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Note

Estimation of ICI 35,868 (Diprivan^{R}) in blood by high-performance liquid chromatography, following coupling with Gibbs' reagent

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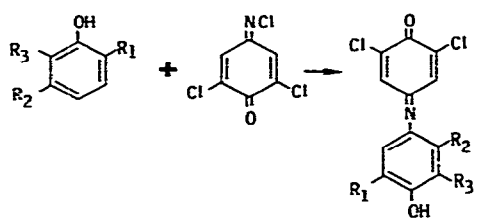
ICI 35,868 (Diprivan^{R}*, 2,6-diisopropylphenol; I, Fig. 1) is a new intravenous anaesthetic agent. In animals [1, 2] and man [3, 4] it rapidly produces anaesthesia of short duration devoid of excitory side effects.



I, II III IV, V

	R_1	R_2	R_3
I and IV	$(CH_3)_2CH$	H	$(CH_3)_2CH$
II and V	$(CH_3)_2CH$	CH_3	H

Fig. 1. Structural formulae of ICI 35,868 (I), thymol (II), Gibbs' reagent (III) and the indophenols IV, V.

Pharmacokinetic studies in animals have shown that anaesthesia was maintained by concentrations in the range 1—4 μg/ml [5]. These studies were monitored by a fluorescence assay procedure with a limit of detection of 300 ng/ml. To define fully the pharmacokinetic parameters in man and thus allow predictions for the use of ICI 35,868 for maintenance of anaesthesia, a more sensitive assay procedure is required.

*Diprivan is a trademark, Property of Imperial Chemical Industries Ltd.

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This paper describes a procedure, based on coupling to Gibbs' reagent [6] and high-performance liquid chromatography (HPLC) of the resultant dye, which can quantify concentrations of ICI 35,868 down to 25 ng/ml. Thymol was included as internal standard to allow for variations occurring during extraction and derivatisation.

MATERIALS AND METHODS

Reagents

Cyclohexane (Spectrosol grade), isopropanol, sodium chloride, potassium dihydrogen orthophosphate and ammonia solution (35%, w/w) were all Analar grade from Hopkin and Williams (Chadwell Heath, Great Britain), Gibbs' reagent was from Koch-Light (Colnbrook, Great Britain).

Thymol and tetramethyl ammonium hydroxide (Fluka, Buchs, Switzerland) trifluoroacetic acid (Fluorochem, Glossop, Great Britain) and acetonitrile and methanol (both HPLC grade from Rathburn Chemicals, Walkerburn, Great Britain) were all used without further purification. Diethyl ether (Analar grade from Hopkin and Williams) was redistilled before use. ICI 35,868 is a liquid at room temperature and was stored in sealed glass ampoules at −20°C. For day-to-day use the contents of an ampoule were transferred to a glass vial fitted with a screw cap containing a PTFE liner. This vial was also stored at −20°C and allowed to come to room temperature before use.

Standard solutions

Stock solutions of ICI 35,868 and thymol (1 mg/ml in methanol) were diluted as necessary with methanol to ensure that a total of not more than 100 μl of methanol was added to blood to give the required concentrations of ICI 35,868 and internal standard. The stock solutions were stable for at least seven days at −20°C but were prepared fresh each day.

Sample preparation

To oxalated whole blood (1 ml) was added internal standard (as described under Quantification) and the sample vortex mixed (Rotamixer, Hook and Tucker, Croydon, Great Britain). After addition of aqueous potassium dihydrogen orthophosphate (0.1 *M*, 1 ml) and cyclohexane (5 ml) the mixture was tumbled (60 rpm, 10 min) in a rotary tumbler (TM/V100, Luckham, Burgess Hill, Great Britain). After centrifugation (700 *g*, 2 min) to separate the phases, an aliquot of the organic layer (4.5 ml) was transferred to a clean tube. To this was added, with gentle agitation, Gibbs' reagent in isopropanol (1 mg/ml, 60 μl) and tetramethylammonium hydroxide solution [1 part 24% (w/v) solution in methanol and 9 parts isopropanol, 50 μl]. The reaction was allowed to proceed at room temperature (20 min) before water (1 ml) was added and the mixture tumbled (15 min) and centrifuged (700 *g*, 3 min). The organic layer was aspirated and discarded, aqueous sodium chloride [25% (w/v), 1 ml] and diethyl ether (5 ml) were added and, after further mixing and centrifugation, an aliquot of the ethereal layer (4.8 ml) was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in a mixture of acetonitrile—water—ammonia (80 : 20 : 0.05; 500 μl).

Towards the limit of quantification of the procedure, i.e. in the range 20—100 ng/ml, background interference can be minimised by reduction of the amount of Gibbs' reagent, to that corresponding to a two-fold molar excess of the anticipated maximum ICI 35,868 concentration.

High-performance liquid chromatography

An Altex pump (Model 110A) was used in conjunction with an automatic injection system (WISP Model 710, Waters Assoc., Milford, MA, U.S.A.) and a column (20 × 0.5 cm) packed with 5-μm Hypersil ODS (Shandon Southern Products, Runcorn, Great Britain) in the manner described by Bristow et al. [7]. The eluting solvent was acetonitrile—water—trifluoroacetic acid (80 : 20 : 0.1) with a flow-rate of 1.5 ml/min. The ultraviolet detector (Spectromonitor III, Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used at 276 nm.

Quantification

Calibration standards were prepared in control whole blood by addition of known amounts of ICI 35,868 and an amount of thymol corresponding to the median concentration of the calibration series under examination. The standards were treated in an identical manner to the unknown samples. The ratios of the peak heights derived from ICI 35,868 and thymol in the standards were used to construct calibration curves from which unknowns were quantified.

RESULTS AND DISCUSSION

ICI 35,868 absorbs in the ultraviolet (λ_{max} 275 nm) but its molar absorptivity is insufficient to allow quantification at the levels occurring in biological fluids after therapeutic doses. The compound also possesses a natural fluorescence, which formed the basis of the procedure previously described for measurement in biological samples [5]. However, the limit of quantification of this method was 300 ng/ml. To allow a full pharmacokinetic evaluation in man it was anticipated that a more sensitive analytical procedure would be required.

Since ICI 35,868 (I) has no *para* substituent, it reacts with Gibbs' reagent (2,6-dichloroquinone-4-chloroimide, III) in the presence of base to yield an intensely blue "indophenol" [2,6-dichloroquinone-4-(2,6-diisopropyl-1-hydroxyphenol)-imide, IV]. The leuco form of this derivative has greatly enhanced UV molar absorptivity compared to the parent compound and when quantified by UV detection after HPLC separation, permits estimation of ICI 35,868 in biological fluids down to 25 ng/ml, which is adequate for monitoring human pharmacokinetic profiles. Thymol (2-isopropyl-5-methylphenol, II) has a similar structure to ICI 35,868 and forms the corresponding "indophenol" (V). Compounds IV and V were stable for at least a week when stored in acetonitrile—water—ammonia.

When these blue dyes are injected under the acidic HPLC conditions they are converted instantly to the colourless leuco forms which have a high absorbance at 276 nm. These reduced forms are unstable under acid conditions, releasing the free phenols. However, the half-life of this decomposition was 30—40 min for both compounds and, since the HPLC column transit time was less than 6 min and the flow-rate was kept constant, this was found not to affect the re-

producibility of the procedure. HPLC chromatograms from blood extracts are shown in Fig. 2.

The results of nine calibration series in the range 25—1000 ng/ml were treated by linear regression analysis, and the *y*-axis intercept (*c*) and the S.E. calculated. A value of *c* plus twice its S.E. was taken as the limit of detection for each series. The mean of these values was found to be 25 ± 3 ng/ml (S.E.M., $n = 9$). This corresponded to the lowest concentration used routinely in calibration series and confirmed that 25 ng/ml was a reasonable lower limit for the estimation of ICI 35,868.

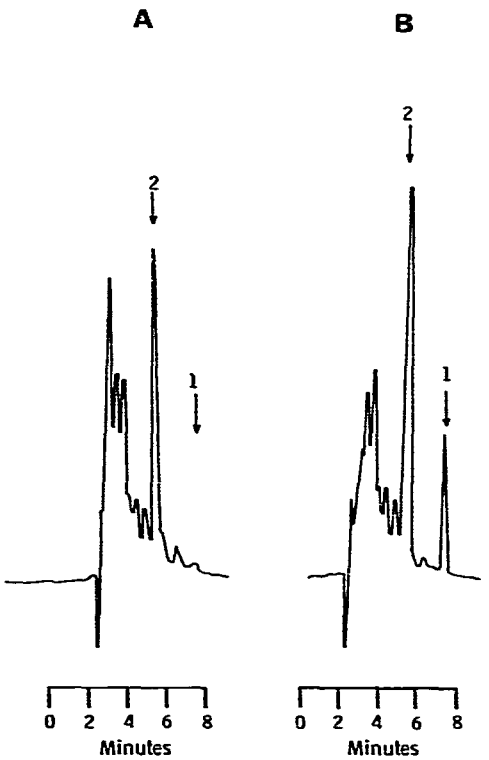

Fig. 2. Typical chromatograms of (A) control blood to which internal standard (100 ng/ml) has been added and (B) control blood to which has been added ICI 35,868 (40 ng/ml) and internal standard (100 ng/ml). The arrows 1 and 2 indicate the retention times of ICI 35,868 and internal standard respectively.

The present HPLC procedure was compared with the fluorimetric procedure in (a) a series of standards prepared by the addition of known amounts of ICI 35,868 to control blood and (b) in samples from animals which had received ICI 35,868. For (a) the concentration range was 0.6—7 μg/ml, for (b) 0.07—2.0 μg/ml. The results of these parallel determinations are presented in Fig. 3 with the exception of those which were less than 0.30 μg/ml which could not be quantified by the fluorescence assay. The regression line $Y = 0.97X + 0.10$ ($r^2 = 0.96$) obtained from the data is also presented. This figure shows that, within the range where both assay procedures are applicable, they produce compa-

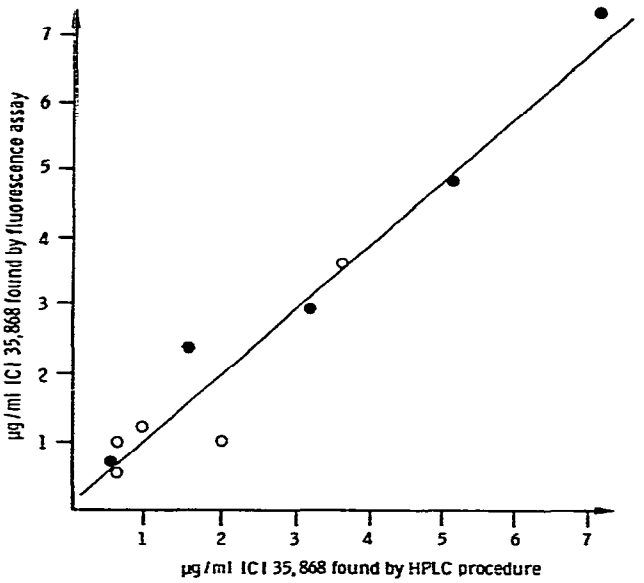

Fig. 3. Comparison of assay results by fluorimetric and HPLC procedures on known samples (0.6, 1.5, 3.0, 5.0 and 7.0 µg/ml, •) and unknown samples (○).

rable results. The accuracy of the HPLC procedure was found to be better in that, for the samples whose ICI 35,868 content was known (series a), the mean percentage found or added was 100 ± 4 (S.E.M., n = 5). For the fluorescence assay the corresponding value was 114 ± 12%.

Within-batch variation of the HPLC procedure was examined in greater detail by quintuplicate analyses of samples to which known amounts of ICI 35,868 had been added. The results obtained are shown in Table I. These show that the HPLC procedure gives an accurate assessment of the ICI 35,868 concentration with an overall mean result of 106 ± 2% (S.E.M., n = 20) of the added amount.

The between-batch variation was assessed by analysis of the same samples on four separate occasions. The mean coefficient of variation was 4.4 ± 0.8% (S.E.M., n = 4), showing excellent agreement between results obtained on different days.

This procedure was used to assess the stability of ICI 35,868 in blood samples stored under various conditions. No decrease in drug concentration was

TABLE I

DETERMINATION OF ICI 35,868 ADDED TO CONTROL BLOOD; WITHIN-BATCH VARIATION

Added (µg/ml)	Found* (µg/ml)	Mean recovery (%)
0.2	0.21 ± 0.01	104
1.0	1.11 ± 0.04	111
4.0	4.32 ± 0.42	108
8.0	7.87 ± 0.32	98

*Each value is mean ± S.E.M. (n = 5).

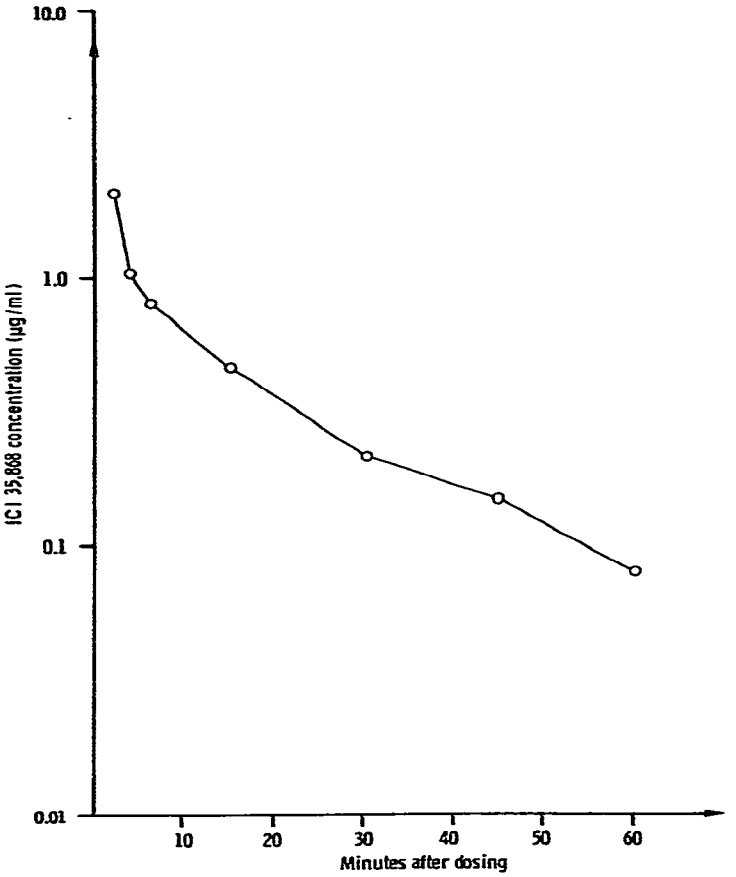

Fig. 4. Concentrations of ICI 35,868 in the blood of a dog after an intravenous dose of 5 mg/kg.

found in samples stored one week at room temperature in the light, one week at 4°C or nineteen weeks at −20°C in the dark. Since diazepam is frequently given as premedication for administration of intravenous anaesthetics, we have subjected to this procedure blood spiked with up to 400 ng diazepam per ml. There was no interference in the measurement of ICI 35,868.

Fig. 4 shows the profile of ICI 35,868 concentrations obtained in a dog after a single intravenous dose of 5 mg ICI 35,868 per kg.

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