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Note

Determination of revenast hydrochloride in human plasma by gas chromatography with nitrogen-selective detection

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Revenast hydrochloride (KC-6300, I, Fig. 1) (1,5-diphenyl-2-[3-(4-(2-pyridyl)piperazin-1-yl)propyl]pyrazolin-3-one hydrochloride) is a potent new anti-allergic compound under development in the Pharmaceutical Division of Kali-Chemie. The substance is rapidly and extensively metabolized in animals resulting in very low plasma levels of the unchanged drug [1]; furthermore, the bioavailability reaches only ca. 1% in monkeys, and the protein binding is high (over 90% in humans [2]. Owing to its (thermo) lability, methods for its analysis must avoid stress to the sample: e.g. high-performance liquid chromatographic (HPLC) and/or gas chromatographic (GC) on-column techniques. The latter is preferred, because the achieved sensitivity in plasma with HPLC turned out to be unsatisfactory for the determination of concentrations in the low ng/ml range in clinical studies.

To provide information on the pharmacokinetic profile of the compound, e.g. the effects of different administration routes, a reliable analytical method had to be developed for quantitative determinations in body fluids (plasma, urine). In this report, a simple, rapid, specific and sensitive method for the quantitative determination of I in plasma is presented [3,4].

EXPERIMENTAL

Chemicals

All chemicals used were analytical grade (E. Merck, Darmstadt, F.R.G.); for the extraction. Extrelut I® columns (E. Merck) were employed. I was synthesized by Laboratoire de Therapeutic Moderne (Suresnes, France) and the internal standard II (Fig. 1) (KC-4183) at Kali-Chemie.

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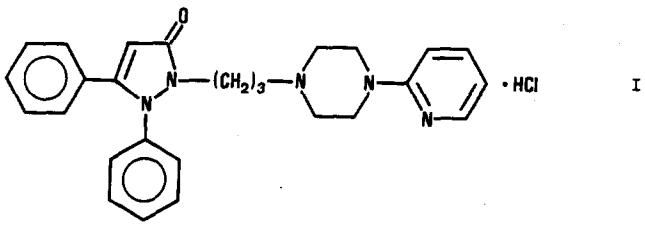
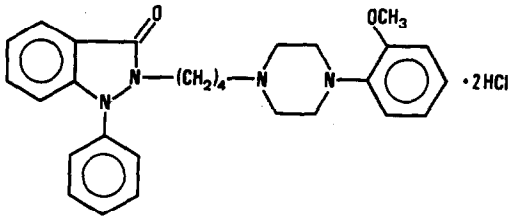


I



II

Fig. 1. Structures of revenast hydrochloride (I) and the internal standard (II).

Extraction method

To a 1-ml plasma sample, 100 μl (100 ng/ml) of an aqueous internal standard (I.S.) solution and 100 μl of a concentrated ammonia solution (25%) were added and vigorously shaken. This solution was placed on the Extrelut column and allowed to equilibrate for 10 min, followed by the elution with 6 ml of diethyl ether. The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C. The resulting residue was redissolved in 50 μl of ethanol, of which a 1-μl aliquot was injected.

TABLE I

PRECISION AND ACCURACY OF THE METHOD FOR I IN PLASMA

Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	Difference (added versus mean values found) (%)	C.V. (%)
2	1.83 ± 0.41	− 8.5	22.4
10	8.33 ± 0.82	−16.7	9.8
50	42.33 ± 0.52	−15.3	1.2
500	469.17 ± 19.5	− 6.2	4.2
1000	1014.83 ± 18.4	+ 1.5	1.8
2000	2071.83 ± 17.2	+ 3.6	0.8
Mean		8.6	6.7

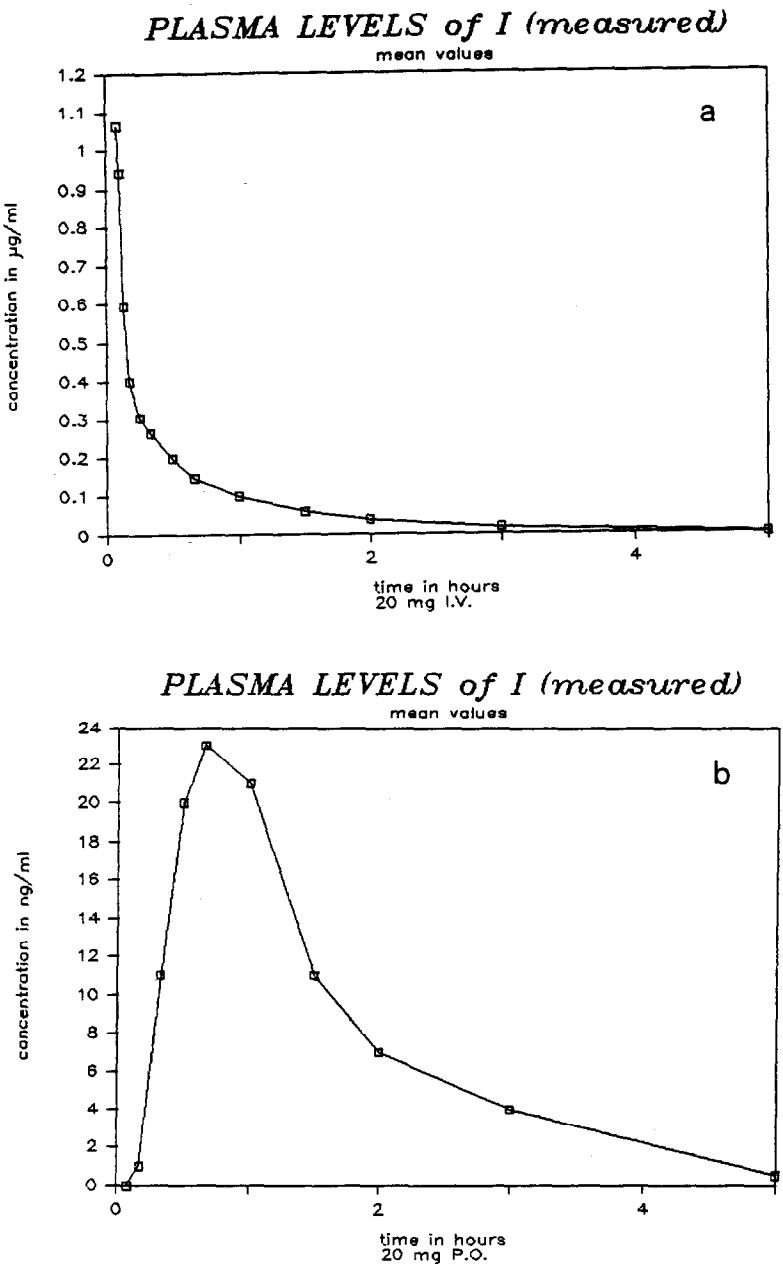


Fig. 2. Mean plasma levels after application of 20 mg of I intravenously (a) or orally (b) to healthy volunteers.

Apparatus

A Hewlett-Packard (Palo Alto, CA, U.S.A.) gas chromatograph (HP 5880 A), equipped with nitrogen-selective detector and an automatic on-column injector (HP 7673 A), was used. Chromatography was performed with a methylsilicone,

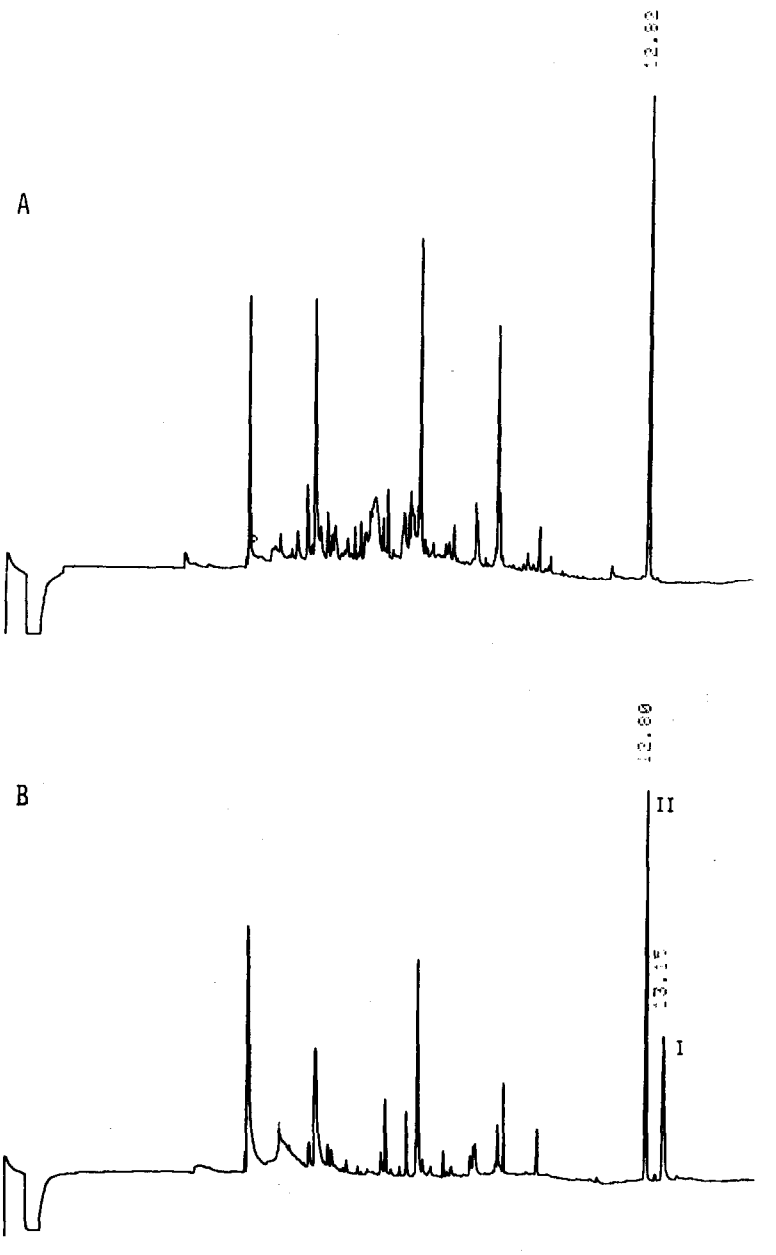


Fig. 3. Chromatograns of (A) plasma blank and (B) plasma sample 1 h after oral application of 20 mg of I to a healthy volunteer.

fused-silica, cross-linked, bonded-phase capillary column (25 m×0.31 mm I.D.) with a film thickness of 0.17 μm, also supplied by Hewlett-Packard.

The temperature of the column was programmed from 50°C (0.5 min) to 330°C (5 min) at 25°C/min, and the detector was kept at 350°C. The flow-rates for

hydrogen, air and helium (auxiliary) were 3, 80 and 30 ml/min, respectively; the carrier gas helium was at 140 kPa.

The approximate retention times for I and II were 13.2 and 12.8 min, respectively.

Preparation of standards

All standards (spike solutions, working standards, etc.) were prepared by diluting a discrete volume of the appropriate stock solution with ethanol. For the preparation of spiked plasma samples, 100 μl of the appropriate spike solution were pipetted into a 10-ml conical glass centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. Then 1 ml of plasma and 100 μl of the 25% ammonia solution were added, and the plasma was extracted as described above.

RESULTS AND DISCUSSION

Linearity and sensitivity

The linearity for the nitrogen-selective detector was checked for both substances, first with ethanolic solutions and then in plasma, as the peak-area ratio (Q) versus concentration in the ranges 0–2000 ng/ml (I) and 0–200 ng/ml (II); a linear relationship could be achieved in both cases. Because no blank value could be detected, the limit of detection (relative sensitivity) could be assessed by the coefficient of variation (C.V.) calculated for the precision: taking a C.V. value of 20% as a maximum acceptable level of imprecision, the limit of detection may be stated as 2 ng/ml; levels below this limit are clearly distinguishable from zero, but should be reported as "not detectable".

The overall inaccuracy of the assay was calculated to be 8.6% (Table I).

Precision and accuracy

The precision for I was evaluated by preparing and measuring, six times each, six samples in the concentration range 2–2000 ng/ml. The data show excellent agreement between replicate measurements; even the lowest concentration could be quantified with adequate precision, but the C.V. exceeding 20% for 2 ng/ml indicates that the detection limit may be reached. The reproducibility of the assay thus ranges between 22.4% at 2 ng/ml and 0.8% at 2000 ng/ml.

Recovery

The recovery from plasma ($n=6$) was determined for I at 10, 100 and 1000 ng/ml and for II at 50, 100 and 200 ng/ml. Calculations were based on comparison of the peak areas of extracted standards and those of blank samples which were extracted, subsequently spiked and then measured. The overall recovery for I reached 86% (82% at 10 ng/ml, 91% at 100 ng/ml and 86% at 1000 ng/ml), for II 92% (90% at 50 ng/ml, 90% at 100 ng/ml and 96% at 200 ng/ml). Therefore we calculated a recovery from plasma of ca. 90% for both compounds.

Specificity

There was zero response in all analytical samples where the drug was not present: "blank values were blank". In the chromatogram there was no peak at the retention time of the substances under consideration in the system chosen, and the identity of the component given the response at the retention time of the analyte was checked by mass spectrometry. In general we carefully watched for possible reasons for unexpected analytical results.

Stability

A batch of 40 ml of pooled drug-free human plasma was spiked with I (100 ng/ml). An aliquot was analysed for its concentration of I. It was divided in a 20-ml batch and twelve single-portions of 1.5 ml and deep-frozen at −20°C. Every week for the following three months the batch and one 1.5-ml portion were thawed and analysed, and the batch was deep-frozen again.

Our investigations show that I is stable in plasma that is kept frozen for three months. However, in plasma that has been subjected to four freeze–thaw cycles becomes "sticky", and contains solid particles when thawed. The associated chromatograms show severe deterioration.

Use in clinical studies

The described method has been used in several studies of the pharmacokinetics and bioavailability of the drug. Fig. 2 shows the mean measured concentrations of I in the plasma of twelve subjects after administration of 20 mg orally or intravenously, respectively. The chromatograms in Fig. 3A and B show that the assay is suitabale for quantitative analysis in biological fluids.

CONCLUSIONS

A GC method for the quantification of I in plasma has been developed which fulfills the requirements of an analytical method for generating data from clinical studies of the pharmacokinetics, bioavailability, etc. of revenast hydrochloride. The described assay has been used in several clinical studies.

ACKNOWLEDGEMENT

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