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Note

Determination of midazolam and the α-hydroxy metabolite by gas chromatography in small plasma volumes

M. SUNZEL

Department of Biopharmaceutics and Pharmacokinetics, Box 580, BMC, S-751 23 Uppsala (Sweden)

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Midazolam is an imidazobenzodiazepine derivative with sedative and hypnotic properties. It is metabolized in the body, and the major metabolite, α-hydroxymidazolam, is also pharmacologically active, although to a lesser extent [1]. The chemical characteristics include the basic properties of one nitrogen (pK_a 6.15), which enables the preparation of salts with good water solubility at a pH below 4. At a physiological pH of 7.5 the free midazolam base shows lipophilicity, with a partition coefficient of 475 (*n*-octanol–phosphate buffer) [2].

To perform reliable pharmacokinetic and pharmacodynamic studies in small laboratory animals, e.g. rodents, it is essential to have a sensitive and accurate method for drug analysis that requires small sample volumes. A number of analytical methods for determining midazolam and α-hydroxymidazolam have been developed. Both gas chromatographic (GC) [3,4] and liquid chromatographic (LC) [5] techniques have been employed. The previously published methods require larger plasma volumes and are more cumbersome than the method presented here.

This paper describes a rapid and sensitive GC method with electron-capture detection (ECD), which requires minute sample volumes for determination of midazolam in plasma, and in extension α-hydroxymidazolam, after derivatization to a trimethylsilyl derivative [6]. The method is applied to determine the disposition in vivo of midazolam in the rat.

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EXPERIMENTAL

Chemicals and reagents

Midazolam, midazolam maleinate, α-hydroxymidazolam, diazepam and 7-chloro-5-(2-chlorophenyl)-1,3-dihydro-1-(2-(dimethylamino)ethyl)-2*H*-1,4-benzodiazepine-2-one (internal standard 2, IS2) were kindly supplied by Hoffman LaRoche (Basle, Switzerland). Structures of midazolam and α-hydroxymidazolam are shown in Fig. 1. Bis(trimethylsilyl)acetamide (BSA) was purchased from E. Merck (Darmstadt, F.R.G.). Acetonitrile, *n*-butyl acetate and other reagents were of analytical grade.

Instruments

A Pye Unicam Series 304 gas chromatograph (Philips, Cambridge, U.K.), equipped with a ^{63}Ni electron-capture detector and a standard paper recorder, was used. The column (1.5 m × 2 mm I.D.) was packed with 3% OV-17 on Gas-Chrom Q 100–120 mesh (Chrompack, Middelburg, The Netherlands). The injection temperature was 285°C, and the detector temperature 300°C. The column temperature was kept at 260°C for the midazolam analysis and was reduced to 245°C when α-hydroxymidazolam was included in the analysis. Nitrogen was used as carrier (40 ml/min) and make-up gas at a total flow-rate of 85 ml/min.

Procedures

Stock solutions of midazolam and α-hydroxymidazolam were dissolved in 95% ethanol and further diluted in distilled water. Stock solutions of internal standards were also dissolved in 95% ethanol and further diluted in *n*-butyl acetate. As internal standards diazepam and IS2 were used. Plasma samples for standard curves were prepared by using 900 μl of pooled blank plasma spiked with 100 μl of stock solutions of the compounds.

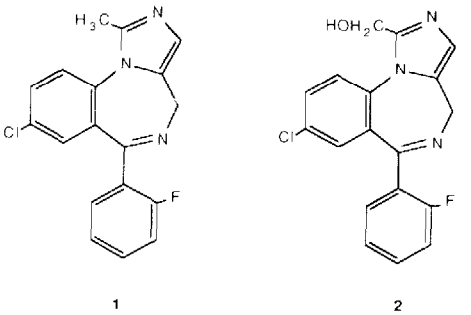

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Fig. 1. Chemical structures of midazolam (1) and α-hydroxymidazolam (2).

A 25% solution of BSA in acetonitrile was prepared on each day of the metabolite analysis.

Injectable solutions for the animal experiments were prepared by dissolving midazolam maleinate in distilled water to the corresponding free midazolam base content.

To carry out the analysis, 200 μl of plasma and 200 μl of *n*-butyl acetate containing internal standard were transferred to 5-ml conical test-tubes. The tubes were sealed with screw-caps and vortex-mixed for 3.25 min or shaken vigorously for 20 min in an Evapo-mix® (Fort Lee, NJ, U.S.A.). The test-tubes were centrifuged for 10 min (2250 *g*), and 2–4 μl of the organic phase were injected into the chromatograph.

When α-hydroxymidazolam was included in the analysis, the procedure was extended. A 100-μl volume of the organic phase was transferred into a new tube and evaporated under a stream of nitrogen. Then 50 μl of BSA–acetonitrile solution were added, the tubes sealed, and the contents thoroughly mixed. The samples were left for 20 min at room temperature for derivatization, and 2–4 μl were injected. The GC column was conditioned daily with several injections of *n*-butyl acetate extract from untreated plasma before analysis of test or standard samples.

Animal experiments

Six male Sprague-Dawley rats (Alab, Sollentuna, Sweden) weighing 230–250 g, were catheterized in the jugular vein and carotid artery under ether anaesthesia the day before the experiment. On the day of the experiment the rats were given a 5 mg/kg midazolam intravenous bolus dose. Plasma samples (0.4 ml) were taken at 2, 5, 10, 15, 60, 75, 90 and 120 min after drug administration. After centrifugation the plasma samples were immediately frozen and stored (−20°C) until analysed. A weighted least-squares non-linear regression program (PCNONLIN) [7] was used to fit a bi-exponential equation to the plasma concentration–time data. The distribution ($t_{1/2\,\lambda 1}$) and the elimination ($t_{1/2\,\lambda 2}$) half-lives were calculated from the exponents of the fitted function.

RESULTS AND DISCUSSION

The retention times for diazepam, midazolam and IS2 were 2.4, 3.8 and 6.8 min, respectively. Owing to the lower column temperature when α-hydroxymidazolam was included in the analysis, the retention times were prolonged to 4.4, 7.0, 8.7 min for diazepam, midazolam and α-hydroxymidazolam, respectively. Typical chromatograms for both procedures and a blank plasma sample are shown in Fig. 2a–c. Samples and standards were quantified by the measurement of peak heights.

Originally IS2 was used as internal standard to enable the analyses of both

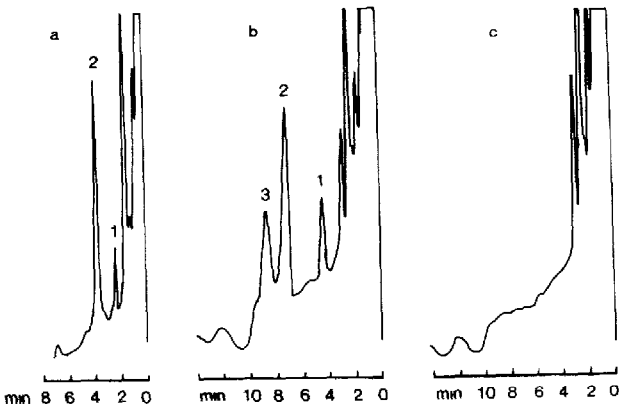

Fig. 2. (a) Chromatogram of the internal standard diazepam (1) (45 ng/ml) and midazolam (2) (300 ng/ml) in a spiked plasma sample (b) Chromatogram of the internal standard diazepam (1) (65 ng/ml), midazolam (2) (300 ng/ml) and the derivatized α-hydroxymidazolam (3) (100 ng/ml) in a spiked plasma sample. (c) Chromatogram of blank plasma prepared according to the metabolite analysis procedure.

diazepam and midazolam according to the procedure [8]. For the midazolam analysis diazepam was preferred to IS2, owing to the shorter retention time, longer storage stability and inertness to the metabolite derivatization procedure when the metabolite was included in the analysis.

The precision for the midazolam analysis was determined to 3.3% at 300 ng/ml ($n=8$) and 4.9% at 25 ng/ml ($n=9$). When the metabolite was included, the precision was 5.5% ($n=6$) and 4.4% ($n=10$) at the same plasma concentrations of midazolam and 3.3% at 100 ng/ml ($n=6$) and 6.1% at 10 ng/ml ($n=10$) for α-hydroxymidazolam. The accuracy was determined by five repeated injections of the same extracted plasma sample and was found to be 3.6% (300 ng/ml) and 4.8% (25 ng/ml) for midazolam and 3.6% (100 ng/ml) and 4.2% (10 ng/ml) for α-hydroxymidazolam. The standard curves were linear in the concentration range 0–500 ng/ml for both compounds, and for higher plasma concentrations of midazolam an additional standard curve showing linearity at 500–3000 ng/ml was prepared. The detection limit was 5 ng/ml for both midazolam and the metabolite, with a coefficient of variation of 7.3 and 7.8%, respectively. The absolute recovery of midazolam was 81%, and 86% for α-hydroxymidazolam.

In order to obtain good precision in the analysis of the small plasma samples, the narrowness of the conical test-tubes, as well as the keeping of the mixing time, proved to be of importance.

When the metabolite was included in the analysis both retention times and sample preparation times were prolonged. It was possible to evaporate the samples with a stream of nitrogen after derivatization. The sealed test-tubes could

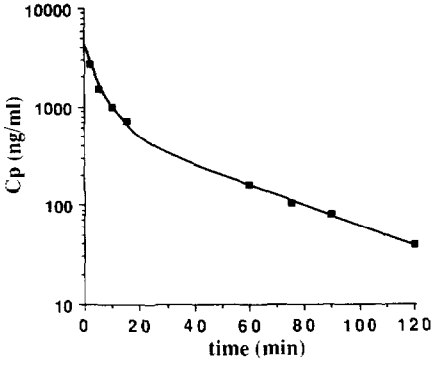

Fig. 3. Plasma concentration–time curve in one rat after a 5 mg/kg intravenous dose of midazolam. The solid squares denote the observed plasma concentrations and the line is the computer fit to the data.

be stored refrigerated overnight, and their contents redissolved and analysed the following day.

The plasma concentration–time curve after an intravenous bolus dose of 5.0 mg/kg midazolam to one rat is shown in Fig. 3. The $t_{1/2\,\lambda 1}$ was determined to be 2.3 ± 0.5 min (mean $\pm$ S.D.) and the $t_{1/2\,\lambda 2}$ to be 24.1 ± 4.2 min (mean $\pm$ S.D.) for the six rats. In three rats α-hydroxymidazolam was not detectable in plasma after the bolus dose. In the remaining three rats the maximum concentration of the metabolite appeared after 75 min and did not exceed 141 ng/ml (range 70–141 ng/ml).

In man, it has been shown that when midazolam is administered intravenously, the metabolite is formed in negligible amounts. However, owing to first-pass metabolism, the metabolite is formed in amounts sufficient to contribute to the overall drug effect when midazolam is administered orally [9]. To investigate if the rat has a similar pattern of metabolism, further studies are required.

CONCLUSION

The described analysis method for midazolam and its metabolite α-hydroxymidazolam requires small sample volumes and is rapid and sensitive with good precision and accuracy. This method makes it possible to perform pharmacokinetic studies in small laboratory animals, thus increasing the number of possible blood samples drawn from one animal and thereby reducing both the cost of and the number of animals used in such studies.

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