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Note

Determination of climazolam in the plasma of farm animals by gas chromatography

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Climazolam, 8-chloro-6-(*o*-chlorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine, is a new representative of the imidazo-1,4-benzodiazepine series (Fig. 1). It has a fast onset of action and exhibits sedative and anticonvulsive activities for a short period of time following parenteral administration in pig and cow. This new drug is currently undergoing clinical trials for use in farm animals and pets, particularly to eliminate stress in pigs during regrouping and transport [1–4].

Several methods have been published for the analysis of imidazo-1,4-benzodiazepine in biological fluids, involving liquid chromatographic [5–7], polarographic [8], radioimmunoassay [9] and gas chromatographic (GC) [10–15] techniques. A low detection limit, down to 5 ng/ml of plasma, was needed for single-dose pharmacokinetic studies and could easily be achieved with GC. Heizmann and Von Alten [10] have developed a GC method with electron-capture detection to assay midazolam in the plasma and urine of humans. As the chemical structure of this benzodiazepine is close to that of climazolam, a similar technique was used to assay the new drug in the plasma of farm animals.

EXPERIMENTAL

Reagents and solvents

Methanol, ethanol, dichloromethane, acetone, titrisol buffers and 1 *M* sodium hydroxide were all p.a. grade from Merck (Darmstadt, F.R.G.). Bi-distilled water was used for the preparation of all the aqueous standard and buffer solutions. Climazolam and its internal standard midazolam, 8-chloro-6-(*o*-fluorophenyl)-

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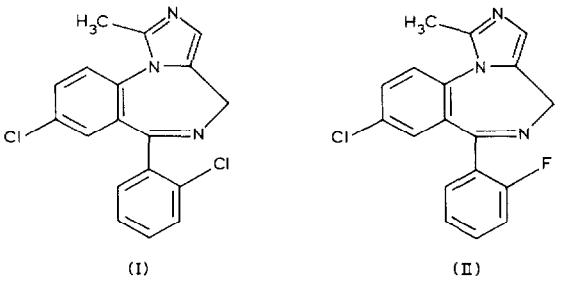

(I) (II)

Fig. 1. Structural formulae of climazolam (I) and the internal standard midazolam (II).

1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine, are compounds from F. Hoffmann-La Roche (Basle, Switzerland).

Chromatography

The system consisted of an HP 5700A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) fitted with a 15-mCi ^{63}Ni electron-capture detector (Hewlett-Packard, Model 18713A), which was coupled to a computing integrator SP 4100 (Spectra-Physics, F.R.G.). The column, a coiled glass tube (1.40 m × 2 mm I.D.), was treated for 5 min with a solution of 10% dimethylchlorosilane in toluene. After it had been washed with toluene, methanol and acetone, the deactivated column was dried at 110°C under a stream of nitrogen (10 ml/min, 1 h) and packed with 3% SP 2250 on 100–120 mesh Supelcoport (article No. 22, Supelco, Bellefonte, PA, U.S.A.). The packed column was conditioned in an oven for 20 h using a temperature programme from room temperature to 310°C in steps of 1°C/min and a nitrogen flow-rate of 35 ml/min. Subsequently, the column was connected to the detector and maintained at 280°C for 24 h. To deactivate the active sites on the stationary phase and to optimize the limit of detection, the column was primed with several injections of plasma extracts prior to the first analysis.

The instrumental parameters during the assay were: carrier gas, nitrogen; flow-rate, 25 ml/min; column oven temperature 290°C; injector temperature, 300°C; detector temperature, 300°C. Under these conditions, the retention times of the internal standard and climazolam were 2.5 and 3.5 min, respectively.

An automatic sampler from Hewlett-Packard (Model 17871A) was used for routine analysis. Weekly changing of the septum, the liner and the silanized glass-wool at the top of the column was necessary to maintain the chromatographic system in a deactivated state and achieve reproducible results.

Solutions and calibration standards

Plasma standard. The maleate salt of climazolam (13.56 mg, corresponding to 10 mg of free base) was dissolved in 100 ml of water in a volumetric flask. Typically, the calibration was carried out using spiked plasma standards which were made up in the following way: 100 µl of the stock solution were added to 25 ml of drug-free plasma giving a concentration of 400 ng/ml; this stock plasma solution

was shaken for 5 min and further plasma standards covering the range 6–200 ng/ml were prepared by subsequent dilution with drug-free plasma. The plasma standards were stored deep frozen in 5–10 ml portions at −20 °C.

A batch of four or five quality-control samples, covering the 10–200 ng climazolam per ml plasma concentration range, was prepared by diluting an appropriate volume of an aqueous stock solution (100 μg/ml) with blank plasma from animals. To obtain optimum control of the assay, plasma standards and quality-control samples were prepared by different persons using different stock solutions.

Internal standard. The maleate salt of midazolam (13.39 mg, corresponding to 10 mg of free base) was dissolved in 100 ml of water. From this solution, dilutions were made in water in order to obtain the final concentrations of 200 ng per 100 μl (solution A) and 50 ng per 100 μl (solution B). Solution A was used for concentrations of climazolam higher than 50 ng/ml and solution B for concentrations of climazolam lower than this value.

Sample preparation

Frozen plasma* was allowed to thaw and warm to room temperature. After equilibration at 37 °C in a water-bath, it was shaken for 5 min on a rotating shaker (Heidolph; 15 rpm). Then, 0.5 ml of plasma were transferred into a conical glass tube (15 ml volume) and mixed with 0.2 ml of 1 *M* sodium hydroxide solution. A 100-μl volume of an aqueous solution of internal standard A or B, depending on the expected concentration of climazolam, was added. After mixing on a vortex mixer (Bender-Hobein), 8 ml of dichloromethane were added. The sample was extracted on a rotary extractor (5 min, 20 rpm) and then centrifuged for 5 min at 1750 *g* and 10 °C. The upper aqueous phase was discarded; the organic phase (7 ml) was transferred to another glass tube and evaporated to dryness at 35 °C under a gentle stream of pure nitrogen. The dry residue was taken up in 100 μl (for concentrations of climazolam below 50 ng/ml of plasma) or 400 μl (for concentrations of climazolam between 50 and 400 ng/ml of plasma) of benzene–acetone–ethanol (8:1:1, v/v/v). The mixture was vortexed for a short time, and 2 μl were injected onto the GC column for analysis.

Calibration

Five to nine calibration standards, covering the appropriate concentration range of the unknown samples, were processed as described above and analysed together with the biological samples. A calibration curve was obtained by a weighted least-squares linear regression of the peak-height ratio of climazolam to internal standard versus the climazolam concentration. The data points were weighted inversely to the concentration. The ratios of the measured peak heights of the compound and the internal standard in the unknown samples were interpolated into this calibration curve to obtain the climazolam concentrations.

All data-processing and calculations were carried out by the Spectra-Physics SP 4100 computing integrator and 4100D Minifile [16].

*Plasma standard (calibration), control plasma (quality control), biological samples (analysis), or drug-free plasma (blank plasma).

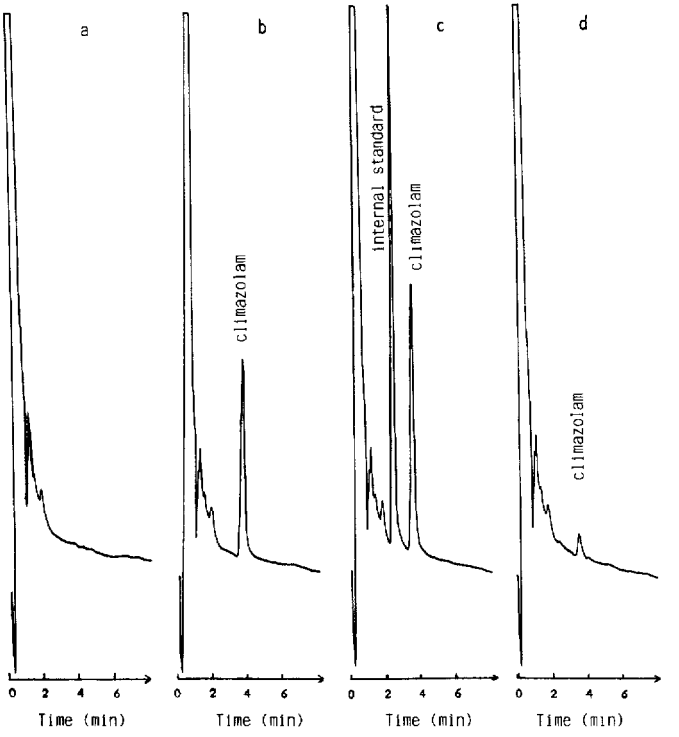

Fig. 2. Chromatograms of calf plasma extracts. (a) Blank plasma; (b) plasma from a calf treated with climazolam (100 ng/ml); (c) as in b, but the internal standard was added before the extraction and the climazolam concentration was 140 ng/ml; (d) as in b, but the climazolam concentration was 10 ng/ml. Chromatographic conditions: column, 1.4 m×2 mm I.D. 3% SP 2250; flow-rate 26 ml/min; column temperature 290°C.

RESULTS AND DISCUSSION

Specificity, choice of the internal standard and chromatographic conditions

The method was developed for the determination of climazolam in plasma of dog, pig, cattle and horse. Several blank plasmas from these species were analysed as described above. In all cases, clean plasma extracts were obtained. Typical chromatograms of calf plasma extracts are shown in Fig. 2; they were collected prior to the intravenous injection of a single dose of 2 mg of climazolam per kg of body weight (Fig. 2a), and at 9 h (Fig. 2b), 8 h (Fig. 2c), and 24 h (Fig. 2d) after the drug administration. The chromatograms b and d were recorded from plasma extracts that were not added with the internal standard before the extraction, in order to detect possible interfering metabolites of climazolam. These results indicated specificity of the assay with respect to other endogenous components in plasma.

The duration of a chromatographic run (ca. 7 min) and the chromatographic resolution was optimized at 290°C. Lower temperatures resulted in an increased peak width and higher limit of determination. Further increase of the temperature decreased the deactivation of the column within a short time which, in turn, impaired the peak shape.

TABLE I

INTER-ASSAY PRECISION AND ACCURACY (PLASMA OF MINIPIG, FOUR REPLICATES)

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)	Accuracy (%)
6.25	6.2	4.1	−0.80
25.0	26.1	2.5	+4.4
100	99.7	3.2	−0.3
400	409	2.3	+2.3

Midazolam differs from climazolam in the replacement of the chlorine atoms in the benzyl ring by a fluorine (see Fig. 1). Small differences of solubility in solvents between the two drugs and a good chromatographic separation make midazolam the internal standard of choice [17].

Recovery

The extraction yield was determined from the difference between the peak height when climazolam was added to plasma and the peak height when the same amount was added to the final extract of a blank plasma. Climazolam was well extracted (78–83%) in the 10–200 ng/ml concentration range, and the extraction yield did not depend on the concentration.

Linearity

Linear correlation between the peak-height ratio and the concentration of climazolam in plasma was obtained over various concentration ranges (6–200, 3–50 and 25–2000 ng/ml). The coefficients of correlation were always higher than 0.99.

Precision and accuracy

Intra-assay precision. Spiked samples of different concentrations were analysed as replicates during one working day. The intra-assay precision was then expressed as the coefficient of variation (C.V.) calculated from the resulting peak-height ratio. The mean value was 2.4% over the 10–200 ng/ml range.

Inter-assay precision and accuracy. Spiked plasma from pig, horse and calf were analysed along with the biological samples as quality controls during routine analysis. Results obtained with the horse and minipig are reported in Tables I and II. They originate from two different technicians who performed the assay on two different sets of GC apparatus. A satisfactory inter-day precision ($\leqslant 5\%$) and accuracy ($\leqslant 5\%$) over a 10–2000 ng/ml plasma concentration range was observed [18].

Limit of quantitation

The limit for quantitation of the assay was defined as the lowest concentration that could be measured with a precision and accuracy of ca. 10% [19–21]. The standard deviation and the accuracy of the limit of determination were measured

TABLE II

INTER-ASSAY PRECISION AND ACCURACY (PLASMA OF HORSE)

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	95% Confidence limit (ng/ml)	Replicates	Accuracy found − added (%)
3.9	4.3 ± 0.3	3.8- 4.8	4	+10.3
19.5	20.4 ± 0.5	19.7- 21.1	4	+ 4.6
375	369 ±20	365 - 372	12	− 1.6
750	745 ±18	741 - 748	12	− 0.7
2250	2260 ±64	2249 -2272	12	+ 0.4

by analysing spiked plasma during four different days. The limit of quantitation under these routine conditions was 4 ng of climazolam per ml of plasma, with a standard deviation of ± 0.3 ng/ml (Table II). This limit was the same for plasma from pig, cattle, horse and dog.

Stability of climazolam in the plasma of minipig

Blank plasmas from minipig, fortified with climazolam and stored in colourless glass vessels under normal laboratory lighting conditions [22], were stable for 24 h (Table III). When stored at −20 °C in a deep freezer, the plasma solutions were stable for at least three months.

Application of the method

The method was applied to the determination of climazolam in the plasma of minipigs, pigs, cattles, horses and dogs treated with a single i.v., i.m. or p.o. administration of climazolam at various dose levels. Fig. 3 is the plasma concentration–time curve of climazolam following a single i.v. dose of 2 mg/kg body weight to a calf. This dose resulted in a good sedation of the animal. The terminal half-life of climazolam in calf was in the range of 5–7 h. These data demonstrated that the present analytical technique is sensitive enough to measure plasma levels for 24 h (three to four half-lives) after a single i.v. injection. It is therefore suitable for monitoring pharmacokinetics in individual animals.

TABLE III

STABILITY OF CLIMAZOLAM IN THE PLASMA OF MINIPIG (FIVE REPLICATES)

Added (ng/ml)	24 h at room temperature		3 months at −20 °C	
	Found (ng/ml)	90% Confidence limit (ng/ml)	Found (ng/ml)	90% Confidence limit (ng/ml)
10.0	9.98	9.65- 10.31	10.05	9.84- 10.26
20.0	20.05	19.61- 20.50	18.4	17.61- 19.20
40.0	40.05	39.25- 40.85	–	–
80.0	81.40	77.05- 85.75	77.9	77.31- 78.50
160.0	156.0	153.6 -158.5	157.8	156 -159.6

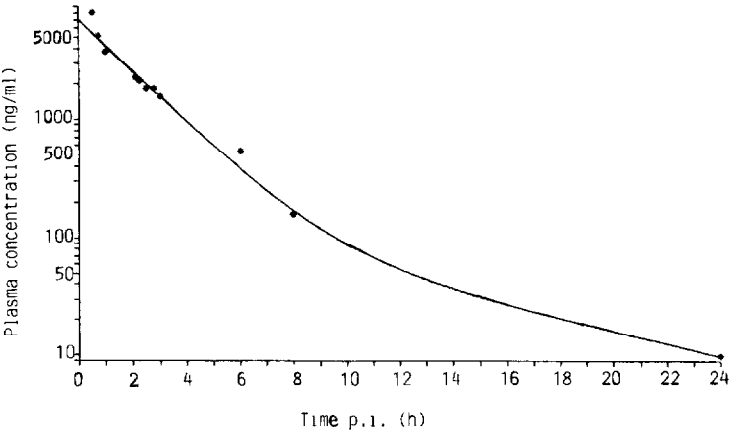

Fig. 3. Plasma concentration–time curve of climazolam following a single i.v. injection of 2 mg/kg body weight administered to a calf.

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