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# MULTIPLE INVERSE ISOTOPE DILUTION ASSAY FOR CADRALAZINE AND FOUR METABOLITES IN BIOLOGICAL FLUIDS

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## SUMMARY

An inverse isotope dilution assay was developed for the specific determination of $^{14}$C-labelled cadralazine and four of its metabolites in biological samples. After addition of unlabelled carrier substances to the sample, metabolite IV was derivatized. The derivative and the unaltered compounds (I, II, III, V) were extracted and separated by high-performance liquid chromatography on silica gel. Quantitation was performed by on-line ultraviolet detection at 274 nm and off-line radiometry by liquid scintillation counting. Endogenous compounds and unknown metabolites did not interfere in the assay. The analysis of water, plasma and urine samples spiked with [$^{14}$C]cadralazine showed mean recoveries between 98.4 and 101.3%. The lower limit of detection was 10 nmol/l (3 ng/ml) for any of the compounds I–V. The method was used for the analysis of plasma and urine samples of rats dosed with [$^{14}$C]cadralazine.

## INTRODUCTION

Cadralazine (I; 2-{3-[6-(2-hydroxypropyl)ethylamino]-pyridazinyl}-ethylcarbazate) is an antihypertensive trial drug acting as a peripheral vasodilator[1]. In studies with $^{14}$C-labelled cadralazine in the rat four metabolites have been qualitatively identified or postulated[2]. They include the 3-methyltriazolo[4,3-*b*]pyridazine derivative (II), the 3-oxotriazolo[4,3-*b*]pyridazine derivative (III), the 3-hydrazino-pyridazine derivative (IV) and the 3-acetylhydrazino-pyridazine derivative (V) (for formulae see Figs. 1 and 2).

A method for the quantification of cadralazine by high-performance liquid chromatography (HPLC) has been developed[3].

The present paper describes a multiple inverse isotope dilution assay for the simultaneous determination of $^{14}$C-labelled cadralazine and four metabolites in biological fluids.

The kinetics of metabolite IV are of special interest, because this substance shows a strong antihypertensive activity when given as such to animals[4]. The free hydrazino group of metabolite IV may react with endogenous aldehydes or ketones,

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as is known from other examples[5–7]. The assay should also include this bound fraction of IV, which can be released from the hydrazones by acid-catalysed hydrolysis.

## EXPERIMENTAL

### *Chemicals*

Cadralazine (I), 6-[(2-hydroxypropyl)ethylamino]-3-methyltriazolo[4,3-*b*]pyridazine (II), 6-[(2-hydroxypropyl)ethylamino]-3-oxotriazolo[4,3-*b*]pyridazine (III), and 3-(2-acetyl)hydrazino-6-[(2-hydroxypropyl)ethylamino]pyridazine (V) were obtained from ISF (Trezzano, Milan, Italy). 3-Hydrazino-6-[(2-hydroxypropyl)ethylamino]pyridazine (IV) was provided by Ciba-Geigy (Basle, Switzerland). [$^{14}$C]Cadralazine labelled in 3,6-position of the pyridazine ring was obtained from ISF (synthesized by Amersham International, Amersham, U.K.) and purified by Ciba-Geigy (Batch No. B-1011-B; specific radioactivity 101.4 kBq/mg = 2.74 $\mu$Ci/mg, radiochemical purity *ca.* 99%).

1,2-Dichloroethane (Chemische Werke Hüls, Marl, F.R.G.) was distilled before use. Benzaldehyde (Fluka, Buchs, Switzerland) was distilled, and 1-ml portions were stored in fused-glass ampoules. Butyl-PBD [2-(4-*tert.*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole], Irgasolv, and Irgascint A 300 were obtained from Ciba-Geigy. All other solvents and chemicals were of analytical reagent grade and were purchased from E. Merck (Darmstadt, F.R.G.). Phosphate buffer (pH 7.2) was prepared by mixing 1/15 *M* potassium dihydrogen phosphate (29 ml) with 1/15 *M* disodium hydrogen phosphate (71 ml). Buffer mixture I contained 0.2 *M* potassium chloride (25 ml), 0.2 *M* hydrochloric acid (4.3 ml) and 2 *M* sodium hydroxide (13 ml), diluted with distilled water to a volume of 100 ml. Buffer mixture II consisted of phosphate buffer pH 7.2 (100 ml) and saturated sodium hydrogen carbonate solution (13 ml).

### *Synthesis of 3-benzylidenehydrazino-6-[(2-hydroxypropyl)ethylamino]pyridazine (VI)*

Compound IV (0.5 g) was dissolved in ethanol (5 ml) and shaken with benzaldehyde (0.5 ml) for 30 min. The ethanol was evaporated, the residue dissolved in water (5 ml), and 0.01 *M* hydrochloric acid was added until a clear solution (pH *ca.* 2) resulted. After extraction with *n*-hexane (3 × 10 ml), the aqueous layer was adjusted to pH 8 with diluted sodium hydroxide and extracted with ethyl acetate (3 × 10 ml). The combined organic phases were evaporated and the residue was crystallized from ethanol–water (8:2). The melting point was 154–155°C (reported in ref. 8: 155–157°C). The purity of the substance was checked by HPLC (see under *Chromatographic conditions*) and by thin-layer chromatography (silica gel 60 $F_{254}$, Merck; $R_F$ = 0.53 in 1,2-dichloroethane–methanol (5:1); $R_F$ = 0.45 in 1,2-dichloroethane–methanol–10% acetic acid (50:10:1)). Elemental analysis: ($C_{16}H_{21}N_5O$) calculated, C = 64.19%, H = 7.07%, N = 23.39%; found, C = 63.92%, H = 6.98%, N = 23.39%.

### *Chromatographic conditions*

Separation of I, II, III, V and of the benzylidenehydrazino-pyridazine derivative (VI) of IV was achieved by HPLC on a 250 × 10 mm I.D. stainless-steel column packed with LiChrosorb Si 60, 10 $\mu$m (Merck). The separation column was protected

by a precolumn (50 × 4.6 mm I.D.) with identical material. For injection, a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 100-μl loop was used. The solvent system, dichloromethane–ethanol–conc. aqueous ammonia–water (86:14:0.4:0.1), was pumped through the column at a flow-rate of 4 ml/min using a Model 110 A solvent pump (Altex, Beckman, Berkeley, CA, U.S.A.). The compounds were detected by a Uvikon Model 725 UV detector (Kontron, Zürich, Switzerland) at 274 nm and range 2 and quantified on-line by an Infotronics Model CRS-204 digital integrator (Shannon, Ireland). The integrator was equipped with a variable-level sensor, controlling collection and integration of the peak fractions.

*Inverse isotope dilution analysis (IDA)*

To a 0.1–2.5 ml sample of urine or plasma a solution of the non-labelled carrier substances I (0.2 mg), II (0.3 mg), III (0.5 mg), IV (0.1 mg) and V (0.3 mg) in 0.5 ml 0.1 *M* hydrochloric acid was added. After 15 min the mixture was treated with 0.2 ml of 2 *M* hydrochloric acid for 30 min at room temperature, to hydrolyse hydrazones of IV. Then 1 ml of buffer mixture I effecting a pH value of 1.5–2, and 0.02 ml of benzaldehyde were added; air was removed by a stream of nitrogen and the mixture shaken for 30 min in the dark. After extraction of the excess of benzaldehyde with *n*-hexane (5 ml, $N_2$, in the dark, 5 min) the pH value of the aqueous phase was adjusted to 7–7.5 with 2.5 ml of buffer mixture II. Residues of *n*-hexane were removed by a stream of nitrogen, and the sample was injected into a Sep-Pak $C_{18}$ cartridge (Waters, Milford, MA, U.S.A.). The cartridge was washed with water (10 ml) and blown dry with nitrogen, and the retained substances were eluted with ethanol (5–6 ml). The ethanol phase was evaporated and the residue dissolved in 0.15–0.2 ml of the solvent system. After centrifugation, a volume of 0.1 ml was injected on the HPLC column and chromatographed as described above.

The eluate fractions corresponding to the integrated peak sections of each of the five compounds were collected automatically in counting vials by a Model 2111 MultiRac fraction collector (LKB, Bromma, Sweden). Counting vials contained 0.05 ml of 2 *M* hydrochloric acid to prevent formation of quenching, coloured decomposition products. The fraction collector and a pneumatically driven three-port valve (Chromatronix, Berkeley, CA, U.S.A.), fixed on the collector, were both actuated by the integrator via a control device with an adjustable time delay (Ciba-Geigy).

About 15 ml of scintillation cocktail were added to each sample, and radioactivity was measured. The amount of each compound in a sample can be determined from the spectrophotometric and radiometric results.

*Calibration of the HPLC method*

Replicate samples containing various amounts of one of the five compounds (I, II, III, V and VI) were injected into the HPLC system. The range of calibration was selected to cover the expected concentrations of samples mixed with the carrier substances. The chromatographic peaks were integrated automatically by the UV detector and integrator. Calibration curves were fitted by means of a computer using a linear or non-linear (quadratic) regression program. The resulting coefficients were used for the calculations in the isotope dilution analysis.

*Radiometry*

Radioactivity in biological samples and chromatographic eluate fractions was measured in a Tri-Carb Model 460 C liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.) with external standardization equipment. The scintillation cocktail used was 0.6% (w/v) Butyl-PBD in toluene–methanol (3:1) for eluates and urine, and Irgascint for plasma, after dissolution in Irgasolv.

*Recovery of [$^{14}$C]cadralazine from spiked biological samples*

Samples of 1 ml of water, dog plasma, rat plasma, dog urine and rat urine were spiked with [$^{14}$C]cadralazine (3.5–6.7 $\mu$mol/l). The spiked samples were carried through the analytical procedure described. The results obtained were compared with the amount added to each respective sample to determine the recovery of [$^{14}$C]cadralazine.

*Pattern of urinary metabolites*

To check the HPLC separation of I, II, III, V and VI for possible interference by unknown metabolites, a urine sample from rats treated with [$^{14}$C]cadralazine (3 mg/kg; p.o.) was submitted to IDA. Unlike the procedure described above, however, the total effluent of the chromatographic column was collected in small fractions of 0.3 ml, which were then counted for radioactivity. The UV absorption profile was compared with the radioactivity pattern.

*Animal experiment*

Five male albino rats [Tif:RAIf(SPF)], weighing 220 g, were obtained from the Tierfarm Sisseln, Switzerland. (For designation of strains see ref. 9). They received single oral doses of 3 mg/kg of [$^{14}$C]cadralazine, dissolved in 1 ml of water–1 *M* hydrochloric acid (99:1). In one rat, blood samples of 0.2 ml were obtained retro-orbitally with heparinized glass capillaries at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after dosing. Blood was centrifuged and the plasma obtained analysed without delay. From the other four rats, urine was collected for 6 h and pooled.

## RESULTS AND DISCUSSION

*Reaction of compound IV with benzaldehyde*

Owing to its hydrazino function, compound IV is not sufficiently stable in aqueous solution. Also, it cannot be easily extracted and chromatographed. In the present method it was, therefore, treated with benzaldehyde to yield 3-benzylidene-hydrazino-6-[(2-hydroxypropyl)ethylamino]pyridazine (VI) (Fig. 1).

Compound VI was first synthesized on a preparative scale, and its structure was confirmed by elemental analysis (see Experimental) and by NMR spectroscopy.

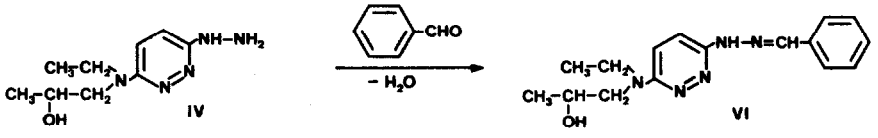


Fig. 1. Reaction of compound IV with benzaldehyde to form the hydrazone derivative VI.

A signal of an uncoupled single proton at 8.06 ppm (in [$^2H_6$]dimethyl sulphoxide) showed the presence of a benzylidene proton. The melting point is in agreement with the value reported by Pifferi *et al.*[8].

In the isotope dilution assay, the derivatization procedure was applied to the biological sample, after addition of the non-labelled carrier substances I–V. The yield of VI was almost quantitative. It has the same retention time in the HPLC system as the reference compound VI. Under the conditions of the assay none of the other carrier substances was attacked, and interfering peaks were not detected in the liquid chromatographic separation.

*Specificity of the HPLC separation*

Using the HPLC system described all five substances were completely separated from each other as shown in Fig. 2.

Analysis of blank samples of plasma and urine from rat and dog demonstrated that endogenous components do not adversely interfere with the UV detection (see Fig. 2). In rat urine there is a certain interference with compound VI which, however, can be overcome by reducing the sample volume. Also good coincidence was observed between the radioactivity pattern and the UV absorption profile, when urine of rats dosed with [$^{14}$C]cadralazine was analysed according to the IDA procedure

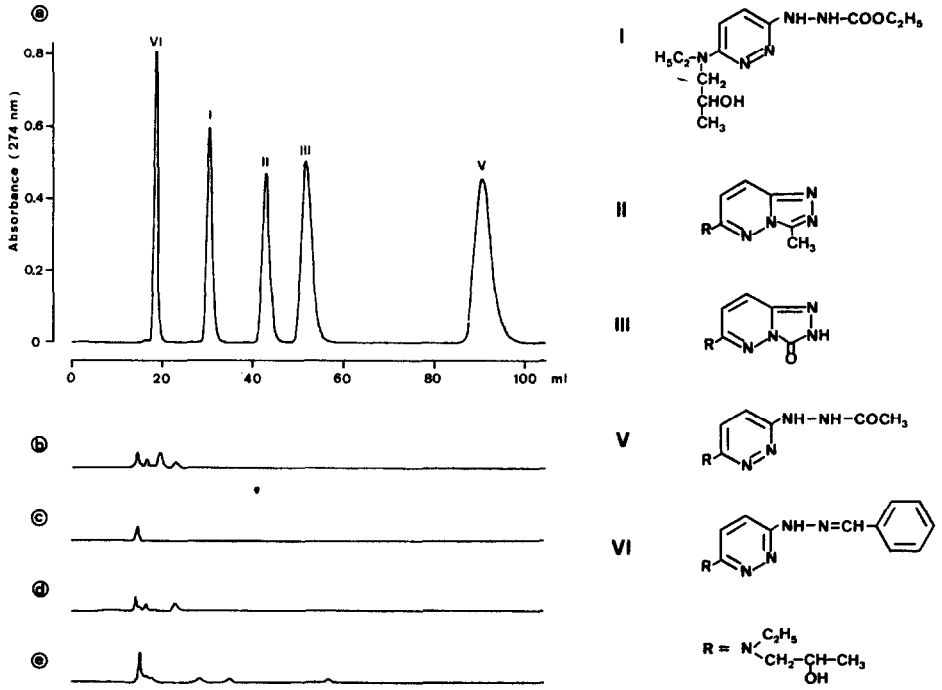


Fig. 2. (a) HPLC separation of the hydrazone derivative (VI) of metabolite IV, of cadralazine (I) and of metabolites II, III and V. Chromatograms from blanks: (b) dog plasma; (c) rat plasma; (d) dog urine; (e) rat urine; as obtained following the IDA procedure. HPLC conditions: column, LiChrosorb Si 60 (250 × 10 mm I.D.); eluent, dichloromethane–ethanol–conc. aqueous ammonia–water (86:14:0.4:0.1); flow-rate, 4 ml/min; UV detection at 274 nm.

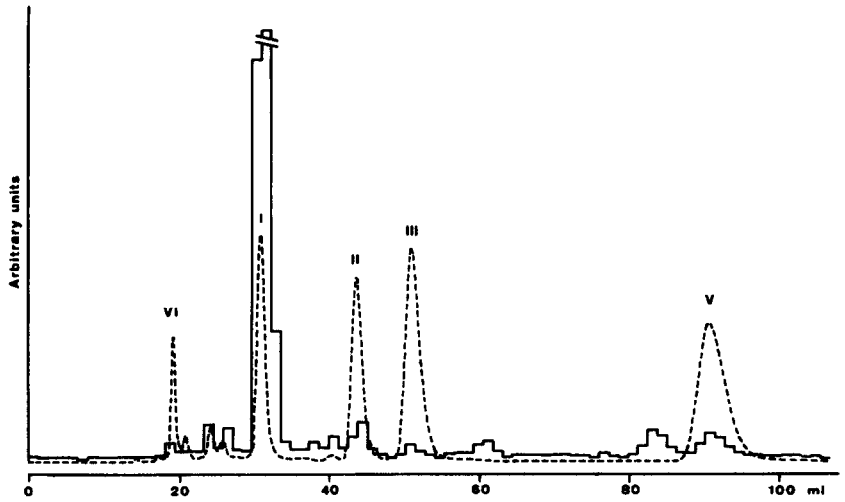


Fig. 3. HPLC of cadralazine (I) and metabolites II–V (IV as derivative VI), in IDA from urine of a rat dosed with [$^{14}$C]cadralazine, monitored by radioactivity (———) and UV detection (---------). HPLC conditions as in Fig. 2.

(see Fig. 3). This indicates that unknown metabolites of cadralazine do not interfere with the HPLC separation.

*Calibration and reproducibility of the spectrophotometric determination*

Linear calibration curves were obtained for compounds II, III and VI. For substances I and V suitable fits were achieved by quadratic regression analysis resulting in non-linear calibration curves. The coefficients of the curves are displayed in Table I, which also lists the coefficients of variation as an indication of the reproducibility of the method.

TABLE I

CALIBRATION AND REPRODUCIBILITY OF UV DETECTOR RESPONSE TO CADRALAZINE (I) AND COMPOUNDS II, III, V, VI

For HPLC conditions see Fig. 2.

| *Compound No.* | *Amount injected (μg)* | *Coefficients of the calculated calibration curves* ($y = a + bx + cx^2$)* | | | | *Coefficient of variation, C.V. (%)**** | *Number of samples* |
|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c* | *r*** | | |
| I | 20–120 | 359 | 117.4 | −0.186 | 0.9995 | ±2.1 | 22 |
| II | 30–150 | 69 | 69.7 | 0 | 0.9999 | ±0.8 | 15 |
| III | 100–260 | 645 | 68.0 | 0 | 0.9998 | ±0.6 | 15 |
| V | 30–150 | 937 | 188.5 | −0.184 | 0.9998 | ±1.2 | 15 |
| VI | 10–50 | 181 | 158.4 | 0 | 0.9996 | ±1.3 | 15 |

* Calibration curves were calculated by linear ($c = 0$) or non-linear quadratic ($c < o$) regression analysis ($y$ = integration units, $x$ = amount injected).

** Coefficient of correlation.

*** For the ratio $x$ (calculated) to $x$ (given); $n$ = 15–22.

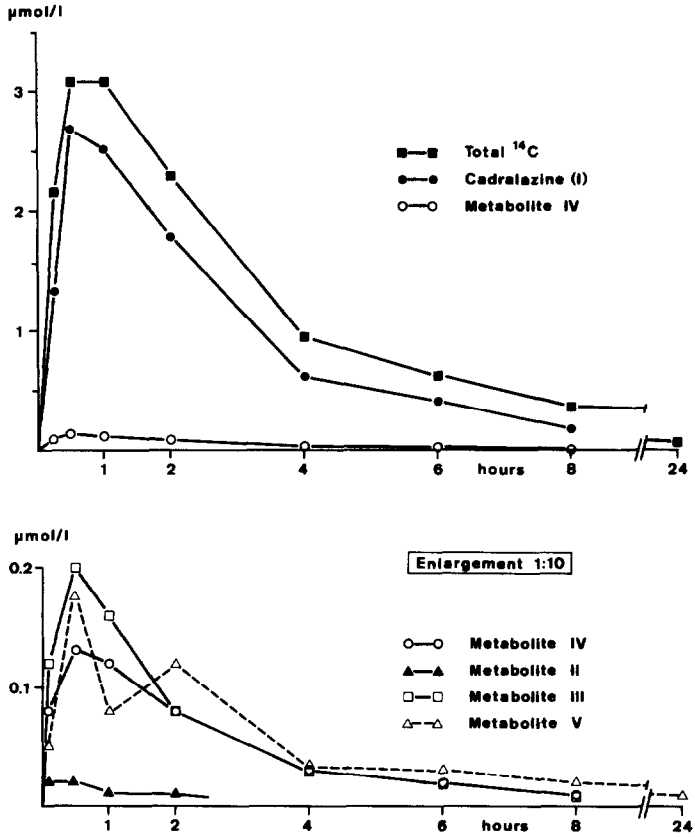


Fig. 4. Plasma concentration profiles of total $^{14}$C-labelled substances, unchanged cadralazine (I) and metabolites II–V in one rat after oral dosage of 3 mg/kg [$^{14}$C]cadralazine.

*Accuracy of the IDA method*

Biological samples were spiked with 3.6–6.7 µmol/l [$^{14}$C]cadralazine and analysed by the IDA assay. The calculated recoveries for cadralazine had means ± C.V. of 99.0% ($n$ = 3) in water, 100.6% ± 1.1% ($n$ = 5) in dog plasma, 99.5% ($n$ = 2) in rat plasma, 101.3% ± 2.8% ($n$ = 5) in dog urine and 98.4% ± 1.9% ($n$ = 4) in rat urine.

TABLE II

EXCRETION (%) OF TOTAL $^{14}$C-LABELLED SUBSTANCES, UNCHANGED CADRALAZINE (I) AND METABOLITES II–V IN POOLED 0–6 h URINE OF FOUR RATS AFTER ORAL DOSAGE OF 3 mg/kg [$^{14}$C]CADRALAZINE

| *Total $^{14}$C-substances* | *I* | *II* | *III* | *IV* | *V* | *Sum I–V* |
|---|---|---|---|---|---|---|
| 100.0 | 79.2 | 1.4 | 0.8 | 0.3 | 3.6 | 85.3 |
| (76.5)* | (60.6) | (1.1) | (0.6) | (0.2) | (2.8) | (65.3) |

* Figures in parentheses give percentage of dose.

The sensitivity of the method would be *ca.* 0.01 μmol/l (3 ng/ml) using a specific radioactivity of 185 kBq/mg (5 μCi/mg) and a 1-ml sample.

*Application of the IDA*

The described method was applied to determine the concentrations of cadralazine and compounds II–V in plasma and urine of rats orally dosed with 3 mg/kg of [$^{14}$C]cadralazine. Fig. 4 shows the plasma concentration curves of the individual compounds and of the total radioactive substances. The results of the analysis of a 0–6 h urine pool from four rats are given in Table II.

Cadralazine accounted for the major fraction of total plasma radioactivity. Concentrations of metabolites III, IV and V were about ten-fold lower than those of the parent compound, but were well above the limit of detection. The concentration of II, however, was near this limit. Unchanged cadralazine was also the main component (79%) in urine. The sum of the metabolites II–V constituted 6% of urinary radioactivity.

## CONCLUSION

The inverse isotope dilution assay described allows one to measure cadralazine and four of its metabolites in biological fluids after administration of radioactively labelled cadralazine. This analysis includes the pharmacologically active hydrazino compound IV, which requires derivatization for its determination. The added carrier substances act as ideal internal standards, thus providing high specificity, accuracy and reproducibility of the quantitative measurements.

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## REFERENCES

1 C. Semeraro, L. Dorigotti, S. Banfi and C. Carpi, *J. Cardiovasc. Pharmacol.*, 3 (1981) 455.
2 L. Simonotti, M. Zanol, F. Parravicini and G. Pifferi, *Eur. J. Drug Metab. Pharmacokin.*, 7 (1982) 39.
3 S. A. Hauffe and J. P. Dubois, *J. Chromatogr.*, submitted for publication.
4 C. Carpi, L. Dorigotti and C. Semeraro, *Brit. J. Pharmacol.*, 72 (1981) 188 P.
5 J. Wagner, J. W. Faigle, P. Imhof and G. Liehr, *Arzneim.-Forsch.*, 72 (1977) 2388.
6 P. A. Reece, I. Cozamanis and R. Zacest, *J. Chromatogr.*, 225 (1981) 151.
7 L. B. Colvin, *J. Pharm. Sci.*, 58 (1969) 1433.
8 G. Pifferi, F. Parravicini, C. Carpi and L. Dorigotti, *J. Med. Chem.*, 18 (1975) 741.
9 M. F. W. Festing and W. Butler (Editors), *International Index of Laboratory Animals*, Medical Research Council, Laboratory Animals Centre, Carlshalton, Surrey, U.K., 1975.