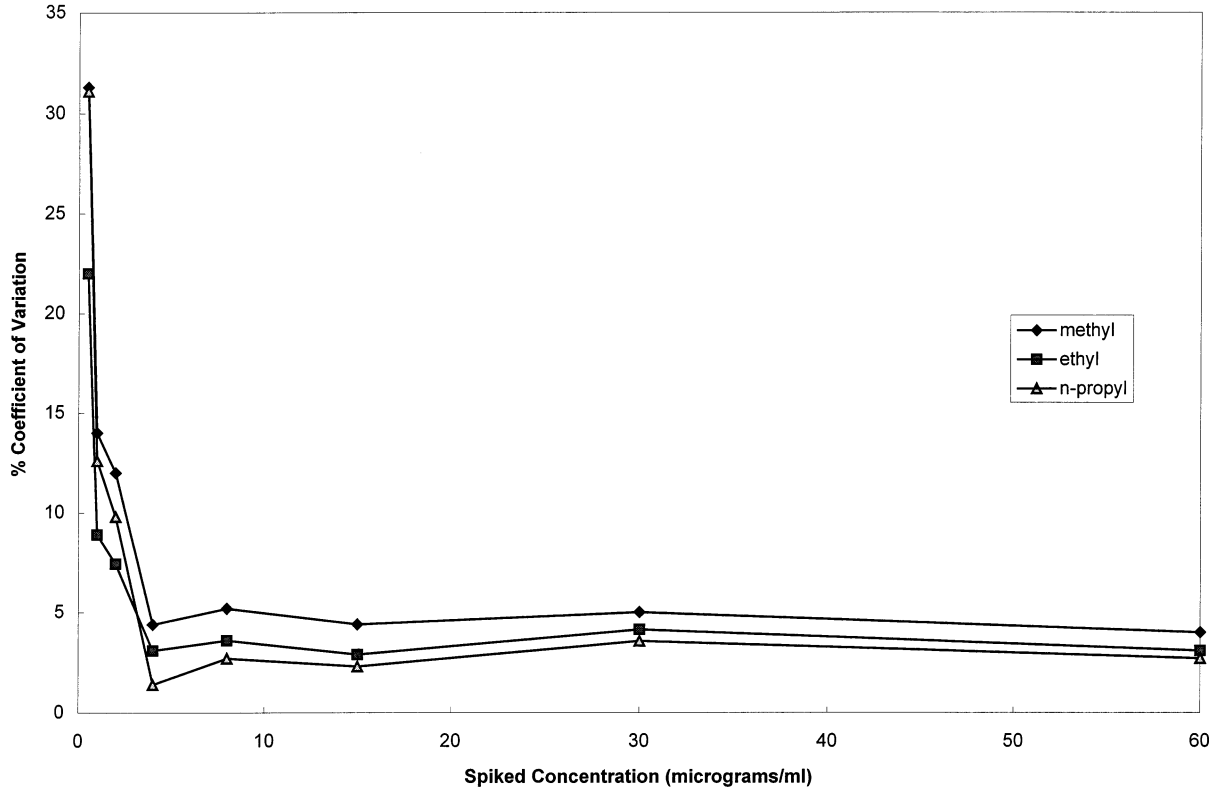

Fig. 6. The percentage coefficient of variation of methyl to *n*-propyl barbituric acids from liver homogenates against concentration, $n = 6$.

bone prevents direct homogenisation, they are susceptible to digestion by sulphuric acid (3.5 M) at 60°C. Previous work by Ballard [10] demonstrated the stability of the congeneric series in sulphuric acid (3.5 M) for 20 h at 60°C.

Tissue homogenate samples characteristically contain a high proportion of proteins and lipids. The analyte must ultimately be selectively extracted from these matrices into one compatible with HPLC. Liquid–liquid extraction is ideal for tissue homogenates because the phases are easily separated. In contrast, solid phase extraction and filtration techniques only work well with solutions due to blockage by particulates.

For adipose tissue a hexane clean-up step was needed to remove excessive lipids that would be extracted with the analytes in procedure 1. For the analysis of the group D mixture in all tissues except adipose, skin and bone, hexane was also used to remove endogenous lipophilic compounds because of interference with the peaks of interest when direct solvent extraction was employed. Endogenous material only interfered when using the most non-polar mobile phase.

Ethyl acetate proved to be the optimal extraction solvent for the compounds in groups A and B, with recovery greater than 70% from most tissues (Tables 2 and 3). The recovery of all congeners from the spleen was consistently lower than that from other tissues. No satisfactory explanation could be found. Other potential solvents were investigated (chloroform, toluene, hexane, heptane) and found to be less efficient than ethyl acetate in extracting methyl to *n*-pentyl. Steiner et al. [11] also used ethyl acetate to extract a number of barbiturates of similar physico-chemical characteristics from muscle, liver and adipose tissue and reported a recovery of each barbiturate of greater than 70%. However, Bailey and Kelner [12] noted that ethyl acetate was

Table 8
Mean percentage recovery from Sprague-Dawley rats at 5 min post dose of i.v. administered barbiturate

Tissue	Methyl	Ethyl	*n*-Propyl	*n*-Butyl	*n*-Pentyl	*n*-Hexyl	*n*-Heptyl	*n*-Octyl	*n*-Nonyl
Dose left (%)	86	78	85	82	78	84	74	63	71
Rat body assayed (%)	98	98	98	98	98	92	92	90	90

For each barbiturate homologue $n = 3$.

prone to form an emulsion with the sample when attempting to extract acidic drugs from water and plasma, they favoured methylene chloride. For more lipophilic homologues (groups C and D), a more polar solvent, t-BME, proved superior to ethyl acetate. Overall recovery was better for the less lipophilic barbiturates (groups A and B), suggesting recovery per se is limited by the ability of the extraction solvent to extract the homologue from the lipid components of the tissue.

Although, the recovery of an individual barbiturate has not been tested, the calibration lines for the multicomponent mixtures are linear suggesting that recovery is likely to be concentration independent. Additionally, the recovery for the bridging compound, i.e. 5-*n*-propyl and 5-*n*-pentyl etc., between each group of barbiturates was reasonably reproducible.

4.3. Assay validation

The assay validation undertaken, although not exhaustive demonstrated that the methodology was suitable for the assay of barbiturates in tissue samples obtained from in vivo experiments. The barbiturate tissue concentrations recorded at 5 min post dose in in vivo rat experiments were used to estimate the percentage of the administered dose remaining in the body at that particular time (Table 8). The percentage of the administered dose left ranged from 86 to 71% for the methyl and *n*-nonyl, respectively. Furthermore, it was as expected, based on a priori knowledge of the pharmacokinetics of the 5-*n*-alkyl-5-ethyl barbituric acids, namely, that the more lipophilic the homologue the more rapidly it is cleared from the body [2].

The percentage inter- and intra-assay variability increases in line with the number of extraction steps. For procedure 1 the inter-assay variability is approximately 10% (1 step) compared to 20% for procedure 3 (3 steps). Procedure 3 was the most complex assay developed; its errors will therefore embrace those in the other methods which have fewer steps and subsequently lower variability. A comparison of the variability between procedure 1 and 3 (Table 7) supports this assumption.

The stability of the barbiturates on storage in the excised tissues, especially the liver, which in vivo is the primary organ of metabolism, may be an issue [13]. Literature evidence suggests this can be disregarded [14,15]. Degradation of pentobarbitone was negligible in spiked liver homogenate left for 2 months at 4 and 25°C [14]. An examination of the stability of thiopental present in liver thawing to room temperature found no appreciable degradation of this barbiturate [15]. Based on these literature findings the samples were assayed within 2 months of the in vivo investigation.

5. Conclusions

The determination of a drug's tissue pharmacokinetics affords a greater insight into the mechanisms of drug disposition than the study of systemic blood concentrations alone. To yield reliable results from such studies, it is essential to employ well characterised and validated analytical methods. The current study developed specific assays for the quantification of nine barbiturate homologues in 15 tissues. Such an extensive assay development has not previously been reported in the pharmacokinetic literature. A simple 1 step liquid–liquid extraction performed well for all but the most lipophilic congeners (*n*-heptyl, *n*-octyl and *n*-nonyl), for which the endogenous tissue components became a problem by co-eluting with the peaks of interest. The latter problem was

resolved using a more complex extraction procedure. The methodology developed reduced a potential 135 individual assays to a more manageable 16. In future, the number of assays could be reduced even further by using more sophisticated chromatographic techniques such as isocratic LC-MS or gradient LC-UV. Overall, the present assay was considered suitable for the purpose of providing barbituric acid concentrations in all the major rat tissues which could then be subsequently used for PBPK modelling [16].

Acknowledgements

Zeneca Pharmaceuticals are acknowledged for funding this study.

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