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Note

High-performance liquid chromatographic method for the determination of carazolol in serum or plasma of pigs

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The β-receptor blocking agent carazolol [1-(4-carbazolyloxy)-3-isopropylamino-2-propanol] is used in veterinary practice as a rapidly acting tranquillizing agent for treatment of stress syndrome in pigs. Stress syndrome can occur during transportation, rehousing, mating and farrowing [1–4]. In order to reduce morbidity and mortality of the pig under stress conditions, carazolol is indicated to be given prophylactically by intramuscular injection.

Little is known about the kinetics of this drug and in order to be able to evaluate residue problems more rationally, further information concerning its basal kinetics is needed. Published studies have used qualitative and semi-quantitative measurements such as thin-layer chromatography (TLC) [5],radioimmunoassay [6] and radiolabeling techniques [7,8]. More recently Engelsma and Simons [9], Rudolph and Steinhart [10] and Keukens and Aerts [11] have described more sensitive high-performance liquid chromatographic (HPLC) methods for the quantification of carazolol in various pig organs, especially the kidneys. These procedures involve extraction with suitable organic solvent, followed by sample clean-up using silica and C_{18} cartridges and fluorimetric determination. Detection limits are rather high: 1, 0.48 and 0.3 μg/kg, respectively.

However, there is still a lack of a specific and sufficiently sensitive method for measurement of concentrations of carazolol in serum and/or plasma. Ap-

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plication of the aforementioned HPLC methods appears to be laborious and has only low sensitivity. Therefore we developed a rapid HPLC method with fluorimetric detection, involving a simple two-step extraction procedure followed by ion-pair reversed-phase separation. This method has been tested in a pilot study on the pharmacokinetics of carazolol following intravenous administration in pigs.

EXPERIMENTAL

Reagents

Carazolol base and the commercially available preparation Suacron® were gifts from Upjohn (Veterinary Department, Ede, The Netherlands). A methanolic stock solution of carazolol standard (50 mg per 100 ml) was prepared and stored at 4 °C. Blank serum samples (pig serum) were spiked using a working standard solution, which was prepared by diluting (100-fold) the standard solution with distilled water.

The extraction medium was prepared by mixing (85:15, v/v) *n*-hexane (analytical grade, Janssen Chimica, Beerse, Belgium) with 2-methyl-2-butanol (analytical grade, E. Merck, Darmstadt, F.R.G.). Phosphate solution (0.05 *M*, pH adjusted to 11) and 5% (v/v) acetic acid solution were prepared using chemicals of analytical grade (E. Merck).

The mobile phase was prepared by mixing 0.1 *M* ammonium acetate buffer in water (pH 4.5), 2-propanol, acetonitrile and sodium dodecylsulphate (SDS) [61% (v/v):25% (v/v):14% (v/v):0.05% (g/v)]. Acetonitrile and ammonium acetate were p.a. quality (E. Merck) and SDS and 2-propanol chromatographic grade (E. Merck). After filtration and vacuum sonication (20 min), the mixture was pumped through the column.

Extraction procedure

In order to minimize photodecomposition exposure to light was limited.

An aliquot of 750 μg of plasma was pipetted in a glass tube and mixed with the same volume of the phosphate solution (pH 11). Then 3.0 ml of the extraction fluid, *n*-hexane–2-methyl-2-butanol (85:15), was added and the mixture was shaken for 30 min at a low speed in order to prevent the formation of an emulsion. Organic and aqueous layers were separated by centrifugation (5 min, 4053 *g*). Of the organic layer 2.0 ml were transferred to another tube, which contained 250 μl of the 5% acetic acid solution. The drug was back-extracted by vortex-mixing for 20 s. After centrifugation for 5 min (4053 *g*) the lower aqueous layer was transferred carefully to an injection vial, from which 100 μl were injected into the HPLC system.

HPLC system

An HPLC system with fluorimetric detection, composed of the following units, was used: a Kratos solvent-delivery system (Spectroflow 400), a Kontron autosampler (MS 1660) equipped with a 100-μl loop, a Hypersil ODS column (Chromsep, Middelburg, The Netherlands, 100 mm$\times$3.0 mm I.D., particle size 5 μm) which was connected to a guard column (C_{18} reversed phase, Chrompack, Middelburg, The Netherlands), a Baird Atomic fluorimeter (Fluoricord, excitation wavelength 330 nm and emission wavelength 360 nm) and a recorder (Kipp, BD-40). The flow-rate of the eluent was 0.8 ml/min.

Peak heights were used for quantitation. The detection limit was calculated as three times the noise of the baseline.

Recovery and reproducibility studies

Linearity was determined with standard solutions over the range 0–100 ng/ml. Calibration curves were obtained by spiking blank pig serum samples (750 μl) with increasing amounts to give serum concentrations in the range 0–70 ng/ml, and subsequent determination.

Recovery efficiencies were calculated by comparing peak heights of the extracted samples (40 ng/ml, $n=7$) and the directly injected standard.

Calibration curves were repeatedly determined for the calculation of the within-run ($n=5$) variation and on different days for the between-run ($n=11$) variation.

The influence of declotting of blood samples was investigated by comparing extraction patterns of plasma and serum samples. Plasma was prepared by dissolving 10 mg of heparin, corresponding to 1600 I.U., in 10 ml of freshly sampled pig blood followed by centrifugation (15 min, 4053 g).

Stability and sample storage

The stability of frozen serum samples was tested by storing spiked samples at $-21\,^{\circ}C$ for periods of one, two, three and four weeks. Apart from this, the influence of freeze–thaw frequency on serum concentrations was investigated. Samples to which a known amount of carazolol had been added were thawed and frozen once, twice, three or four times, and carazolol concentrations were determined.

Decomposition as a result of light sensitivity was investigated by analysis of a freshly prepared standard solution and comparison with a solution that had been exposed to daylight at room temperature for eight days.

In vivo studies

Carazolol, Suacron, in a dose of 0.025 mg/kg, was given intravenously to cannulated (external jugular vein), castrated male pigs ($n=5$, mean weight 100 kg) via the inserted cannula. Blood samples (5 ml) were taken at regular times during 450 min after administration. Immediately after sampling and

centrifugation, serum was transferred to a glass tube and all samples were stored at −21 °C until assay. High plasma levels could be determined only after appropriate dilution according to the range of the calibration graph.

RESULTS

Chromatograms of blank and spiked (35 ng/ml) serum are shown in Fig. 1. Carazolol was eluted from the column with a retention time of 6.5 min. A sufficient separation of the peak from the blood constituents was realized. No difference was observed between extracts of plasma and serum.

The recovery of carazolol from spiked serum was 93.7 ± 1.5% (mean ± S.D., $n=7$) at a level of 40 ng/ml.

Standard curves showed correlation coefficients of $r=0.999$ ($n=5$) or higher with a y-intercept that was not significant. Reproducibility of the analytical method was demonstrated by the coefficients of variation for the slopes of the calibration lines. Between- and within-day variations were 2.5 and 6.8%, re-

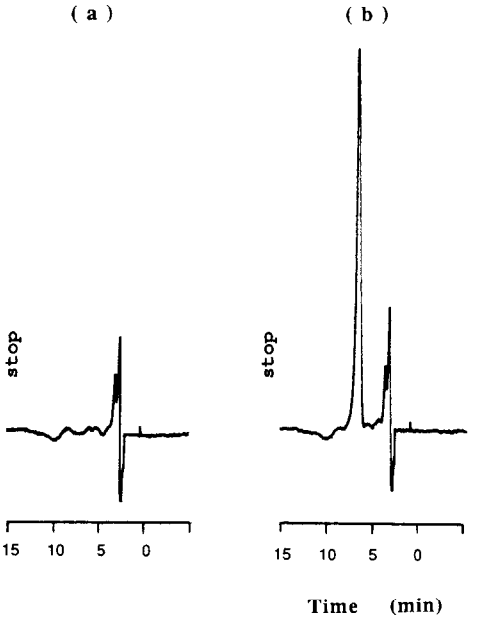

Fig. 1. Typical chromatograms of extracts from (a) blank serum and (b) serum spiked with carazolol (35 ng/ml). Retention time of carazolol: 6.5 min.

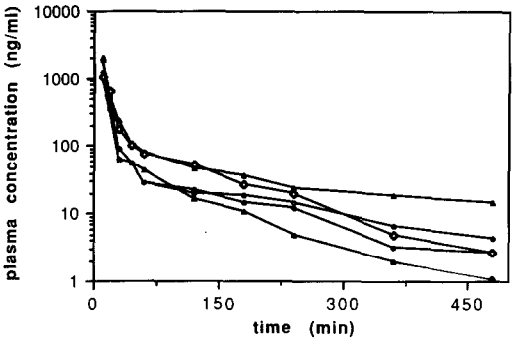

Fig. 2. Plasma concentration-time courses of carazolol in five pigs (mean weight 100 kg) following intravenous administration of 2.5 mg of carazolol (5 ml of Suacron).

spectively. The lowest detectable amount was 0.2 ng, which corresponds to a serum concentration of 0.6 ng/ml.

Repeated freezing and thawing of the samples for more than one cycle diminished carazolol concentrations, whereas freezing samples for a period of up to four weeks did not influence serum concentrations.

Analysis of the standard solution that had been exposed for eight days to daylight and room temperature revealed a decrease of ca. 40% in the concentration compared with that in the freshly prepared solution.

Plasma profiles after intravenous administration of carazolol to pigs are shown in Fig. 2. Plasma concentrations range from 2 to 2000 ng/ml, and concentrations in plasma fell very rapidly during the distribution or α-phase. β-Phase elimination half-lives, as calculated from these curves, were 137 ± 71 min, and elimination rate constants 0.006 ± 0.003 min^{-1} (mean $\pm$ S.D.). Volumes of distribution were 0.45 ± 0.16 l/kg.

CONCLUSION

The described method for determination of carazolol in serum or plasma is rapid, sensitive, reproducible and simple. Following a simple two-step extraction procedure the compound was separated and detected by HPLC with fluorescence detection. No internal standards were used. The method described allowed a very sensitive determination of carazolol: with 750 μl of plasma, concentrations as low as 0.6 ng/ml could be measured.

Carazolol samples are stable in the freezer but are sensitive to decomposition under the influence of light and repeated freezing and thawing.

The applicability of the method has been demonstrated by analysis of plasma concentrations for intravenous kinetic studies in pigs.

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