Preliminary Characterization of Aqueous Extracts of *Piper methysticum* (Kava, Kawa Kawa)

By MAUREEN J. O'HARA*, WILLIAM J. KINNARD, and JOSEPH P. BUCKLEY

An extract of *Piper methysticum* Forst. prepared by steam distillation was separated by means of differential solubility into two fractions, F_1 and F_2. These fractions had different physical and chemical characteristics, and known α-pyrones were identified only in subfraction F_2. Both fractions decreased spontaneous motor activity in doses which did not alter forced motor activity of mice. Fraction F_2, dihydromethysticin, desmethoxy-yangonin, and kawain exhibited potent antiserotonin activity on the isolated rat uterus, whereas F_1 appeared to be devoid of antiserotonin activity. F_1, F_2, and dihydromethysticin did not alter serotonin brain levels in mice.

PIPER METHYSTICUM Forst. (*Piperaceae*) has been used for hundreds of years as an intoxicating beverage in ceremonial rites by natives of many of the islands of the South Pacific. Pharmacological studies of substances isolated from Kava resin were published as early as 1886 (1); and recently, Keller and Klohs (2) have reviewed the chemistry and pharmacology of *P. methysticum*. The pure crystalline α-pyrones, isolated from the roots of the plant which possess sedative-type activity, are chloroform or ether soluble but insoluble in water. Furgiuele *et al.* (3) reported that aqueous extracts of *P. methysticum* possessed ataractic-type activity. The extraction of an aqueous slurry of pulverized kava root by steam and subsequent lyophilization produced a substance which was pharmacologically the most active water soluble extract. This extract depressed spontaneous motor activity in mice without appreciably altering forced motor activity. It also markedly reduced irritability of rats having bilateral septal lesions, inhibited the conditioned avoidance response in rats, and blocked EEG arousal patterns in cats. The purpose of this present study was to further characterize the pharmacologically active aqueous extracts.

EXPERIMENTAL

The plant material used was obtained from the S. B. Penick & Co., New York, N. Y., and consisted of finely pulverized root of *P. methysticum* (*Piperaceae*). Fraction LE-I was obtained by steam extraction of the plant material as described by Furgiuele *et al.* (3). When this LE-I fraction was reconstituted in a concentration of 10 mg./ml. in distilled water, some of the material was insoluble and formed a fine suspension. Preliminary studies were thus conducted to compare the activity of the suspension with an equal volume of its filtrate. Sixteen male albino mice were divided into eight groups; four groups received 50 mg./Kg. of LE-I i.p., and four groups received an equal volume of the filtrate. Spontaneous activity was determined through the use of photocell activity cages in the manner described by Furgiuele *et al.* (3). Both LE-I and the filtrate obtained from LE-I markedly depressed spontaneous activity of the mice. The suspension decreased spontaneous activity by 50% and an equal volume of the filtrate decreased activity by 75%.

Preparation of Water Soluble Subfractions F_1 and F_2.—LE-I was suspended in distilled water in a concentration of 20 mg./ml. and shaken for 3 min. The mixture was then filtered through Whatman No. 1 filter paper, the filtrate shaken with 2 equivalent vol. of chloroform, the remaining aqueous solution lyophilized, and the resulting amorphous solid labeled F_1. The residue from the initial filtration was washed and made up to 15 ml. with distilled water, shaken for 3 min., and filtered. This filtrate was also washed with 2 equivalent vol. of chloroform, the resulting aqueous solution lyophilized, and the amorphous solid labeled F_2. Both fractions were examined for the presence of nitrogen using the standard sodium fusion method. The presence of aldehydes and ketones in subfractions F_1 and F_2 were determined as follows. Two milligrams of the subfraction was dissolved in 2 ml. of distilled water added to 3 ml. of 2,4-dinitrophenylhydrazine reagent (3 Gm. of 2,4-dinitrophenylhydrazine in 15 ml. of concentrated sulfuric acid). The resulting solution was then added to 20 ml. of water and 70 ml. of 95% ethanol and filtered. Aldehydes and ketones react to form insoluble dinitrophenylhydrazone. Ultraviolet absorption spectra were obtained for F_1 and F_2, using a Beckman DB spectrophotometer. F_1 and F_2 were prepared in concentrations of 0.1 mg./ml. in distilled water.

Thin-Layer Chromatography.—One hundred micrograms each of LE-I, F_1, and F_2 were applied to Silica Gel G plates and the spotted plates developed with a skelly B–ethyl acetate (7:8) solvent, air-dried, and sprayed with sulfuric acid.

Spontaneous and Forced Motor Activity Studies.—The effects of subfractions F_1 and F_2 on spontaneous motor activity of male albino Swiss-Webster mice were evaluated in photocell activity

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TABLE I.—SOME PHYSICAL AND CHEMICAL CHARACTERISTICS OF FRACTIONS F_1 AND F_2 FROM THE LYOPHILIZED STEAM DISTILLATE OF KAWA KAWA

Property	F_1	F_2
Physical state	Amorphous solid	Amorphous solid
Color	Amber	Yellowish white
Odor	Aromatic	Aromatic
Sodium fusion	Nitrogen absent	Nitrogen absent
Ignition	Burned with a sooty flame, small residue	Burned with a sooty flame, no residue
Solubility	8 mg./ml.	0.5 mg./ml.
Av. yield	60 mg./200 mg. LE-I	18 mg./200 mg. LE-I
2,4-Dinitrophenylhydrazine	No dinitrophenylhydrazone formed	Red needle shaped crystals of insoluble dinitrophenylhydrazone formed

cages as previously described (4). Fifty minutes after receiving an intraperitoneal injection of either F_1, F_2, or saline, groups of five mice were placed in photocell activity cages and a 15-min. count taken 10 min. later. Each fraction was investigated at four dose levels, using four groups per dose. The effects of F_1 and F_2 on forced motor activity were studied in 60 male albino Swiss-Webster mice, weighing 18 to 25 Gm., randomly divided into groups of six. Each group was trained to walk a 1.5-in. diameter, hardwood rod, rotating at 15 r.p.m. The apparatus used has been described in more detail by Watzman *et al.* (5). The mice were exposed to two training periods each on 2 consecutive days. The morning of the third day, each mouse received 0.1 ml. of 0.9% normal saline solution intraperitoneally, 1 hr. prior to a 3-min. trial period. The afternoon of the third day, each of four groups received a dose of one of the subfractions intraperitoneally, 1 hr. before a 3-min. trial period. The fifth group received a saline injection.

Antiserotonin Activity.—Dombro and Woolley (6) suggested that structural analogy to serotonin might reside in the cinnamamide portion of a class of potent antiserotonin cinnamamide derivatives described by Krapcho *et al.* (7). They demonstrated that simple cinnamamide derivatives devoid of the anilide moiety were also potent serotonin antagonists *in vitro*. These studies suggested that certain crystalline α-pyrones isolated from *P. methysticum* should be investigated for antiserotonin activity. These tests were conducted on quiescent virgin rat uteri in the estrus stage as determined by microscopic examination of vaginal smears. A uterine horn was suspended in a 10-ml. tissue bath containing modified deJalon's solution (8), containing $CaCl_2$, 20 mg./L., oxygenated with 95% of O_2 and 5% of CO_2, and maintained at 37.5°. Uterine contractions were recorded on a smoked kymograph drum *via* a muscle lever giving a magnification of approximately 3×. All substances were added in volumes not exceeding 0.15 ml., and the α-pyrones investigated were administered in a suspension in 1% methylcellulose, 1500 cps. The minimal amount of serotonin creatinine sulfate necessary to induce the maximal contraction of the uterine horn was initially determined and usually varied between 0.5 and 1.0 mcg./10 ml. bathing solution. This dose was then added to the bath every fourth minute; following the contraction, the tissue was washed with fresh deJalon's solution. After two equivalent responses to serotonin were obtained, a given quantity of the substance under study was added to the bath, 30 sec. prior to the next dose of serotonin. If inhibition occurred, further doses of the antagonists were not added until the serotonin response had returned to normal. In studies with α-pyrones, control responses to an equivalent volume of methylcellulose were obtained.

Effects on Brain Serotonin Content.—Dihydromethysticin, 100 mg./Kg., F_1, 100 mg./Kg., F_2, 100 mg./Kg., chlordiazepoxide, 5 mg./Kg., and reserpine phosphate, 1.25 mg./Kg., were each administered to five mice; 1 hr. later the animals were sacrificed by stunning and exsanguination. The brains were removed intact, weighed, and transferred to a glass homogenization tube containing sufficient 0.1 *N* HCl to make a total volume of 3.0 ml. The tissue was homogenized with a motor driven Teflon homogenizer and the homogenate rinsed with 7.0 ml. of double distilled water and transferred to a 30-ml. centrifuge tube containing 3.0 ml. of borate buffer. Serotonin was extracted using the method of Bogdanski *et al.* (9), as modified by Aprison *et al.* (10) and serotonin concentration determined as described by Udenfriend *et al.* (11) using a Turner fluorometer.

RESULTS

Water Soluble Subfractions F_1 and F_2.—The physical-chemical characteristics and average yield of F_1 and F_2 are summarized in Table I. Subfraction F_1 was found to be 16 times more water soluble than F_2. Nitrogen could not be detected

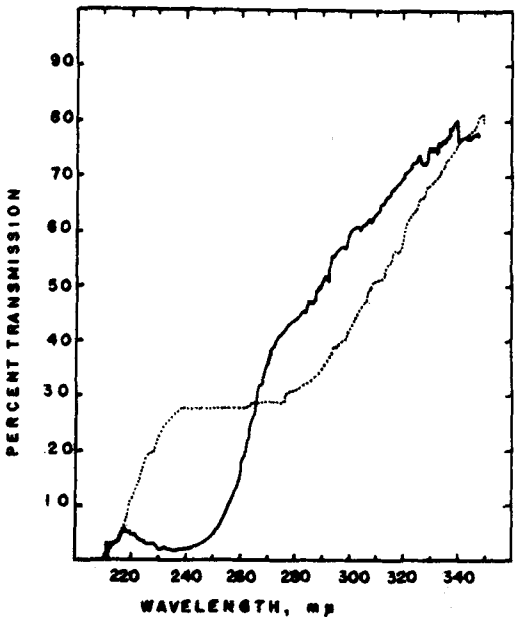

Fig. 1.—Ultraviolet absorption spectra of fractions F_1 and F_2 of an aqueous kawa extract. Key: ————, F_2; . . . . . , F_1.

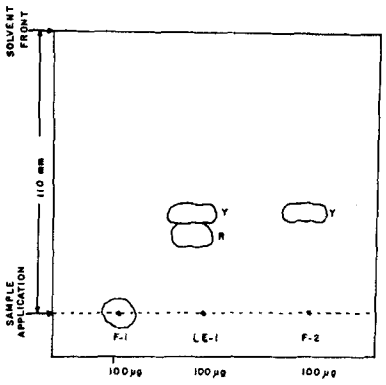

Fig. 2.—Thin-layer chromatogram of certain aqueous extracts of kawa.

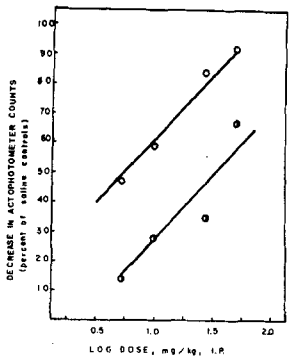

Fig. 3.—Log dose-response curves comparing the effects of F_1 and F_2 on the spontaneous activity of albino mice as measured by the photocell-activity cage method. Key: ○, F_2; ◑, F_1.

in either fraction, and aldehydes and/or ketones were detected in F_2 only.

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectra of F_1 and F_2 are found in Fig. 1, the major difference being that F_2 shows 26% more absorption than F_1 and 240 mμ excitation wavelength.

Thin-Layer Chromatography.—Thin-layer chromatograms using skelly B–ethyl acetate solvent system demonstrated a marked difference between constituents in F_1 and F_2 (Fig. 2). This solvent system has been reported by Furgiuele *et al.* (3) to separate pyrones from LE-I. The chromatograms obtained in this study are very similar to those reported previously in that trace amounts of methysticin or dihydromethysticin appeared to be present in LE-I but were not present in either F_1 or F_2. Traces of other α-pyrones appeared to be present in F_2; and since F_1 remained at the point of application, it would appear that this subfraction is free of all known α-pyrones.

Spontaneous and Forced Motor Activity Studies.—Both F_1 and F_2 depressed spontaneous motor activity of mice, and these effects were dose-related (Fig. 3). The estimated ED_{50} for F_1 was 31.6 mg./Kg. and for F_2, 5.4 mg./Kg. F_1 and F_2, in doses ranging from 5 to 50 mg./Kg. i.p., which were previously shown to markedly inhibit spontaneous motor activity, did not alter the forced motor activity of albino mice, as there was no apparent change in rotarod performance of these mice receiving saline and experimental substances.

Antiserotonin Activity.—The antagonism of dihydromethysticin to the serotonin-induced contraction of the isolated rat uterus is shown in Fig. 4, where each point represents the average response of six preparations to that dose. This antagonism appears to be dose-related and specific, since no antagonism of responses to either bradykinin or acetylcholine can be demonstrated (Table II). Desmethoxy-yangonin was the most potent of the α-pyrones tested, and the ED_{50} (that dose per 10 ml. of bathing fluid required to inhibit the serotonin response by 50%) was 32 mcg. The ED_{50} of kawain was 100 mcg. and F_2, 225 mcg. F_1 did not antagonize the serotonin-induced contractions in doses ranging from 100 to 400 mcg./10 ml. (Figs. 5 and 6).

Effects on Brain Serotonin Content.—The mean brain level of serotonin was determined to be 1.0783 mcg./Gm. of wet weight. Dihydromethysticin, F_1, F_2, and chlordiazepoxide did not alter brain level of endogenous serotonin in mice (range between 99 and 101% of control) 1 hr. after intraperitoneal administration (time of maximal depressant activity). Reserpine, on the other hand, reduced brain serotonin levels by 27%.

DISCUSSION

Two water soluble, apparently distinctive fractions of the steam distillate of *P. methysticum* Forst. were subjected to preliminary psychopharmacological investigations. Both fractions showed depression of spontaneous motor activity in albino mice without concomitant effects on forced motor activity. Although fraction F_1 was a much weaker depressant on a w/w basis than subfraction F_2, data have been presented indicating that this fraction is apparently free of known α-pyrones, yet is in a potency range which suggests it should be subjected to thorough phytochemical followed by

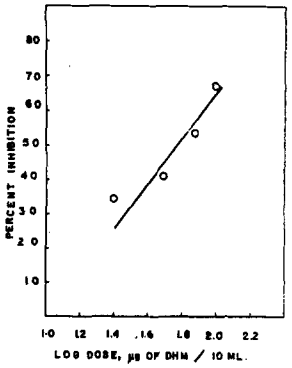

Fig. 4.—Log dose-response curve showing dihydromethysticin antagonism of serotonin on the isolated rat uterus.

TABLE II.—RESPONSES OF AN ISOLATED RAT UTERUS TO DIHYDROMETHYSTICIN SHOWING SPECIFICITY OF ANTAGONISM TO SEROTONIN

Serotonin, mcg./10 ml.	Acetylcholine, mcg./10 ml.	Bradykinin, ng.[a]/10 ml.	DHM, mcg./10 ml.	Contraction, cm.
1.0	0.0	0	0	4.5
1.0	0.0	0	100	1.4
0.0	5.0	0	0	7.2
0.0	5.0	0	100	7.2
0.0	0.0	400	0	7.8
0.0	0.0	400	100	7.8

[a] ng., nanogram.

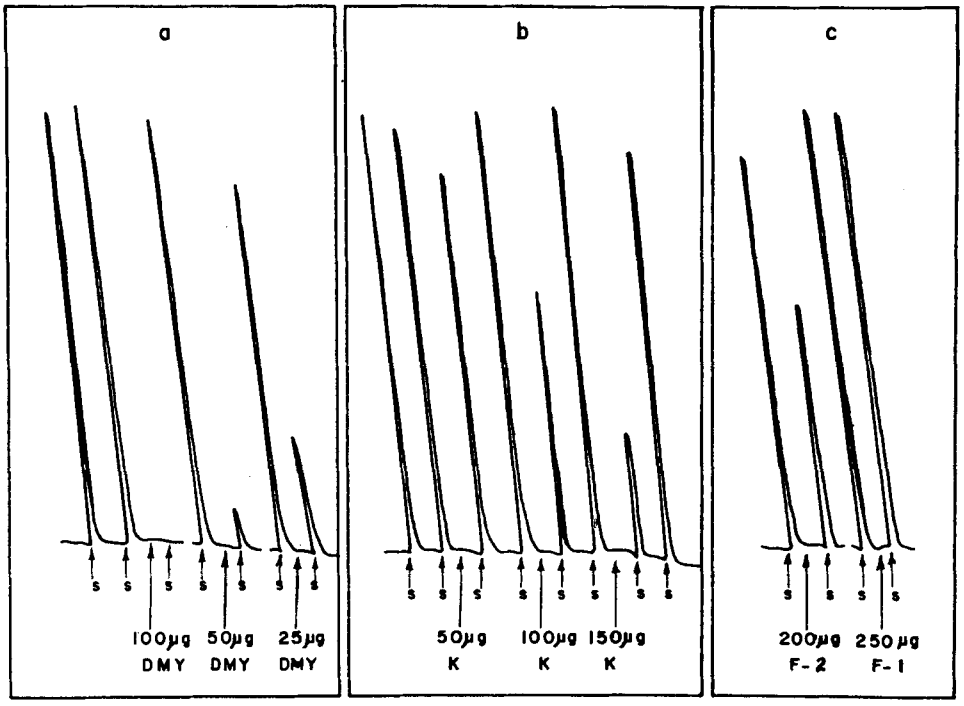

Fig. 5.—Kymograph tracings showing the effects of (*a*) desmethoxy-yangonin (DMY), (*b*) kawain (K), and (*c*) F_1 and F_2 on serotonin-induced contractions (s) of the isolated rat uterus.

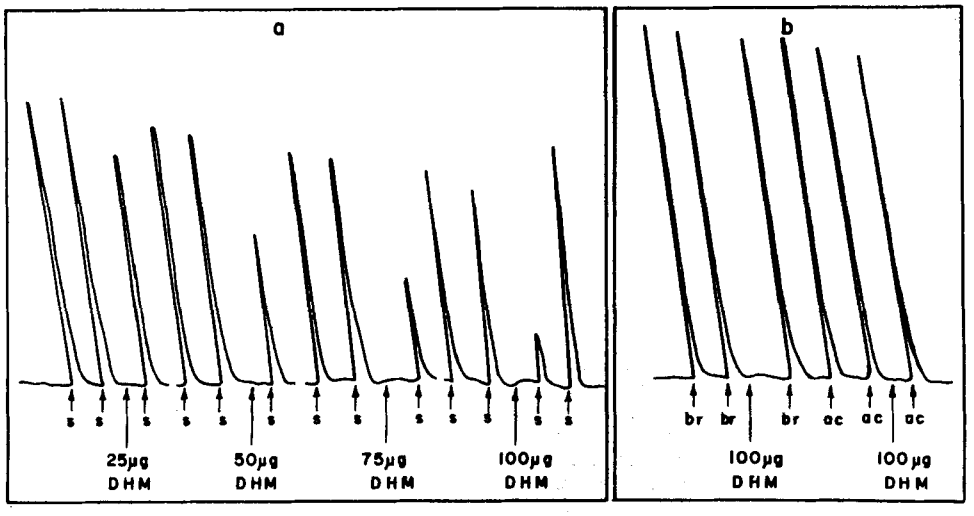

Fig. 6.—Kymograph tracings showing the effects of dihydromethysticin (DHM) on (*a*) serotonin (s) and (*b*) bradykinin (br) and acetylcholine (ac) induced contractions of the isolated rat uterus.

an intensive psychopharmacological investigation. Data have been reported indicating a dose-related antagonism of the serotonin-induced contractions of the rat uterus by dihydromethysticin, desmethoxy-yangonin, and dihydromethysticin, which exhibit potency comparable to *N*-(β-dimethylaminoethyl)-cinnamamide (6). The α-pyrones isolated from kawa kawa possessing antiserotonin activity have a cinnamoyl moiety which may be responsible for this particular pharmacological action. This involves the assumption that the replacement of the cinnamamide nitrogen by a carbon atom in the α-pyrone does not abolish this particular action. A similar effect has been reported by Ariëns (12), who showed that replacement of a nitrogen by a carbon in a homologous series of anticholinergic compounds did not abolish this cholinergic antagonism. The antiserotonin studies further substantiated the difference in activity between F_1 and F_2, in that F_1 did not affect the serotonin response while F_2 was antagonistic to it. This preliminary study suggests the

presence of some component(s) in the more water soluble subfraction F_1 possessing CNS depressant and possibly ataractic activity.

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Evaluation of Certain Hypotensive Agents VII

Tetramethylpiperidine and Benzothiadiazinate Derivatives

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The hypotensive activity of four tetramethylpiperidine and four benzothiadiazinate derivatives was investigated in anesthetized rats and dogs. The active tetramethylpiperidine derivatives exerted their effects by ganglionic blockade. EX 4922, 1-hydrazinophthalazine 3,4-dihydro-6-nitro-7-sulfamoyl-1,1,3-trioxo-2H-1,2,4-benzothiadiazinate, was hypotensive in rats, cats, and dogs, the rat being the most sensitive species. The data obtained suggest that the response is due to α adrenergic blockade and a direct depressant action on vascular smooth muscle. EX 4526, 2,2,6,6-tetramethylpiperidine 3,4-dihydro-6-nitro-7-sulfamoyl-1,1,3-trioxo-2H-1,2,4-benzothiadiazinate, was also hypotensive in rats, cats, and dogs. Pre- and postganglionic conduction along sympathetic nerves was depressed, but the pressor effects to exogenous epinephrine were potentiated. A direct relaxation of vascular smooth muscle was also observed.

THE HYPOTENSIVE activity of a series of 26 compounds structurally related to the ganglionic blocking agent pempidine (1,2,2,6,6-pentamethylpiperidine) has been reported by Buckley *et al.* (1). The present report extends this series of compounds synthesized by Robertson *et al.* (2) to include evaluations of four additional compounds. The hypotensive effects of four benzothiadiazinate derivatives are also reported. The compounds have structural similarities to chlorothiazide and 7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide) (3), a nondiuretic benzothiadiazine which lowers arterial pressure by a direct action on vascular smooth muscle (4). The structures of the compounds investigated are found in Table I.

EXPERIMENTAL

Hypotensive Activity in Rats.—Hypotensive activity was evaluated using the method described by Bickerton *et al.* (5). Normotensive Wistar rats, anesthetized with urethan, 1.25 Gm./Kg. i.p., were prepared for blood pressure recording from a carotid artery onto a slowly revolving kymograph *via* a mercury manometer. Compounds were administered into an exposed femoral vein and solutions prepared so that the dose could be administered in a volume of 1.0 ml./Kg. Normal saline was used as a solvent for EX 4272, EX 4629, EX 4827, and EX 4916. A 50% solution of dimethylacetamide (DMAC) was used as the solvent for EX 4348, EX 4526, EX 4826, and EX 4922.

Hypotensive Activity in Dogs.—The experimental compounds were further investigated for hypotensive activity in dogs anesthetized with sodium pentobarbital, 35 mg./Kg. i.v. Blood pressure was recorded from a femoral artery onto a slowly moving kymograph. Pressor responses to a 10-sec. bilateral carotid occlusion (BCO), epinephrine, 1–2 mcg./Kg., norepinephrine, 1–2 mcg./Kg., and angiotensin II, 1 mcg./Kg., were obtained prior to and after administration of an experimental compound into an exposed femoral vein.

Cat Nictitating Membrane Preparation.—The cat nictitating membrane–superior cervical ganglia preparation was utilized to assess the effects of the more active compounds on ganglionic transmission. Cats of either sex were anesthetized with sodium pentobarbital, 35 mg./Kg. i.p. Blood pressure was recorded from a femoral artery onto a Grass polygraph using a Statham (P23AC) transducer. Platinum stimulating electrodes were placed under the pre- and postganglionic fibers of the superior cervical nerve and contractions of the nictitating membrane recorded *via* a Grass FT03 force displacement transducer. The nerves were bathed in warm mineral oil to prevent drying. Responses of the nictitating membrane to submaximal stimulation of the pre- and postganglionic nerves and to 5–10 mcg. of epinephrine i.v. were obtained prior to and following intravenous administration of EX 4348, EX 4526, EX 4629, EX 4826, EX 4916, or EX 4922.

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