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LIQUID CHROMATOGRAPHIC ASSAY OF HEPTAMINOL IN SERUM AND ITS ORAL PHARMACOKINETICS IN THE DOG

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SUMMARY

In order to investigate the pharmacokinetics of heptaminol in dogs, a high-performance liquid chromatographic assay of the drug was devised and it was evaluated in a general purpose validation design through analysis of variance. Heptaminol and its internal standard *n*-propylamine were salted out from plasma together with acetonitrile, the previously proposed "solvent demixing" extraction procedure. Both amines were derivatised in acetonitrile with the *o*-phthaldialdehyde, 2-mercaptoethanol procedure of Roth. The adducts were quantitated by reversed-phase high-performance liquid chromatography on Radial-Pak® cartridges with ultraviolet detection. Peak height ratios were linearly related to concentrations up to 250 μmol l^{-1} with a 2% coefficient of variation. Sensitivity was 3.5 μmol l^{-1} (signal-to-noise ratio of 5).

Means of the usual pharmacokinetic parameters in four dogs were: elimination half-life 3.75 h, apparent distribution volume 2.18 l kg^{-1} and total clearance 0.402 l kg^{-1} h^{-1}, similar to the results obtained in humans by other authors using radiolabelled heptaminol.

INTRODUCTION

Heptaminol (6-amino-2-methyl-2-heptanol) is a cardiotonic drug used in Europe since 1953. Current drug regulations now require pharmacokinetic investigations which were not mandatory at that time and which were never done since then. In charge of animal studies, we had to devise a liquid chromatographic assay suitable for plasma levels lower than 10 mg l^{-1} (55 μmol l^{-1}).

We applied to heptaminol the solvent demixing extraction procedure [1] which we had found convenient and reproducible for valproic acid [2] and major anticonvulsants [3]. Low ultraviolet (UV) absorbance necessitated derivatisation. We adopted derivatisation with *o*-phthaldialdehyde (OPT) and

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mecaptoethanol according to the procedure of Roth [4] and already applied to pharmaceutical forms of the drug with UV detection by Nicolas et al. [5]. The whole assay procedure was evaluated through analysis of variance (ANOVAR) in an experimental design fitted to this general purpose, variations of which we have already used profitably in previous instances [2, 3].

EXPERIMENTAL

Materials

Reagents and solvents were of analytical grade: potassium chloride and disodium hydrogen orthophosphate from Rhône-Poulenc (France); *n*-propylamine from Merck (F.R.G.); *o*-phthaldialdehyde and 2-mercaptoethanol from Fluka; acetonitrile for far UV from Fisons (Loughborough, U.K.) through Touzart et Matignon (France). Heptaminol hydrochloride was from Finorga and tablets were from Richard (Sauzet, France).

The chromatographic apparatus was a Spectra-Physics SP 8000, equipped with a Valco loop injector (injected volume 10 μl). The column was a Radial-Pak cartridge from Waters, filled with C_{18} bonded reversed-phase, particle size 10 μm. The detector was a Model 770 variable-wavelength spectrophotometric detector from Schoeffel (F.R.G.).

Extraction procedure

Heptaminol was extracted from plasma or water into acetonitrile by a solvent demixing [1] procedure. To a 1.0-ml plasma sample were added 0.1 ml of 1 *M* sodium hydroxide and 1.0 ml of acetonitrile containing the internal standard at fixed concentration (*n*-propylamine, 3.6 mg l^{-1}), then the mixture was briefly mixed. An excess of solid potassium chloride was then poured in, followed by vigorous vortex-mixing and centrifugation (20°C, 1500 *g*, 15 min). A 500-μl aliquot of the acetonitrile supernatant was transferred into a second tube for pre-column derivatisation.

For assay validation, a blank plasma and water were used and the added acetonitrile contained both the internal standard at the fixed concentration and heptaminol hydrochloride at one of the following concentrations: 2.5, 5 or 10 mg l^{-1} (13.75, 27.5 or 55.0 μmol l^{-1}). Calibrations were made with the blank plasma and the highest heptaminol concentration.

Derivatisation and chromatography

The derivatisation solution was 0.075 mol l^{-1} *o*-phthaldialdehyde and 0.14 mol l^{-1} 2-mercaptoethanol in acetonitrile. To the 500-μl aliquot of acetonitrile extract, 200 μl of the derivatisation solution and 500 μl of a 1 *M* sodium hydroxide solution were added, and the two unmixed phases were briefly shaken together on a vortex mixer. The acetonitrile supernatant of the derivatisation mixture was injected through a 10-μl sample loop. The isocratic mobile phase was acetonitrile—disodium hydrogen orthophosphate buffer, pH 7, 12.5×10^{-3} *M* (50:50, v/v), flow-rate 1.5 ml min^{-1}, detection at 330 nm [5].

Method validation

The experiment was designed for an analysis of variance both factorial and

nested. The two fixed factors studied were heptaminol concentration (three levels) and sample composition (two levels: water and plasma).

For the nested analysis of extraction and chromatographic measurements, each water or plasma extract was duplicated and each derivatised duplicate was chromatographed twice. The acetonitrile solutions were also derivatised in duplicate in the same way as the extracts for evaluation of the extraction yield. Table I shows the structure of the validation design.

TABLE I

STRUCTURE OF THE FACTORIAL/NESTED VALIDATION DESIGN

	Factor: drug concentration			Total R_i
	$X'_1 = -1$	$X'_2 = 0$	$X'_3 = 1$	
Factor: sample composition				
Water		Y_{ij11}, Y_{ij12} → E_{ij1}; Y_{ij21}, Y_{ij22} → E_{ij2} → K_{ij}		R_1
Plasma				$R_{r=2}$
Total C_j	C_1	C_2	$C_{c=3}$	G

Analysis of variance

Table II shows the corresponding variance analysis. Data y (peak ratios) were input as their decimal logarithms, Y, in order to warrant homoscedasticity under the hypothesis of a constant coeffient of variation.

Regression analysis

The hypothesis to be tested (assay linearity) that Y is proportional to the drug concentration x, i.e. $y = ax$, results in a linear relationship of unit slope $Y = X + \log a$, where $Y = \log y$, $X = \log x$ and the expected regression coefficient $b = 1$.

Calculations were simplified by using a coded abscissa

$$X' = 1 + \frac{\log x - \log \mathrm{Hi}}{\log 2}$$

where Hi = 10 mg l^{-1} is the highest of the three concentrations in ratio 1:2, and which is 1 when $\log x = \log \mathrm{Hi}$, 0 when $\log x = \log (\mathrm{Hi}/2)$, and -1 when $\log x = \log (\mathrm{Hi}/4)$.

Using the coded abscissa, regression calculations came down to: expected regression coefficient $b' = \log 2 = 0.30103$; sum of squares $SX' = \Sigma X'^2 = 8$ for each individual regression, 16 for common regression; sum of products $SYX' = \Sigma YX' = K_{3j} - K_{1j}$ for each regression, $C_3 - C_1$ for common regression.

TABLE II

ANALYSIS OF VARIANCE OF THE VALIDATION DESIGN (TOTALS E, K, R, C AND G AS SHOWN IN TABLE I)

Variance component	Sum of squares	Degrees of freedom
Correction factor	$C = G^2/4rc$	
Total	$ST = \sum^{4rc} Y^2 - C$	$NT = 4rc - 1 = 23$
Between all extracts	$SE = \frac{1}{2}\sum^{2rc} E^2 - C$	$NE = 2rc - 1 = 11$
Factorial		
Between cells	$SK = \frac{1}{4}\sum^{rc} K^2 - C$	$NK = rc - 1 = 5$
Between compositions	$SR = \frac{1}{4c}\sum^{r} R^2 - C$	$NR = r - 1 = 1$
Between concentrations	$SC = \frac{1}{4r}\sum^{c} C^2 - C$	$NC = c - 1 = 2$
Regression (common)	$SL = \frac{(C_3 - C_1)^2}{16}$	1
Interaction	$SRC = SK - SR - SC$	$NRC = NK - NR - NC = 2$
Nested		
Intra-cell	$SI = ST - SK$	$NI = NT - NK = 3rc = 18$
Betweeen duplications of measurements	$SM = ST - SE$	$NM = NT - NE = 2rc = 12$
Between duplications of extracts	$SX = SI - SM$	$NX = NI - NM = rc = 6$

Overall calculation procedure (all on logarithmic values)

(1) Homoscedasticity of log values was tested through Bartlett's test [6]. If not significant at the probability level $P = 0.1$, then:

(2) Variability of extraction was tested through a one-sided F test of SE vs. SM. If not significant at $P = 0.1$, then the intra-cell mean square was taken as the error variance s^2.

(3) Analysis of variance was performed on the whole data and separately on data from water and from plasma extractions.

(4) Separate regressions were calculated. Departure from linearity was tested versus the common error variance s^2 through a one-sided F test of (in each row) $SC - SL$ with 1 and 18 degrees of freedom (DF). If not significant at $P = 0.1$, departure from parallelism was tested through a two-sided t test as

$t = \frac{|b_1' - b_2'|}{\sqrt{s^2_{\Delta b}}}$, 18 DF, where $s^2_{\Delta b} = s^2 \frac{2}{SX'}$. If not significant at $P = 0.1$, then:

(5) Common regression was calculated and departure of the common slope b' from expected theoretical value 0.30103 was tested through a two-sided t test as

$t = \frac{|b' - 0.30103|}{\sqrt{s^2_b}}$, 18 DF, where $s^2 = s^2_b \frac{1}{SX'} = s^2/16$

(6) The intra-assay coefficient of variation (C.V.$_i$) was calculated through the already proposed approximations [2]

$$\text{C.V.}_i \simeq \frac{dm}{m} = \text{d ln } m = 2.306 \text{ d log } m \simeq 2.3026\, s$$

which assimilate ($\simeq$) standard deviations with differentials and arithmetic with geometric means m, and which work as long as C.V. is not too large, say lower than 10%.

(7) The inter-assay C.V.$_e$ was determined separately. Two plasma samples (T2 and T8) were taken from the same dog 2 and 8 h, respectively, after heptaminol ingestion and were assayed in five separate assay sessions.

Pharmacokinetics in the dog

Four female beagle dogs (13, 16, 13 and 12.6 kg body weight) ingested 300 mg (two tablets) of heptaminol as 376.5 mg of heptaminol hydrochloride. This represented five times the usual unitary dosage for men on a mg/kg basis. Blood samples were taken 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 24 h later and plasmas were assayed for heptaminol. Pharmacokinetic parameters were calculated through fitting to an open two-comparment model.

RESULTS

Chromatography

Fig. 1 shows the chromatogram obtained from a blank plasma to which heptaminol and internal standard were added as described in Experimental at concentrations of 2.5 mg l^{-1} (17.25 μmol l^{-1}) and 3.6 mg l^{-1} (60 μmol l^{-1}), respectively.

Extraction yield

The volumetric yield of acetonitrile demixing (demixed/added volume) was estimated as 0.7. Comparison of peak heights on chromatograms obtained from calibration solutions and from plasma or water extracts, when combined with the volumetric yield, resulted in the following values of extraction yields: from water, drug = 0.634, internal standard = 0.662; from plasma, drug = 0.653, internal standard = 0.534.

Method validation

Homoscedasticity of log values was not ruled out by Bartlett's test. Variation from extraction was found not significant: the comparison SE vs. SM resulted in $F(6/12) = 1.52$, $P_{1\alpha} = 0.25$.

Linearity of the regression line was not denied: for water extracts $F(1/9) = 3.052$, $0.10 < P_{1\alpha} < 0.25$; for plasma extracts $F(1/9) = 0.136 < 1$, $P_{1\alpha} \gg 0.50$.

Parallelism was not denied: $t_{18} = 1.5725$, $0.10 < P_{2\alpha} < 0.20$.

The slope of the common logarithmic regression line did not differ significantly from expected value 0.30103: $t_{18} = 0.8109$, $0.40 < P_{2\alpha} < 0.50$.

Interaction between concentrations and sample composition was not significant: $F(2/18) = 2.546$, $0.10 < P_{1\alpha} < 0.20$.

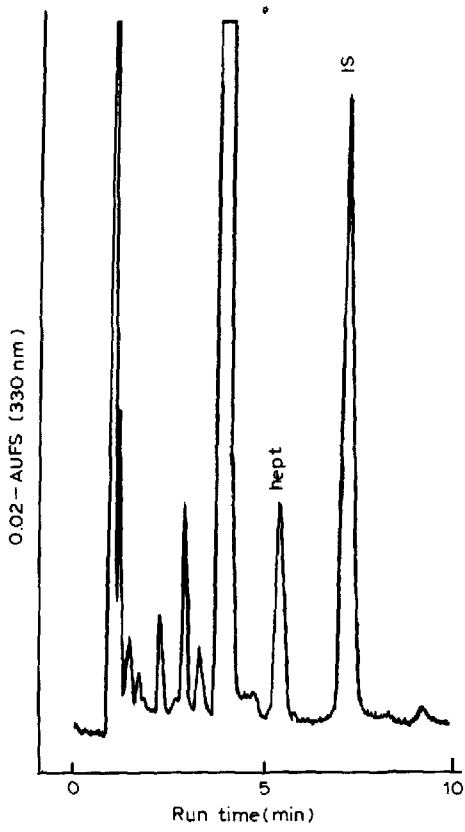

Fig. 1. Chromatogram of *o*-phthaldialdehyde adducts of heptaminol (hept) and propylamine (IS) in plasma.

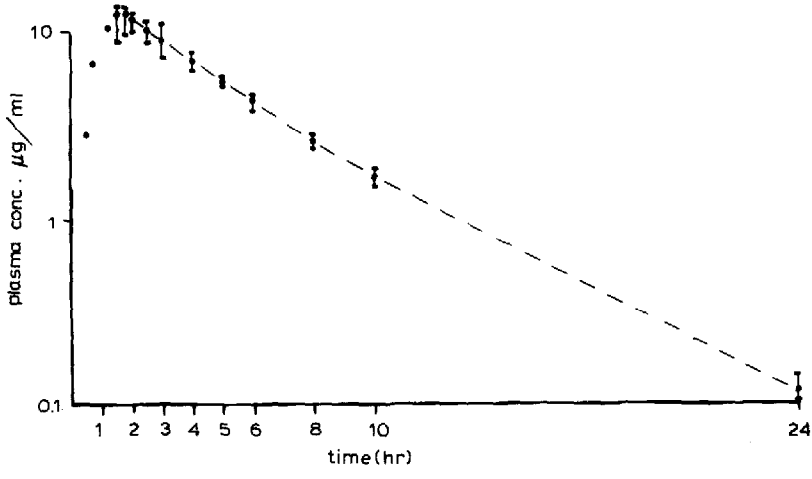

Fig. 2. Plasma concentrations of heptaminol versus time (mean ± S.D. from four dogs).

The intra-assay coefficient of variation was $C.V._i = 2.125\%$.

The five-fold separate determination of two plasma samples from the same dog resulted in: for T2, mean = 9.580 mg l^{-1}, $C.V._e = 3.3448\%$; for T8, mean = 1.857 mg l^{-1}, $C.V._e = 3.4603\%$.

TABLE III

PHARMACOKINETIC PARAMETERS OF HEPTAMINOL IN FOUR FEMALE BEAGLE DOGS (A—D)

	A	B	C	D	Mean
Body weight (kg)	13	16	13	12.6	13.65
Half-time (h)					
First (resorption)	0.319	0.042	0.050	0.009	0.105
Second (distribution)	0.724	1.112	1.338	1.423	1.149
Final (elimination)	3.417	3.828	3.455	4.316	3.754
T_{max} (h)					
Observed	1.5	1.5	1.75	0.75	1.375
Calculated	1.5	1.0	1.15	0.75	
C_{max} (mg l^{-1})					
Observed	16.04	14.29	18.04	19.71	17.02
Calculated	14.80	17.02	19.85	17.09	
Total clearance (l $kg\ h^{-1}$)	0.374	0.421	0.429	0.385	0.402
Distribution volume (l kg^{-1})	1.844	2.330	2.142	2.401	2.179
K_a (h^{-1})	2.17	13.346	13.676	70.796	25.747
K_{el} (h^{-1})	0.49	0.37	0.327	0.259	0.3615

Dog pharmacokinetics

Fig. 2 is a graph of the mean serum concentration ± S.E.M. through time. Table III lists the corresponding calculated pharmacokinetic constants.

DISCUSSION

Extraction

No extraction method has been published for heptaminol to our knowledge. We extended successfully to this drug the solvent demixing technique which we are currently using for the quantitation of anticonvulsants.

The extraction yield is somewhat low, but highly reproducible as judged from the non-significant between-extract component of the error variance (see Method validation). This was to be expected, since solutes are salted out together with the organic phase and they partition between plasma water and acetonitrile at the molecular level instead of equilibrating through an interface. Good reproducibility has been observed also for valproic acid [2] and major anticonvulsants [3].

As mentioned elsewhere [1], the solvent demixing procedure is well adapted to routine use, since it allows calibrations to be performed by means of acetonitrile solutions of drug and internal standard instead of using spiked plasmas. This is true only under the assumption that plasma proteins, when denaturated by acetonitrile, reach the same adsorption equilibrium when the solutes are initially in the aqueous phase as when they are initially in the acetonitrile phase. Long experience with anionic drugs has shown us that this assumption

holds, and systematic studies now in progress show the same for cationic or highly lipophilic drugs.

Comparison of peak ratios shows that values obtained with plasma extracts are about 20% higher than with solutions or aqueous extracts. Comparison of peak heights shows that this discrepancy is due to a lower extraction yield of the presently used internal standard from plasma than from water, whereas heptaminol is equally extracted from both. Ideally, a better internal standard would be worth searching for, all the more as demixing technique makes such an optimisation easy to perform. However, in the present application of the method, this difference was considered sufficiently small that so significant systematic difference could reasonably be expected between different plasmas.

Derivatisation and chromatography

Derivatisation with *o*-phthaldialdehyde and mercaptoethanol according to the procedure of Roth [4] appears well suited to the assay of primary amines involving acetonitrile demixing. The reaction takes no measurable time to go to completion at room temperature in this solvent.

The UV absorbance spectrum of *o*-phthaldialdehyde adducts allows spectrophotometric detection at a wavelength where very few if any plasma components are detectable. The high absorbance affords a sensitivity evaluated at 3.5 μmol l^{-1}, corresponding to a signal-to-noise ratio of 5, which proved sufficient for the present study. Presumably, fluorescence measurements would afford higher sensitivity, but linearity of fluorescence emission would have to be checked.

Method validation

Lack of significance of Bartlett's test allowed for analysis of variance and unweighted regression to be made on logarithmic values. It may also be inferred from this test that measurements have a constant C.V. in the range of concentrations studied.

Lack of significance of comparison of S*E* vs. S*M* shows that extraction adds no measurable contribution to the instrumental error of chromatographic measurements. In other words, it has a better reproducibility than the measurements. Common regression analysis was valid since both lines could be considered straight and parallel. The slope of the common regression line of log values vs. log concentrations did not differ from unit value, which means that measured values are linearly related to concentrations in the range 0—10 mg l^{-1} (0—55 μmol l^{-1}). Results without internal standard not reported here show on visual inspection that linearity remains good up to 40 mg l^{-1}. Parallelism of the lines and absence of significant statistical interaction between concentrations and sample composition confirm that sample composition does not modify the assay relationship other than through logarithmic translation (i.e. arithmetic proportionality).

As can be seen, the validation design proposed here affords a wealth of precise information concerning analytical performance in return for the comparatively modest 24 individual determinations. We use it now whenever working up an analytical procedure [2, 3].

The hierarchical "intra-cell" organization of duplications allows evaluation of successive steps involved in the method (here extraction and chromato-

graphy of extracts). This nested pattern could be extended if desired to more than two levels (e.g. extraction, back-extraction and measurements) at the expense of doubling the number of measurements for every additional level.

Logarithmic transformation of values allows homogeneity of precision in the range of concentrations under study to be checked; otherwise, displaying a C.V. value has little meaning.

The factorial part of the design allows estimation of the contribution of selected factors to variability in addition to checking linearity. It was restricted here to the effect of sample composition, essentially plasma proteins which are known to affect extraction of basic drugs and which turned out indeed to affect differently the extraction yield of internal standard and heptaminol. A useful statistical factor is the repetition of the whole design such as described here in order to estimate a between-assay component of variance which is ascribable to variations in daily calibrations and which combines with the intra-assay component into the total inter-assay variability. Four-fold repetition of sessions was performed in the evaluation of an HPLC assay of anticonvulsants for clinical purposes [3] at the expense of four times more measurements. We restricted the present less-demanding work to the usual determination of the same two dog plasma samples in five assay sessions. The values so obtained of inter-assay C.V. are consistent with fairly equal intra-assay and between-assay variances.

Pharmacokinetics

The pharmacokinetics in the dog were compatible with an open two-compartment model, the half-lives being 1.14 h for the distribution phase and 3.7 h for the elimination phase. The average apparent distribution volume was as large as 2.18 l kg^{-1}, which agrees with the large tissue distribution found in rats [7]. Finally, the high 0.4 l kg^{-1} h^{-1} clearance approximates to renal plasma flow in mammals and is consistent with excretion through tubular secretion, as already described in man [8].

The whole administered dose of heptaminol was found in urine within 24 h, and 82—87% within 10 h. Presumably this corresponded to the parent drug. The rat has been shown to hydroxylate heptaminol on methyl side-chains at a rate of 4—7% [7]. Whether man metabolizes it is unknown. Indeed, our results do not support that metabolism even reaches such a low rate in the dog. A hydroxylated metabolite could be derivatised in the same way as heptaminol, but would presumably have a much shorter retention time in reversed-phase chromatography and could not be mistaken as heptaminol.

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