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Note

High-performance liquid chromatographic assay of k-opioid selective benzeneacetamide derivatives (U50488, U69593 and PD117302) in rat plasma and brain

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The search of a strong analgesic without the dependence liability of morphine has led to the discovery of specific agonists of the opioid receptor subtypes (μ, δ and k). k-Opioid agonists are relatively potent and, unlike analgesics acting at μ receptors (e.g. morphine), have minimal addiction liability, respiratory depressant and constipating effects [1–3]. In contrast to k-ligands of the benzomorphan family (e.g. ethylketocyclazocine, bremazocine), the benzeneacetamide derivatives U50488 (I), U69593 (II) and PD117302 (III) (see Fig. 1 for structures) appeared to be entirely free of μ-agonist properties with little or no μ-antagonist properties [4–8]. These compounds are therefore the most selective k-compounds synthesized to date and appear to offer therapeutically useful opioid analgesics with minimal abuse liability in humans. However, no information is available on their absorption, distribution and elimination in the rat, a species widely used to study these compounds, or indeed in humans. This paper describes a simple and selective high-performance liquid chromatographic (HPLC) method for determining I, II and III in rat plasma and brain. To illustrate the application of the assay, these compounds were quantified in plasma and brain of rats treated intravenously with the respective equieffective analgesic doses.

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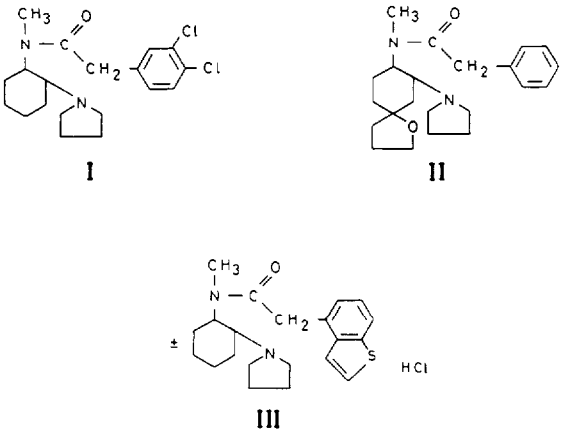


Fig. 1. Chemical structures of U50488 (I), U69593 (II) and PD117302 (III).

EXPERIMENTAL

Chemicals

(±)-*trans*-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinylcyclohexyl)]benzene-acetamide methanesulphonate (I) and (5,7,8)-(−)-N-methyl-[7-(1-pyrrolidinyl-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide (II) were kindly supplied by The Upjohn Company (U.S.A.); (±)-*trans*-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzo[*b*]thiophene-4-acetamide hydrochloride (III) was kindly supplied by Parke-Davis Research Unit (Cambridge, U.K.). Acetonitrile (HPLC grade) was supplied by Omnia Res. (Milan, Italy). Other reagents (analytical-reagent grade) were hexane and acetone (E. Merck, Darmstadt, F.R.G.) and heptane, chloroform and formic acid (Farmitalia-Carlo Erba, Milan, Italy).

Chromatographic system

An HPLC system (System Gold, Beckman Instruments, San Ramon, CA, U.S.A.) comprising a programmable solvent module (Model 126) and a variable-wavelength UV detector (Model 166) was used with a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe chart recorder (Houston Instruments, Austin, TX, U.S.A.). For the analysis of I and III the mobile phase was acetonitrile–0.01 *M* potassium dihydrogenphosphate, adjusted to pH 2.9 with orthophosphoric acid (35:65, v/v) using III as internal standard for the assay of I and vice versa. For the analysis of II the mobile phase was acetonitrile–0.01 *M* potassium dihydrogenphosphate adjusted to pH 2.9 with orthophosphoric acid (31:69, v/v) using III as internal standard. The mobile phase was filtered through a

Millipore filter (0.4 μm) and degassed before use. The flow-rate was set at 1.5 ml/min. Compound III was detected at 230 nm, I and II at 210 nm.

Standard solutions

Standard solutions of I, II and III, expressed as free base (50 and 5 μg/ml), were prepared in acetonitrile and stored at 4 °C. These solutions were used for the preparation of the calibration curves and standards.

Extraction procedure

To 0.5–1 ml of heparin-treated plasma in a polypropylene tube, 20–30 μl of the appropriate internal standard solution were added and the samples were extracted with 4–8 ml of hexane by shaking for 15 min on an automatic shaker. After centrifugation (1500 *g*, 10 min, room temperature) the organic phase was separated and evaporated to dryness under a nitrogen stream. The residues were dissolved in 150 μl of mobile phase and 10–80 μl were injected into the HPLC system.

Brains (ca. 1 g) were homogenized (6 ml/g) in cold acetone–1 *M* formic acid (85:15, v/v). After centrifugation (12 000 *g*, 20 min, 4 °C), the supernatant was washed twice with 10 ml of heptane–chloroform (90:10, v/v). The phases were separated by centrifugation (1500 *g*, 10 min, room temperature) and the aqueous phase, after adjustment of the pH to 7.4–8 with 4 *M* potassium carbonate, was extracted with 9 ml of hexane, then processed as described for plasma.

Calibration and quantification

Drug-free plasma and brain samples spiked with known amounts of the compounds under investigation were analysed concurrently with each set of unknown samples. Calibration graphs were constructed by plotting the ratio of the peak height of the compounds to that of the internal standard and comparing the amounts of the compounds added. Percentage recoveries were calculated by comparing the peak heights of the compounds after plasma and brain extraction with the peak heights obtained by direct injection of their standard solutions.

Animals and drug treatment

Male CD-COBS rats (Charles River, Como, Italy) weighing 250–300 g were used. They were injected intravenously with 2 mg/kg I or 1 mg/kg II and III in a dose volume of 2 ml/kg through a lateral tail vein. Heparinized blood and brains were collected 5 min after drug treatment. Compounds I and III were dissolved in saline (0.9% NaCl, w/v), and II was dissolved in 50 μl of 1 *M* HCl, then diluted with saline (4 ml) and the pH of the solution was adjusted to 7.0 with 30–40 μl of 1 *M* NaOH.

RESULTS AND DISCUSSION

Chromatographic separation and UV detection

Using the chromatographic method described, I, III and II presented symmetrical and well resolved peaks without any serious interference (Figs. 2 and 3). The retention times were ca. 6 and 9 min for III and I, respectively, using 35% (v/v) of acetonitrile in the mobile phase, and 4 and 9 min, respectively,

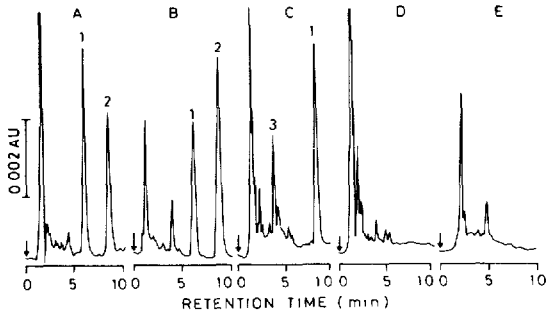


Fig. 2. Chromatograms of extracts from a drug-free plasma (1 ml) spiked with (A) I (50 ng), (B) III (25 ng) and (C) II (100 ng), and from drug-free plasma at (D) 210 nm and (E) 230 nm. Volumes of 80 μl (A) and 40 μl (B,C,D,E) were injected into the liquid chromatograph. Peaks: 1 = III; 2 = I; 3 = II.

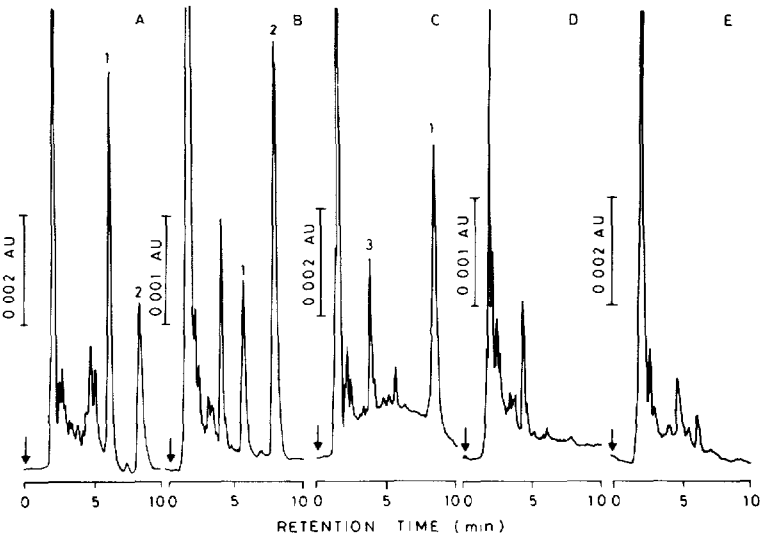
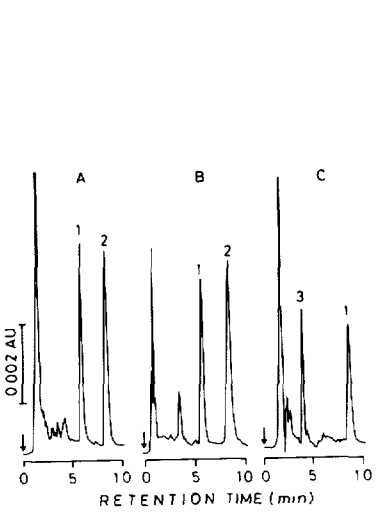


Fig. 3. Chromatograms of extracts from a drug-free brain (ca. 1 g) spiked with (A) I (50 ng), (B) III (25 ng) and (C) II (100 ng), and from drug-free brain at (D) 230 nm and (E) 210 nm. Volumes of 80 μl (A) and 40 μl (B,C,D,E) were injected into the liquid chromatograph. Peaks: 1 = III; 2 = I; 3 = II.



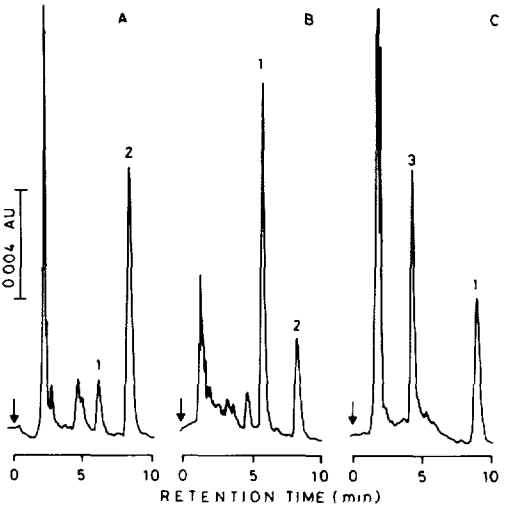


Fig. 4. Chromatograms of extracts from the plasma (0.5 ml) of a rat treated with (A) I, (B) III and (C) II. Volumes of 40 μl were injected into the liquid chromatograph. Peaks: 1 = III; 2 = I; 3 = II.

Fig. 5. Chromatograms of extracts from the brain (ca. 1 g) of a rat treated with (A) I, (B) III and (C) II. Volumes of 20 μl were injected into the liquid chromatograph. Peaks: 1 = III; 2 = I; 3 = II.

for II and III using 31% (v/v) of acetonitrile. Several wavelengths were examined in order to attain optimal detection of the compounds. Compound III showed maximum response at 230 nm, and for I and II a good response without any appreciable interference was observed at 210 nm. Figs. 4 and 5 show typical chromatograms of plasma and brain samples from rats treated with I, II and III.

Recovery, linearity and sensitivity of the method

All three compounds investigated were efficiently extracted from plasma and brain at pH 7.4–8 with hexane. The recoveries during the study in the rat are summarized in Table I. In the 25–2000 ng range, I, II and III were extracted reproducibly with mean recoveries of 88.2 ± 4.3, 73.5 ± 6.4 and $93.5 \pm 4.4\%$, respectively, from plasma and 75.6 ± 2.9, 74.7 ± 5.4 and $81.7 \pm 5.0\%$ from brain, respectively. Linearity was checked by measuring six or seven concentrations (in duplicate) of I, II and III in the 25–2000 ng/ml or ng/g range in two different experiments. The equations of the regression lines obtained from the calibration curves in plasma and brain are listed in Table II. All regression lines were linear in the concentration range stated. Under these conditions, the limits of detection (corresponding to a signal-to-noise ratio of ca. 4:1, with an injection volume of 80 μl) were 10, 30 and 2 ng/ml or ng/g in plasma and brain for I, II and III, respectively.

TABLE I

RECOVERY OF THREE BENZENEACETAMIDE DERIVATIVES, I, II AND III, FROM RAT PLASMA AND BRAIN

Each value is the mean of four determinations obtained in two different experiments. Lowest and highest values are given in parentheses.

Sample	Amount added (ng)	Recovery (%)		
		I	II	III
Plasma	25	83.7 (81.1-86.6)	–	95.6 (90.5-98.4)
	50	86.0 (84.6-87.1)	68.4 (61.8-69.9)	93.8 (91.4-96.0)
	100	85.6 (84.1-88.6)	76.5 (70.6-84.6)	90.4 (86.2-99.6)
	250	89.6 (84.4-95.5)	75.8 (71.4-80.1)	94.6 (92.0-97.2)
	500	91.5 (83.8-95.0)	77.7 (76.4-80.4)	94.9 (87.9-98.8)
	1000	91.2 (85.1-95.0)	76.2 (67.0-83.0)	91.5 (86.5-97.5)
Brain	25	74.2 (71.3-79.1)	–	80.1 (77.7-82.2)
	100	75.7 (73.7-77.0)	79 6 (73.8-84.1)	80.5 (78.5-82.6)
	250	78.8 (75.9-81.0)	76.2 (68.0-82.6)	87.5 (81.5-93.3)
	500	74.4 (72.4-77.3)	72.5 (67.5-75.0)	78.9 (77.0-82.2)
	1000	73.5 (71.8-76.6)	73.2 (67.8-79.9)	81.3 (74.8-91.4)
	2000	76.2 (75.0-77.0)	71.5 (68.5-74.5)	–

TABLE II

TYPICAL CALIBRATION CURVES OF ASSAYS IN PLASMA AND BRAIN

Each point is the mean of four determinations in two different experiments.

Test compound	Concentration range (ng/ml or ng/g)	Plasma equation	Brain equation
I	25-2000	$y=0.0054x+0.0182$ $r=0.9992$	$y=0.0046x+0.0611$ $r=0.9996$
II	50-2000	$y=0.0052x-0.0064$ $r=0.9997$	$y=0.0052x-0.0482$ $r=0.9999$
III	25-1000	$y=0.0068x+0.0189$ $r=0.9999$	$y=0.0081x+0.0004$ $r=0.9999$

Animal study

The HPLC method was used to determine the plasma and brain levels of these compounds in rats given an intravenous dose with ca. 50% analgesic effect [7,9]. Plasma and brain concentrations of I, II and III found at the peak of the analgesic effect (5 min) are summarized in Table III. The compounds differed in plasma and brain distribution: in plasma, after the same dose (1 mg/kg), the levels of II were double those of III, whereas I (2 mg/kg) reached approximately the same concentrations as III. Conversely, high concentra-

TABLE III

PLASMA AND BRAIN LEVELS OF I, II AND III IN RATS

Animals were killed 5 min after drug administration. Each value is the mean of four animals. Lowest and highest values are given in parentheses.

Compound	Dose (intravenously) (mg/kg)	Concentration (ng/ml or ng/g)	
		Plasma	Brain
I	2	240.5 (208.4-279.3)	2629.6 (1728.5-3267.7)
II	1	566.5 (440.4-547.3)	920.8 (798.4-1095.6)
III	1	191.9 (142.6-232.4)	458.8 (340.6- 557.2)

tions of I were found in the brain and this fact was reflected by the brain-to-plasma ratio, i.e. 11 for I and only 2 for II and III.

In summary, the HPLC method reported here for the determination of three benzeneacetamide derivatives with k-opioid agonist activity in rat plasma and brain is rapid, sensitive and selective. Using UV detection at 210 and 230 nm and a C_{18} reversed-phase column the method can quantitate 10 ng/ml of plasma or ng/g of tissue for I, 30 ng for II and 2 ng for III. Chromatographic analysis is relatively fast (10 min) and the extraction procedure is rapid and simple. The procedure is particularly useful for pharmacokinetic studies of these compounds in laboratory animals.

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