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Note

Simultaneous determination of haloperidol and reduced haloperidol by gas chromatography using a megabore column with electron-capture detection: application to microsomal oxidation of reduced haloperidol

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A new method has been developed for the simultaneous determination of haloperidol (HAL) and reduced haloperidol (RHAL) utilizing a gas chromatographic (GC) megabore-column procedure. Analytical methods have been described for HAL and RHAL by high-performance liquid chromatography (HPLC) [1–5], radioimmunoassay [6–8], gas chromatography–mass spectrometry [9] and GC [10–12]. While many HPLC assays exist which are sensitive and reliable for the simultaneous determination of HAL and RHAL this is not so for the GC methods. The method is compared with a traditional packed-column system with derivatized and underivatized samples. Detection was obtained of as little as 1 or 5 pmol per injection for HAL and RHAL, respectively.

HAL is a potent neuroleptic and RHAL is a metabolite of HAL that can be converted back to HAL in vivo in humans [9,13] and in vivo and in vitro in the rat [14]. In addition to a new method for the detection of RHAL and HAL, we have described the first evidence of the in vitro conversion of RHAL to HAL catalyzed by human liver microsomes.

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EXPERIMENTAL

Reagents and materials

HAL and RHAL were purchased from Janssen Pharmaceutica (Olen, Belgium). Magnesium chloride, D-glucose 6-phosphate (G6P), β-nicotinamide-adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate dehydrogenase, type XI (G6PD), crystalline bovine serum albumin-fraction V (BSA) and potassium chloride were bought from Sigma (St. Louis, MO, U.S.A.). Flurazepam was obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Reagent- or HPLC-grade methanol and hexane came from Caledon (Georgetown, Canada), isopropyl alcohol from Mallinckrodt (Paris, KY, U.S.A.) and toluene from Fisher Scientific (Fairlawn, NJ, U.S.A.). Triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and trifluoroacetic anhydride (TFAA) was from Pierce (Rockford, IL, U.S.A.).

Gas chromatography

Chromatography was performed with a Shimadzu gas chromatograph (GC-9A) fitted with a ^{63}Ni electron-capture detector. One of the columns used was a glass column (1.7 m×3 mm I.D.) packed with 3% OV-17 on Chromosorb W [10]; injector and detector temperatures were 300°C, the column temperature was 270°C (for underivatized samples) or 265°C (for derivatized samples) and the nitrogen flow-rate was 40 ml/min. The other column used was a fused-silica megabore column (15 m×0.53 mm I.D.) with a 1.0-μm coating of DB17 from J&W Scientific (Folsom, CA, U.S.A.); the injector temperature was 280°C, the column temperature 237°C and the nitrogen flow-rate 20 ml/min.

Sample preparation

HAL and RHAL were injected underivatized onto the packed column (1–4 μl) and megabore column (0.5–2 μl) in methanol, and derivatized onto the packed column (1–4 μl) in toluene. Derivatization, based on previous acylation methods [15–17], was as follows: HAL, RHAL and flurazepam were mixed in 500 μl of toluene, 100 μl of 0.05 *M* TEA (catalyst in excess) and 10 μl of TFAA were added, and the mixture was heated at 60°C for 30 min. After cooling 1 ml water was added and the solution was shaken for 1 min. A 1-ml volume of 5% aqueous ammonia was then added to the mixture and shaken for a further 1 min. After vigorous vortexing the mixture was centrifuged and the upper toluene layer was injected onto the column.

Microsomal oxidation studies

The oxidative conversion of RHAL to HAL in vitro was studied by incubation of RHAL with human liver microsomes in the presence of an NADPH-generating system. Washed liver microsomes (WM) were prepared according to a previous method [18]. The incubation mixtures (final volume 0.5 ml)

contained phosphate buffer (0.2 *M*, pH 7.4), 2 μmol G6P, 0.2 μmol $NADP^+$, 0.2 U G6PD, 1 μmol magnesium chloride, varying amounts of RHAL in 10 μl methanol and 200 μl (0–5 mg protein per 0.5 ml incubate) WM. The mixture was shaken gently for 30 min at 37°C in air. The reaction was terminated by removal onto ice and the addition of 50 μl cold 5 *M* sodium hydroxide. Flurazepam in water (internal standard) and 5 ml of hexane–ispropyl alcohol (95:5) were added and the mixture was vortex-mixed. The organic layer was then removed and dried under air. At the time of chromatography the sample was resuspended in 300 μl of methanol before injection. A five-fold reduction of this assay (0.1 ml final volume) is possible as only 0.5–2.0 μl of resuspended methanol (60 versus 300 μl) is required.

RESULTS

Chromatographic data

The detection limits on column (signal-to-noise ratio >3) for system 1 (packed column, underivatized sample) were 40 pmol HAL and 200 pmol RHAL, for system 2 (packed column, derivatized sample) 8 pmol HAL and 40 pmol RHAL and for system 3 (megabore column, underivatized sample) 1 pmol HAL and 5 pmol RHAL. The relative chromatographic electron-capture detection (ECD) responses for equal amounts of HAL and RHAL (HAL/RHAL) was for system 1 7.8, for system 2 4.8 and for system 3 3.0. Separation of HAL and RHAL was complete for the derivatized samples with retention times of 2.27 and 4.32 min for HAL and RHAL, respectively, while underivatized samples on the same packed-column system were incompletely separated with retention times of 11.6 and 13.5 min. Representative chromatograms of the separation of HAL, RHAL and flurazepam on systems 1 and 3 are demonstrated in Fig. 1A and B.

TFAA derivatization was sometimes incomplete as indicated by HAL or RHAL peaks at the retention times consistent with the underivatized compound.

Megabore-column chromatography–ECD

Calibration graphs were prepared by comparing the peak height of varying amounts of RHAL or HAL and a constant amount of flurazepam. We checked the linearity of the megabore-column–ECD system over a wide concentration range (5.0–500 μ*M*). Linear regression analysis on the calibration graphs obtained for RHAL and HAL indicated that the correlation coefficients were 0.9996 and 0.9987, respectively, and the intercepts were near the origin (0.06 and 0.10, respectively). The equations of the lines were peak-height ratio = 0.073RHAL + 0.06 and peak-height ratio = 0.088HAL + 0.10, where RHAL and HAL were measured in μ*M*.

Coefficients of variation for HAL and RHAL (100 μ*M*) determinations

($n=6$) were 5.24 and 4.66%, respectively. Chromatographic interferences from a variety of compounds were tested. The retention times of these compounds are: 2-amino-5-chlorobenzophenone, 0.78 min; oxazepam, 1.57 min; medazepam, 1.29 min; diazepam, 2.42 min; trifluroperidol, 3.00 min; nordiazepam, 3.32 min; temazepam, major peak, 4.02 min, minor peak 5.63 min; aldrin, 10.00 min; pipamerone 11.89 min.

Applications: assay conditions

The recovery was determined by comparing the GC response of RHAL or HAL (1) extracted from the assay mixture and (2) spiked into methanol, with that of flurazepam (not extracted). The recoveries were between 96 and 109% for samples with and without tissue with high (250 μM) or low (10 μM) concentrations of HAL and RHAL. To compensate for any variation in the extraction the internal standard was added to the samples prior to extraction. The extracted and dried samples were stable at room temperature for at least five days and in methanol for at least 12 h.

Fig. 1 shows chromatograms of microsomal incubation extracts from a sample with (Fig. 1D) and without (Fig. 1C) substrate (RHAL). The retention times of flurazepam, HAL and RHAL are 4.96, 8.32 and 10.07 min, respec-

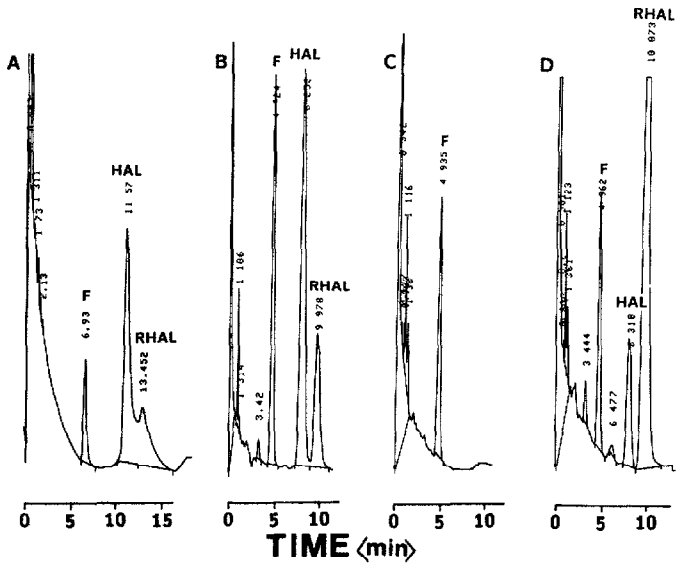

Fig. 1. (A and B) Chromatograms obtained from two of the systems tested indicating separation of equal amounts of HAL and RHAL. (A) Underivatized HAL and RHAL, 400 pmol, packed column (attenuation 4); (B) underivatized HAL and RHAL, 234 pmol, megabore column (attenuation 5). (C and D) Chromatograms obtained from the extracts of 0.5-ml incubations of (C) RHAL, 0 μM, plus WM plus NADPH Generating System (GS) and (D) RHAL, 200 μM plus GS plus WM. F = Flurazepam, internal standard (12 ng).

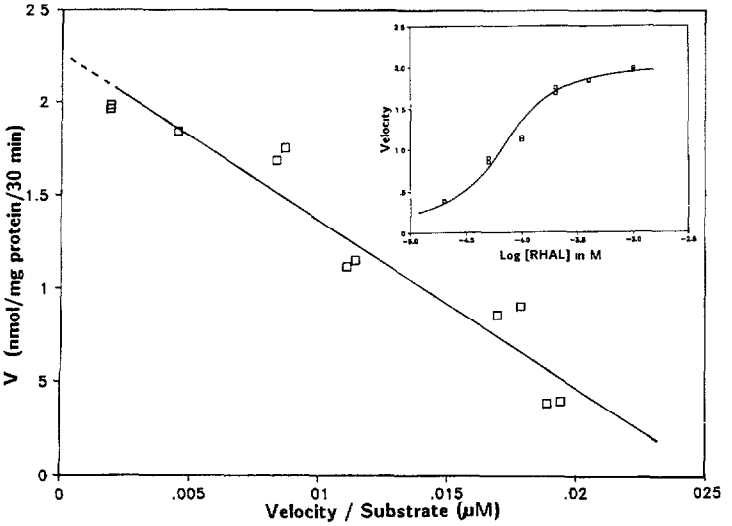

Fig. 2. Eadie-Hofstee plot of RHAL kinetics in human liver microsomes (K21). RHAL concentration varied from 20 to 1000 μM. Velocity is expressed in nmol HAL per mg protein per 30 min (30 min incubation time). Inset, relationship between velocity and the log of substrate.

tively. No interfering endogenous compounds are apparent in the incubates (Fig. 1C). The relative retention times (HAL/flurazepam and RHAL/flurazepam) were the same from a standard solution (1.68 and 2.03) and from an extracted incubated microsomal sample (1.68 and 2.03). The GC responses of HAL to RHAL (HAL-to-RHAL peak-height ratios) are the same for a standard solution (3.07) as for an extracted sample (including microsomes without incubation, 3.09). Identical samples were prepared and analyzed on the same day in order to evaluate the within-day variation. The coefficient of variation for the within-day variation ($n=6$) was 4.62 and 5.82% for HAL and RHAL (100 μM), respectively.

The proposed method was applied in a RHAL metabolism study. The metabolism of RHAL to HAL was followed using human liver microsomes (liver designated K21 [18]) as the enzyme source. The Eadie–Hofstee plot, which plots velocity/substrate versus velocity for the production of HAL from RHAL, can be seen in Fig. 2. The inset shows a plot of velocity versus log substrate concentration. The K_M and V_{max} values in these liver microsomes were 88 μM and 2.25 nmol/mg of protein per 30 min, respectively, and in another liver (K19) the values were 179 μM and 5.69 nmol/mg of protein per 30 min, respectively.

The in vitro formation of HAL from RHAL by human liver microsomes was linear with respect to time for 45 min at varying substrate concentrations and linear with respect to microsomal protein content until at least 10 mg/ml. The microsomal enzyme preparation was stable at $-80\,^{\circ}C$ for at least 40 days. Con-

version of RHAL to HAL was dependent on the enzyme source and NADPH being included in the incubation.

DISCUSSION

From our initial findings we discovered that the megabore-column system was superior to a packed-column system in conjunction with either a derivatized or an underivatized sample. When considering the detection limits the megabore column is more sensitive than either of the packed column systems. In addition the ratio of peak heights of HAL to RHAL (3.0 versus either 7.8 underivatized or 4.8 derivatized) favours the megabore-column system. The additional advantages of an underivatized system are obvious if time for sample preparation is also considered. Occasionally incomplete derivatization was observed and over time degradation products of the TFAA derivatives appear.

The megabore-column system provides a rapid (less than 13 min), sensitive (low picomole range) and reliable system for the simultaneous analysis of HAL and RHAL. The extraction procedure described here led to good, reproducible recoveries of both HAL and RHAL. Chromatographic behaviours of assayed, extracted samples and pure compounds were virtually identical for RHAL and HAL. Peak-height ratios of both HAL and RHAL to internal standard showed a linear relationship with an incubate concentration over a range of at least 5–500 μM (highest concentration tested) and were consistently linear.

It has been observed in vivo in humans [9,13] and in rats [14] that RHAL is converted to HAL. The method presently described was employed to measure the conversion of RHAL to HAL using human liver microsomes as the enzyme source. It can be concluded from these findings that the human liver microsomes are able to catalyze NADPH-dependent conversion of RHAL to HAL in vitro.

The GC analytical method described here, utilizing the megabore-column system versus the conventional packed-column system, provides a rapid, sensitive and reliable method for the simultaneous analysis of HAL and RHAL. This method has a number of practical applications, one of which has been described.

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