DETERMINATION OF ISF-2405, AN ACTIVE METABOLITE OF CADRALAZINE, IN PLASMA AND TISSUES BY RADIOIMMUNOASSAY

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A radioimmunoassay has been developed which makes possible sensitive and specific determination of an active metabolite of cadralazine, (±)-6-ethyl(2-hydroxypropyl)amino]-3-hydrazinopyridazine (ISF-2405), in plasma and tissues. On account of its lability in a sample solution, ISF-2405 in biological samples was selectively derivatized to a stable form, (±)-6-ethyl(2-hydroxypropyl)amino]-3-(3,5-dimethyl-1-pyrazolyl)pyridazine (ISF-3349), by treatment with acetylacetone. The concentration of ISF-2405 was determined by the assay of the resultant ISF-3349. Antiserum against ISF-3349 was elicited in guinea pigs immunized with the ISF-3349 derivative coupled with bovine serum albumin. The obtained antiserum was highly specific for ISF-3349, and did not cross-react with either cadralazine or its metabolites. [Pyrazole-4-3H]labeled ISF-3349 with a specific activity of 13.9 Ci/mmol was used as the radioligand. Assays of ISF-2405 in plasma and tissues were possible over the concentration range from 0.4 to 6.4 ng/ml with 1 ml of plasma, and from 2 to 64 ng/g with 100 mg of tissue. Plasma and blood vessel levels of ISF-2405 in rats after single oral administration of cadralazine have also been determined by the present method.

Keywords — cadralazine; cadralazine metabolite; active metabolite; (±)-6-ethyl(2-hydroxypropyl)amino]-3-hydrazinopyridazine (ISF-2405); derivatization; radioimmunoassay; plasma level; tissue level; blood vessel level

INTRODUCTION

Cadraladine, ethyl (±)-6-ethyl(2-hydroxypropyl)amino]-3-pyridazinecarbazate (Fig. 1) is a new long-lasting antihypertensive vasodilator. In the in vitro study utilizing isolated rabbit aorta, cadralazine itself did not show vasodilating activity, while one of its metabolites, (±)-6-ethyl(2-hydroxypropyl)amino]-3-hydrazinopyridazine (ISF-2405, Fig. 1), antagonized the contractile response induced by several vasoconstrictive agents. This indicates that the antihypertensive effect of cadralazine is mainly attributable to the pharmacological activity of ISF-2405. Consequently, pharmacokinetic studies of the active metabolite are necessary in order to elucidate the characteristics of cadralazine. Schütz et al. developed the inverse isotope dilution assay for ISF-2405 in biological samples, and reported the plasma levels and urinary excretion of ISF-2405 in rats and humans after oral administration of [14C]cadralazine. Their method is sensitive and has the advantage that the unchanged drug and four metabolites including ISF-2405 can be assayed simultaneously. However, since the administration of the radioisotope-labeled cadralazine is needed for the determination, applications of their method are limited. In addition, because their derivatization of ISF-2405 was performed under acidic conditions, both ISF-2405 and hydrazone metabolites of ISF-2405 (e.g. pyruvic
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Acid hydrazone of ISF-2405) were derivatized to ISF-3349, which caused the overestimation of ISF-2405 levels. Therefore, we attempted to develop a sensitive method by which ISF-2405 could be determined specifically without the administration of the labeled drug. In the method we have developed, labile ISF-2405 is selectively derivatized to a stable form, (±)-6-ethyl(2-hydroxypropyl)amino]-3-(3,5-dimethyl-1-pyrazolyl)pyridazine (ISF-3349, Fig. 1), with acetylacetone in a neutral solution, and the yielded ISF-3349 is detected by radioimmunoassay. We describe here in detail the procedure used in the present method, and the application of this method to a pharmacokinetic study in rats after oral administration of cadralazine.

Experimental

Chemicals and Reagents — Cadralazine, ISF-2405 and cadralazine-related compounds (ISF-2567, ISF-2874, ISF-2876, ISF-3182, ISF-3345, ISF-3346, ISF-3472, ISF-3473, ISF-3623) were kindly supplied by ISF Laboratories (Milan, Italy). ISF-3349 was synthesized by the method of Simonotti et al. in [3H]ISF-3349 (labeled at the C5-position of the pyrazole ring) with a specific activity of 13.9 Ci/mmol, which was synthesized by the reduction of 4-bromopyrazolyl derivative of ISF-3349 with 5% Pd-C catalyst and tritium gas, was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.), and its radiochemical purity was checked by thin-layer chromatography on silica gel.

Preparation of Immunogen — Immunogen was prepared as shown in Chart 1.

1) Synthesis of (±)-6-{[Ethyl(2-(methoxycarbonylmethoxy)propyl]amino]}-3-(3,5-dimethyl-1-pyrazolyl)pyridazine (I): ISF-3349 (free base), prepared from ISF-3349·HCl (6.13 g), was dissolved in 30 ml of absolute toluene. The solution was added to a solution of CH3ONa (450 mg of Na in 17 ml of methanol), and methanol was removed by evaporation. To the residual solution was added a solution of methyl bromoacetate (3.0 g) in 18 ml of absolute toluene, and the mixture was refluxed for 30 min. The reaction mixture was cooled with ice water and extracted with 2% HCl. The aqueous layer was made alkaline with saturated Na2CO3 and extracted with CH2Cl2. The organic layer was dried over anhydrous Na2SO4 and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel.

CHART 1. Synthesis of Immunogen (II-BSA)
(67.5 g). After 200 ml of n-hexane–ether (4:1) and 600 ml of n-hexane–ether (7:3) were passed through the column, all fractions eluted with 1400 ml of n-hexane–ether (3:2) and 200 ml of n-hexane–ether (1:1) were collected and evaporated. I (2.2 g) was obtained as pale yellow oil. EIMS m/z: 347 (M+). 1H-NMR (δ in CDCl3): 1.20 (3H, t, J = 7 Hz, NCH2CH3), 1.25 (3H, d, J = 6 Hz, NCH2CH2CH3), 2.27 (3H, s, pyrazole-3-CH3), 2.61 (3H, s, pyrazole-5-CH3), 3.68 (3H, s, COOCH3), 4.09 (2H, s, OCH2), 3.2–4.3 (5H, m, NCH2CH3 and NCH2CH2CH3), 6.00 (1H, s, pyrazole-4-H), 7.03 (1H, d, J = 10 Hz, pyridazine-4-H), 7.80 (1H, d, J = 10 Hz, pyridazine-5-H).

(2) Synthesis of (±)-6-[Ethyl2-(carboxymethoxy)propyl]amino]-3-(3,5-dimethyl-1-pyrazolyl)pyridazine (II): I (2.2 g) was dissolved in 96 ml of 10% HCl, and refluxed for 3 h. The reaction mixture was evaporated under reduced pressure. The residue was made alkaline with 28% ammonia water, and the solvent was evaporated in a vacuum. The residue was purified by column chromatography on silica gel (23 g). After 100 ml of n-hexane, 400 ml of n-hexane–ether (1:1) and 200 ml of n-hexane–ether (1:3) were passed through the column to remove impurities, all fractions eluted with 400 ml of n-hexane–ether (1:3), 600 ml of ether and 300 ml of acetone were collected and evaporated. II (1.68 g) was obtained as pale yellow oil. EIMS m/z: 333 (M+). 1H-NMR (δ in CDCl3): 1.17 (3H, t, J = 7 Hz, NCH2CH3), 1.22 (3H, d, J = 6 Hz, NCH2CH2CH3), 2.25 (3H, s, pyrazole-3-CH3), 2.53 (3H, s, pyrazole-5-CH3), 4.08 (2H, s, OCH2), 3.2–4.3 (5H, m, NCH2CH3 and NCH2CH2CH3), 6.00 (1H, s, pyrazole-4-H), 7.10 (1H, d, J = 10 Hz, pyridazine-4-H), 7.78 (1H, d, J = 10 Hz, pyridazine-5-H), 10.88 (1H, s, COOH).

(3) Synthesis of (±)-6-[Ethyl2-(succinimylcarboxymethoxy)propyl]amino]-3-(3,5-dimethyl-1-pyrazolyl)pyridazine (III): N,N'-Dicyclohexylcarbodiimide (0.66 g) dissolved in 4 ml of absolute tetrahydrofuran was added to a solution of II (1.07 g) and N-hydroxysuccinimide (0.37 g) in 5 ml of absolute tetrahydrofuran in an ice bath. After being stirred for 2 h in an ice bath, the mixture was stirred at room temperature for 2 h and filtered. The filtrate was evaporated under reduced pressure, and the residue was dissolved in ethyl acetate. The resultant solution was washed with water, dried over anhydrous Na2SO4, and evaporated to give III (1.37 g) as pale yellow oil. FDMS m/z: 340 (M+). 1H-NMR (δ in CDCl3): 1.23 (3H, t, J = 7 Hz, NCH2CH3), 1.26 (3H, d, J = 6 Hz, NCH2CH2CH3), 2.03 (3H, s, pyrazole-3-CH3), 2.63 (3H, s, pyrazole-5-CH3), 2.83 (4H, s, succimino-H), 3.3–4.3 (5H, m, NCH2CH3 and NCH2CH2CH3), 4.60 (2H, s, OCH2), 6.03 (1H, s, pyrazole-4-H), 7.05 (1H, d, J = 10 Hz, pyridazine-4-H), 7.82 (1H, d, J = 10 Hz, pyridazine-5-H).

(4) Synthesis of Immunogen (II-BSA): A solution of III (313 mg) in 20 ml of absolute dioxane was added dropwise to BSA solution (800 mg in 80 ml of 0.04 M phosphate buffer, pH 7.0), and the reaction mixture was stirred at room temperature for 3 h. Then the mixture was dialyzed against running water at 4°C overnight and concentrated to about 10 ml by ultrafiltration. The resultant solution was passed through a column of Sephadex G-25 and lyophilized to give II-BSA (780 mg) as a fluffy powder.

Spectrophotometric analysis indicated that 17 mol of II was covalently coupled to 1 mol of BSA.

Immunization — Male Hartley guinea pigs weighing 300–350 g were immunized with the immunogen once every 2 weeks for 3 months. The immunogen was dissolved in sterile isotonic saline and emulsified with an equal volume of Freund’s complete adjuvant. Two-tenths milliliter of emulsion containing 1.5 mg of the immunogen was injected intradermally into several sites on the back of a guinea pig. Blood was collected by cardiac puncture 7 d after each or the final booster injection, and the serum was separated and stored at −20°C.

Procedure for Determination of ISF-2405 in Plasma — Both plasma samples and control plasma for standard curve were prepared according to the following procedures.

(1) Derivatization Procedure: Plasma samples were immediately separated at 4°C from freshly obtained blood. To 1 ml of plasma in a glass vial were added 0.1 ml of 0.05 M acetic acid buffer (pH 5.0) containing 3% BSA or standard solution of ISF-2405, and 0.1 ml of acetylacetone. The mixture was vortex-mixed for 30 s and stood for 1 h at room temperature. The treated plasma was kept frozen until analyzed.

(2) Extraction Procedure: The treated plasma
in a glass vial was transferred to a centrifuge tube, and the vial was washed twice with 0.9 ml of 0.075 M phosphate buffer (pH 7.4). The washings were combined with the treated plasma. Ethylacetate (10 ml) was added to the centrifuge tube, and the tube was shaken for 15 min and centrifuged. The organic layer (9 ml) was evaporated to dryness, and the residue was dissolved in 1 ml of 0.075 M phosphate buffer (pH 7.4) containing 1% BSA. The solution was added to a Bond Elut® C8 cartridge (Analyticchem International Inc., Harbor, Ca., U.S.A.) which had previously been wetted with 4 ml of methanol and subsequently conditioned with 2 ml of water. The cartridge was washed with water (2 ml) three times, and the retained substances were eluted with methanol (2 ml). The eluate was evaporated to dryness, and the residue was dissolved in 0.05 ml of methanol and 1.8 ml of 0.075 M phosphate buffer (pH 7.4) containing 0.3% BSA, 0.03% NaN₃ and 0.2% BGG (buffer A). The resulting solution (assay solution) was used for radioimmunoassay.

(3) Radioimmunoassay: To duplicate plastic tubes were added 0.8 ml of the assay solution, 0.1 ml of [³H]ISF-3349 solution (200 pg/0.1 ml) and 0.1 ml of the diluted antiserum (1/7000). The mixture was vortex-mixed and stood for 15 h at 4 °C. The antibody-bound [³H]ISF-3349 was separated from free [³H]ISF-3349 by adding 1 ml of 30% PEG solution and centrifuging at 3500 rpm for 30 min. The supernatant (1.7 ml) was pipetted into a counting vial containing scintillation cocktail (11 ml) and the radioactivity was counted in a scintillation spectrometer (Packard Tri-Carb Model 2450). The percentage of antibody-bound [³H]ISF-3349 to the total count initially added was calculated from the counted activity of free [³H]ISF-3349.

Antiserum titer was determined by adding 0.1 ml of various antiserum dilutions to an incubation mixture consisting of 0.1 ml of [³H]ISF-3349 solution and 0.8 ml of the buffer A instead of the assay solution.

A standard curve was obtained by a logit-log plot of the relative percentage (B/Bo) of bound labeled drug against the concentration of unlabelled ISF-2405.

Antibody specificity was evaluated by measuring the inhibition of the antibody-ISF-3349 binding caused by increasing amounts of various compounds including the metabolites of cadralazine.

Procedure for Determination of ISF-2405 in Tissues — The concentration of ISF-2405 in liver, kidney, spleen, lung, heart and aorta were able to be determined by means of this procedure. Tissues were taken from each rat immediately after sacrifice. Tissues (except aorta) were homogenized with 3 volumes of 0.075 M phosphate buffer (pH 7.4) in a glass-Teflon or glass-glass homogenizer at 0—4 °C. To a glass vial were added 0.4 ml of the homogenate, 0.6 ml of 0.075 M phosphate buffer (pH 7.4), 0.1 ml of 0.05 M acetate buffer (pH 5.0) containing 3% BSA or standard solution of ISF-2405, and 0.1 ml of acetylacetone. The mixture was vortex-mixed for 30 s and stood for 1 h at room temperature. The treated samples were kept frozen until analyzed.

Owing to the small weight of the samples, aorta was treated by the different way from other tissues. Aorta taken from each rat was weighed and homogenized with 2 ml of 0.075 M phosphate buffer (pH 7.4), 0.1 ml of 0.05 M acetate buffer (pH 5.0) containing 3% BSA or standard solution of ISF-2405, and 0.1 ml of acetylacetone in a glass-glass homogenizer at 0—4 °C. The homogenate (1.8 ml) was transferred to a glass vial and kept frozen until analyzed.

The extraction procedure and radioimmunoassay were carried out as described in the preceding section.

FIG. 2. Dilution Curve of Antiserum. The percentage of [³H]ISF-3349 (200 pg bound to the antibody is plotted against the dilution of the antiserum represented on a semilogarithmic scale.
TABLE I. Specificity of Anti-ISF-3349 Antiserum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Cross-reactivity (%)</th>
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<tr>
<td>ISF-3349</td>
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<tr>
<td>Cadralazine metabolites</td>
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</tr>
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<td>ISF-2405</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>Pyruvic acid hydrazine of ISF-2405</td>
<td><img src="image" alt="Structure of Pyruvic Acid Hydrazine of ISF-2405" /></td>
<td>&lt;0.01</td>
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<td>ISF-2876</td>
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<tr>
<td>ISF-3346</td>
<td><img src="image" alt="Structure of ISF-3346" /></td>
<td>&lt;0.01</td>
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</table>

The values are expressed in percentage cross-reactivity which is defined as $X/Y \times 100$, where $X$ is the amount of unlabeled ISF-3349 and $Y$ is the amount of the test compound required to produce 50% inhibition of binding of $[^3H]ISF-3349$ to the antiserum.

Animal Study — Since antihypertensive vasodilator drugs act directly on vascular smooth muscle to produce vasodilation, aorta levels of ISF-2405 as well as plasma levels were determined in rats after oral administration of cadralazine by means of our method. Fasted male Wistar rats, weighing 200–260 g, were used for the study. They received single oral doses of 3 mg/kg of cadralazine, dissolved in 3 ml of water-1 N HCl (99:1). Blood and aorta were
taken at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after administration. The plasma and aorta samples were treated according to the procedures described above.

RESULTS

Antibody Titer

A typical titer curve of the antiserum is shown in Fig. 2. Dilution of the antiserum chosen for the assay was 1:70000 in final dilution, which gave about 60% binding.

Specificity of the Antiserum

The cross-reactivity of the antiserum with cadrnazine and cadrnazine-related compounds is listed in Table I. Data are expressed in terms of percentage cross-reactivity. None of the compounds showed an appreciable cross-reaction with the antiserum.

Derivatization

The derivatization of ISF-2405 in plasma and tissues was carried out at various concentrations of ISF-2405. The yields of ISF-3349 were determined and found to be almost constant (ca. 70%) over the concentrations tested (< 1000 ng/ml). On the other hand, the acetylated treatment of pyruvic acid hydrazone of ISF-2405 gave scarcely any ISF-3349 (0.7%), and the presence of the metabolite in biological samples did not interfere with the assay of ISF-2405. Cadrnazine and other metabolites did not yield ISF-3349 by the treatment.

Sensitivity of the Assay

A typical standard curve obtained with 1:70000 antiserum dilution and 200 pg of the labeled ligand is shown in Fig. 3. ISF-2405 can be reliably assayed in a concentration range from 0.4 to 6.4 ng/ml with 1 ml of plasma.

In the case of measurements of tissue levels, ISF-2405 can be assayed in a concentration range from 2 to 64 ng/g with 100 mg of tissue.

Intra- and Inter-Assay Variations

Intra- and inter-assay variations were estimated from the repeated assay of plasma and tissues. The coefficients of the variations were less than 15% over the concentrations tested.

Plasma and Aorta Levels of ISF-2405 in Rats

Figure 4 shows the mean plasma and aorta levels of ISF-2405 in rats after single oral administration of cadrnazine at a dose of 3 mg/kg. Plasma levels of ISF-2405 reached a maximum of 7.6 ng/ml at 0.5 h after administration, followed by an elimination half-life of 1.2 h, where-as aorta levels of ISF-2405 reached a maximum of 38.9 ng/g at 2 h after administration, followed by an elimination half-life of 11.6 h.

DISCUSSION

A radioimmunoassay for the sensitive and specific determination of ISF-2405 in plasma and tissues was developed by converting ISF-2405 to the stable form ISF-3349 and preparing a highly specific anti-ISF-3349 antibody. Owing to its hydrazino group, ISF-2405 is easily degraded in an aqueous solution. When ISF-2405 is incubated with plasma for 1 h, it reduces to ca. 20% of the initial value at 25 °C and ca. 50% of that at 4 °C. Accordingly, to determine its concentration in biological samples, ISF-2405 has to be derivatized to a stable form immediately after sampling. We derivatized ISF-2405 to the stable form ISF-3349 by treatment with acetylated in a neutral solution. The resultant ISF-3349 was separated from an excess amount of acetylated using a Bond Elut® C8 cartridge, and then measured by radioimmunoassay. The antiserum used for the assay was highly specific for ISF-3349, and did not cross-react with either cadrnazine or its metabolites. The yields of ISF-3349 in the derivatization procedure were constant, and pyruvic acid hydrazone of ISF-2405 did not give ISF-3349 significantly by this procedure. Therefore, both the
specificity and reproducibility of our assay are good. The determinations of ISF-2405 in plasma and tissues are possible over the concentration range from 0.4 to 6.4 ng/ml with 1 ml of plasma, and from 2 to 64 ng/g with 100 mg of tissue, by the present assay.

Plasma levels of ISF-2405 in rats receiving cadralazine were determined by the present method. The mean plasma level of ISF-2405 in rats which received orally 3 mg/kg of cadralazine was maximal (7.6 ng/ml) at 0.5 h after administration, followed by a rapid elimination with half-life of 1.2 h. Figure 5 shows this mean plasma level of ISF-2405 together with the mean plasma level of unchanged drug in the same samples determined by the method of Hauffe et al. The pattern of mean plasma level of ISF-2405 was almost the same as that of unchanged drug (elimination half-life 0.9 h), but the values of mean plasma levels of ISF-2405 were as low as about 1% of those of the unchanged drug. Schütz et al. reported the plasma levels of ISF-2405 in rats determined by the inverse isotope dilution assay. Although the time to reach the maximal level obtained by their study agreed with our result, the maximal level of ISF-2405 determined by their assay was 3 to 4 times higher than that determined by our method, and the elimination of ISF-2405 from plasma reported by them was slower. Since their derivatization was performed under acidic conditions, they assayed both ISF-2405 and the acid labile hydrazones of ISF-2405 with endogenous ketones (e.g. pyruvic acid). Shepherd et al. reported that the plasma levels of pyruvic acid hydrazone of hydralazine in patients who received hydralazine were higher than those of the unchanged drug, and that the elimination of the metabolite from plasma was several times slower than that of hydralazine. They also showed that the plasma levels of hydralazine and its elimination half-life appeared higher and longer if the plasma levels of hydralazine were measured after the acid treatment. Consequently, the discrepancy between our results and those of Schütz et al. may be explained in terms of the presence of hydrazone metabolites of ISF-2405 in rat plasma.

Since the active metabolite, ISF-2405, was rapidly eliminated from plasma in spite of the long-lasting pharmacological effect of cadralazine, we took notice of the tissue distribution of ISF-2405 subsequently. In general, blood vessel is considered to be a target tissue of antihyper-
Determination of ISF-2405

FIG. 5. Mean Plasma Levels of Cadralazine and ISF-2405 in Rats after Single Oral Administration of Cadralazine at a Dose of 3 mg/kg.

Each point represents the mean ± S.E. from 3 animals.

- ● - , cadralazine concentration; - ○ - , ISF-2405 concentration.

tensive vasodilator drugs, and excised vessels (aorta, mesenteric artery, etc.) are used for the evaluation of these drugs in vitro. Therefore, we determined aorta levels of ISF-2405, for the aorta was a comparatively accessible blood vessel. The mean aorta level of ISF-2405 in rats receiving 3 mg/kg of cadralazine orally was maximal (38.9 ng/g) 2 h after administration, followed by a slow elimination with a half-life of 11.6 h. Fujii et al. investigated the tissue distribution, including that in the aorta, of [14C]cadralazine in rats, compared with those of [14C]hydralazine and [14C]budralazine. The aorta levels of radioactivity in [14C]cadralazine-administered rats were continuous, while those in [14C]hydralazine-administered rats were more continuous in spite of the short-term pharmacological effect of hydralazine. This indicates that the pattern of the aorta levels of radioactivity is not always consistent with that of the pharmacological effect. The aorta radioactivity levels were also measured in the distribution studies of hydralazine or budralazine, but those of the active substances have never been investigated. In the present study, we found that the aorta levels of the active substance ISF-2405 decreased slowly. Our results suggest that the long-lasting pharmacological effect of cadralazine may be related to the distribution pattern of ISF-2405 in blood vessels, a target tissue of antihypertensive vasodilator drugs. Further studies should be carried out to elucidate the mechanism of the long-lasting effect of cadralazine, and to reveal the relationship between the pharmacological effect and the plasma level. The present method should be useful for these pharmacodynamic and pharmacokinetic studies in animals as well as for pharmacokinetic studies in humans.

Acknowledgement

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