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Note

Combined high-performance liquid chromatography and enzyme immunoassay for active metabolites of a sleep inducer (rilmazafone), a ring-opened derivative of benzodiazepines, in human plasma

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Determination of drug metabolites in body fluids is important for pharmacology and toxicology, but it is not easy to develop sensitive and specific assays [1–3]. Most of the metabolites in blood can be separated successfully by high-performance liquid chromatography (HPLC), but sometimes the sensitivity of online UV detection or fluorescence detection is inadequate and/or there is a lot of interference from other blood substances at low concentration [4, 5]. Conventional immunoassay techniques are very sensitive, but it is difficult to determine these metabolites separately because antibodies raised to one may cross-react with others [3, 6, 7]. In this study, we combined the high resolving power of HPLC with the high sensitivity of enzyme immunoassay (EIA) for simultaneous determination of benzodiazepine metabolites in human plasma. This combination has previously been used for individual compounds rather than mixtures [8–15].

The parent drug, a sleep inducer, is a new ring-opened derivative of benzodiazepines, 5-[(2-aminoacetamido)methyl]-1-[4-chloro-2-(*o*-chlorobenzoyl)phenyl]-N,N-dimethyl-1*H*-1,2,4-triazole-3-carboxamide (rilmazafone, I in Fig. 1) hydrochloride dihydrate. It is inactive, but is rapidly metabolized to active forms. Use of ^{14}C-labelling has shown that several metabolites are formed in vivo [16], and a radioreceptor assay has shown that five of them (II–VI in Fig. 1) are active [17].

We used HPLC to separate II–V extracted from human plasma, and detected them all simultaneously using one EIA system. Metabolite VI was left in the

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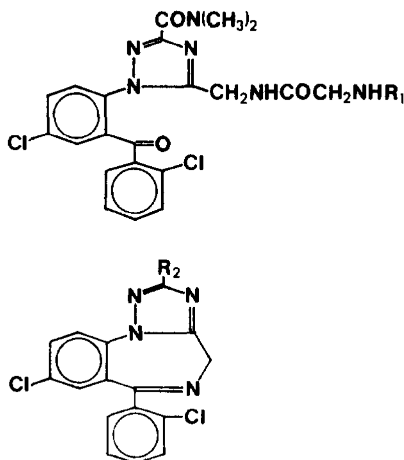

Fig. 1. Structures of the parent drug rilmazafone [I, $R_1=H$ (upper structure)], its active metabolites [II, $R_2=CON(CH_3)_2$; III, $R_2=CONHCH_3$; IV, $R_2=CONHCH_2OH$; V, $R_2=CONH_2$; VI, $R_2=COOH$ (lower structure)] and related compounds [VII, $R_1=COCH_2CH_2COOH$ (upper structure); VIII, $R_2=CONHCH_2CH_2NH_2$ (lower structure)].

plasma phase and assayed by the EIA method reported previously [18]. We used EIA rather than radioimmunoassay (RIA) because of high sensitivity, non-radioisotopic procedures and excellent stability of enzyme-labelled compounds. This is the first report concerned with combined HPLC–EIA.

EXPERIMENTAL

Authentic samples of the parent drug and the metabolites (I–VI) were gifts from Dr. S. Takahashi of our institute [16, 17, 19]. All chemicals were of analytical grade, unless specified.

A 500 μl sample of human plasma or serum was extracted with 3.0 ml of dichloromethane by shaking for 10 min. A 2.5-ml sample of the organic phase was evaporated to dryness at 20–25°C. The residue was dissolved in 300 μl of 50% (v/v) acetonitrile. A 250-μl sample of the solution was then injected into the HPLC instrument, which was a Shimadzu HPLC system, Model LC-4A, fitted with a Rheodyne syringe-loading sample injector with a 500-μl loop.

Metabolites II–V in plasma extracts were fractionated using a Nucleosil C_{18} (particle size 5 μm, Machery-Nagel, Düren, F.R.G.) column (200 mm×4.6 mm I.D.) eluted under isocratic conditions with 35% (v/v) acetonitrile (HPLC grade, Merck, Darmstadt, F.R.G.) in water for 13 min followed by gradient elution. The concentration of acetonitrile was increased exponentially from 13 to 25 min. A value of the v/v percentage of acetonitrile at t min was $K=K_2-(K_2-K_1)(e^{-a(1-(t-13)/T)}-1)/(e^{-a}-1)$ where $K_1=35\%$, $K_2=100\%$, $a=-2$ and $t=25$–13 min. The flow-rate was 1 ml/min and the column temperature was not controlled (room temperature 20–25°C). Fractions of the eluate were collected every 30 s into 75 mm×12 mm I.D. glass tubes, directly evaporated

to dryness under vacuum at 50 °C and redissolved in 500 μl of an EIA buffer: 0.01 *M* phosphate buffer (pH 7.3) containing 0.15 *M* sodium chloride, 1m*M* magnesium chloride, 0.05% (w/v) sodium azide and 1% (w/v) bovine serum albumin (BSA) (recrystallized and lyophilized; Sigma, St. Louis, MO, U.S.A.).

A labelled antigen, β-D-galactosidase-labelled VII, was prepared by the mixed anhydride method [18, 20]. For VII, a mixture of I (320 mg) and succinic anhydride (70 mg in 5 ml of acetonitrile) was stirred overnight at 20–25 °C. The solvent was evaporated and the residue was recrystallized from ethyl acetate–diethyl ether giving 250 mg (64.6%) of VII (m.p. = 113–115°C). Calculated for $C_{25}H_{24}Cl_2N_6O_2 \cdot \frac{1}{2} H_2O$: C = 51.38; H = 4.31; N = 14.38. Found: C = 51.63; H = 4.30; N = 14.35. NMR (C^2HCl_3) δ (ppm): 2.26–2.87 (m, 4H, $-CH_2CH_2-$); 3.13 (s, 6H, $N(CH_3)_2$); 3.93 (d, 2H, $J = 6$ Hz, $COCH_2NH$); 4.47 (d, 2H, $J = 6$ Hz, $Tr-CH_2-NH$); 7.2–7.9 (m, 10H, aromatic H, NH and OH).

Rabbit antiserum (K-10) was against the conjugate of VIII with BSA by the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide method [18, 21]. VIII was synthesized from an ethyl ester of VI [19] (8.7 g in 435 ml of dichloromethane) by adding dropwise ethylenediamine (87 ml) in ethanol (174 ml) at 45 °C for 3 h. The mixture was allowed to stand overnight, concentrated and extracted with dichloromethane. The solvent was evaporated and the residue was taken up in ethanol and treated with oxalic acid to precipitate 9.3 g (83.4%) of VIII, which was recrystallized from diluted ethanol (m.p. = 190 °C). Calculated for $C_{19}H_{16}N_6Cl_2O \cdot C_2H_2O_4 \cdot \frac{1}{2}H_2O$: C = 49.04; H = 3.72; N = 16.34; Cl = 13.79. Found: C = 48.54; H = 4.01; N = 16.62; Cl = 14.18. NMR (DMSO-d_6) δ (ppm): 2.80–3.13 (m, 2H, CH_2NH_2); 3.37–3.70 (m, 2H, $CH_2CH_2NH_2$); 4.95 (s, 2H, $=N-CH_2-$), 7.16–8.17 (m, 7H, aromatic H), 8.96 (b.t, 1H, $J = 7$ Hz, NH). IR (Nujol) cm^{-1}: 1680, 1628.

The development of the EIA technique has been reported in detail previously [18]. In summary, 100 μl of the labelled antigen solution (ca. $1 \cdot 10^{-5}$ μU per 100 μl) were pipetted into each assay tube, which contained 500 μl of samples from HPLC or various concentrations of standard solutions of V (6.3–200 pg/500 μl). Next, 100 μl of the diluted antiserum (1:20 000) were added to each tube, and the mixture was incubated for 16 h at 20–25 °C. Separation into bound and free fraction was performed by 100 μl of the immobilized anti-rabbit second antibody [1 mg/ml suspension of Immunobead (Bio-Rad Labs., Richmond, CA, U.S.A.)], and then the enzyme activity of the bound fraction in each tube was measured using a fluorescent substrate [18, 20].

RESULTS AND DISCUSSION

A reversed-phase system using the ODS column was found to be satisfactory for separation of the metabolites under study. The mobile phase for the combined HPLC–EIA method was chosen to exclude non-volatile components, such as buffer salts or ion-pairing reagents, that might interfere with EIA. It is relatively simple to select appropriate HPLC conditions for the combined HPLC–EIA method because most of EIA methods do not detect any other substances in plasma extracts except cross-reactive compounds. The separation conditions were inves-

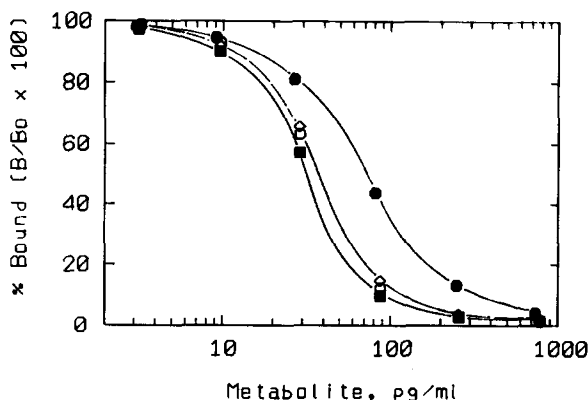

Fig. 2. Standard displacement curves of the EIA. ●, II (55.7%); ■, III (118%); ○, IV (105%); ◇, V (100%). % Cross-reactivities regarding V as 100 on a weight basis are shown in parentheses.

tigated using authentic samples of II–V, which were detected at 254 nm. Under isocratic conditions of 35% (v/v) acetonitrile, the retention time (t_R) of II was long and the peak width was broad. Therefore, after investigation of gradient elution, the best separation was obtained under the conditions as described in Experimental. In addition, t_R of I was longer than those of the four metabolites.

An EIA for the combined HPLC–EIA method needs to be sensitive and broadly specific to all the metabolites. Fig. 2 shows the standard displacement curves of II, III, IV and V on the EIA. When EIA was performed for these metabolites after HPLC separation, a standard displacement curve for V was incorporated each time, and the measured values for II, III and IV were corrected for their cross-reactivities. These standard curves did not vary during the course of one year since the enzyme-labelled antigen was very stable.

The exact techniques involved in combined HPLC–EIA are very important to the assay performance. Initial attempts to collect the whole peaks of each metabolite on the basis of their expected retention times gave low reproducibility. It is likely that the best moment to change fractions between peaks varies according to the peak profile. We collected the eluate directly in the EIA assay tubes at intervals of 0.5 min, which excluded pipetting errors on transferring and eliminated any inaccuracy in the fraction collector.

When normal human plasma was measured by means of this assay procedure, no peaks were detected by EIA. Plasma samples from volunteers who had been administered the parent drug, I, were analysed and the HPLC–EIA profile is shown in Fig. 3. We used a microcomputer to calculate the plasma concentration of each metabolite by (1) calculating the amount of immunoreactivity in each tube using the EIA standard displacement curve of V from the fluorescent intensity value by log–logit second-order regression, (2) summing the immunoreactivities within each peak and (3) correcting the sum for sampling factors, cross-reactivity and recovery factor (described below).

The plasma samples that were prepared by the addition of known amounts of authentic metabolites were measured by combined HPLC–EIA, and the recoveries were assessed. The relationships between added (x ng/ml) and measured

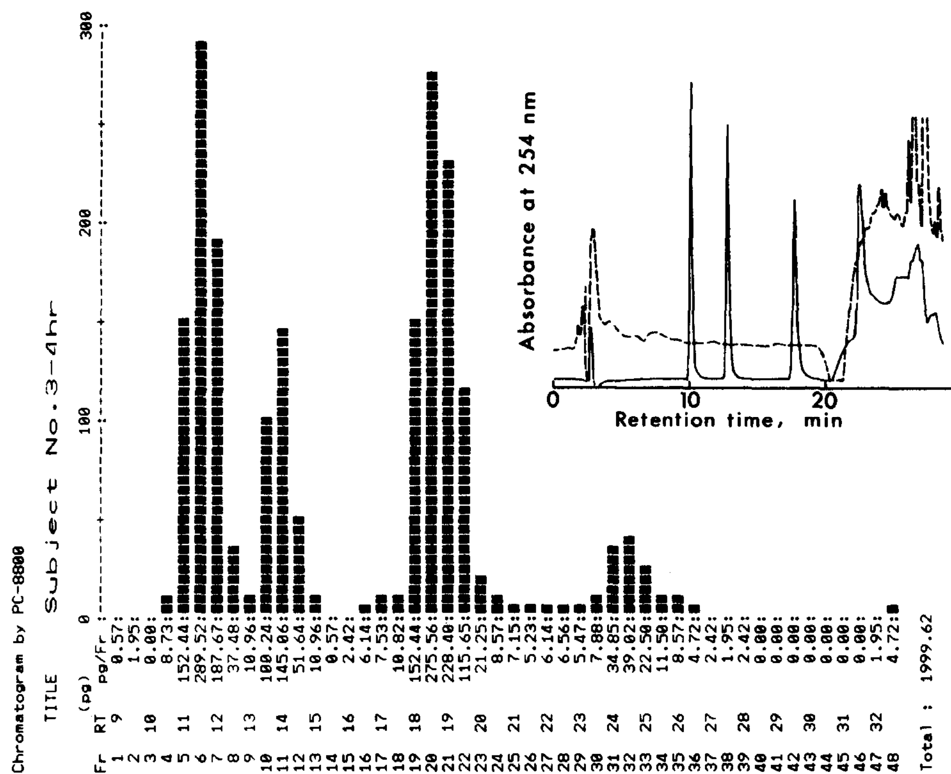

Fig. 3. Combined HPLC-EIA of a human plasma sample 4 h after oral administration of 2 mg of the parent drug. The UV traces (top right) were obtained from this sample (dashed line) and from the authentic samples of metabolites II, III, IV and V (solid line). The retention times determined for the latter were 22.3, 17.4, 10.7 and 12.9 min, respectively; the amounts injected were 58 ng of II and 62 ng each of III, IV and V. The retention times determined by EIA were ca. 1 min longer, owing to the dead volume of the connection tubing.

(y ng/ml) metabolite concentrations were linear: for II, $y=0.96x+0.009$, coefficient of variation (C.V.) = 13.5%, $n=23$, for III, $y=0.97x+0.009$, C.V. = 15.1%, $n=22$; for IV, $y=0.87x+0.007$, C.V. = 16.8%, $n=21$; for V, $y=0.96x+0.010$, C.V. = 15.6%, $n=23$. The recoveries calculated from the slope of each regression line were excellent for II, III and V, and the differences between the added and measured values were not significant according to regression analyses. Correction for incomplete recovery was necessary only for IV. The limits of determination for II, III, IV and V were estimated to ca. 0.24, 0.12, 0.12 and 0.12 ng/ml of plasma, respectively, from their precision profiles. The low sensitivity of II was due to its low cross-reactivity on the EIA. Below these concentrations the precision became worse, more than 20% of the C.V. At a concentration of ten-fold lower than each limit of determination, the metabolite was not detectable in any HPLC fraction by the EIA (detection limit).

The precisions of inter-assay variation were estimated as shown in Table I. Surprisingly, most of the variations, C.V. < 15%, were as small as those of commercial RIA kits. This was due to the adoption of a suitable EIA system with an

TABLE I

INTER-ASSAY PRECISIONS OF THE COMBINED HPLC-EIA METHOD

Metabolite	Level	*n*	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
I	Low	6	0.30 ± 0.05	18.2
	Medium	6	0.86 ± 0.06	6.8
	High	6	1.60 ± 0.14	8.7
III	Low	6	0.25 ± 0.02	9.1
	Medium	6	0.99 ± 0.12	11.9
	High	5	2.35 ± 0.34	14.4
IV	Low	5	0.22 ± 0.01	5.7
	Medium	5	0.90 ± 0.05	5.0
	High	5	2.17 ± 0.37	17.0
V	Low	6	0.24 ± 0.02	8.1
	Medium	6	1.01 ± 0.10	10.3
	High	5	2.41 ± 0.30	12.6

assay range appropriate for these samples and due to the use of an assay procedure that omitted manual pipetting after injection into the chromatograph.

We have determined the concentrations of these metabolites in ca. 500 plasma samples. The assay sensitivity and specificity did not change even in mass routine assays for a year, which proved the reliability of this method. This method compares favourably with previously reported assay methods for benzodiazepines and their metabolites [2]. If necessary, the sensitivity of the EIA could have been increased by a different combination of antiserum and labelled antigen [18].

Some researchers have tried to determine the concentrations of these metabolites in body fluids by using radiolabelled compounds or by gas chromatography–mass spectrometry (GC–MS) [16]. However, the former method is not suitable for making measurement in human body fluids, and the pyrolysis of some metabolites occurred on GC. The combined HPLC–EIA method overcomes these defects and has very wide applicability. The method does have some limitations, however. Because the determination range of EIA is narrower than those of other conventional HPLC detection methods, simultaneous assay is not generally applicable to plasma samples containing some metabolites in very high concentrations and others in low concentrations. In addition, it is difficult to search for all unknown metabolites using this method without prior information about separation behaviour on HPLC and cross-reactivity on EIA. We think that these limitations are not serious, and that the combined HPLC–EIA method is very useful for pharmacological analysis because of its excellent specificity, high sensitivity, good reproducibility and wide applicability.

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