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Determination of the retinobenzoic acid derivative Am580 in rat plasma by high-performance liquid chromatography

B. Dimitrova[a], S. Caccia[b,*], E. Garattini[b], G. Guiso[b]

[a]*Higher School of Medicine, Faculty of Pharmacy, Department of Chemistry, 2 Dunav Str., Sofia 1000, Bulgaria*
[b]*Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy*

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Abstract

A specific liquid chromatographic method for the determination of 4-[[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbonyl]amino]benzoic acid (Am580) in rat plasma is described. The procedure includes one-step isolation of the compound and the internal standard (naphthol AS) from protein precipitated with acetonitrile, resolution on a reversed-phase column (Supelcosil LC18-DB, 5 μm) with water–acetonitrile–methanol–*n*-butanol (45:40:14:1, v/v) containing 65 m*M* ammonium acetate as elution system and UV absorbance detection at 280 nm. The assay was linear over a wide range (25–5000 ng ml^{-1}) and the limit of quantitation was 25 ng ml^{-1} using 0.2 ml of plasma. It was precise and reproducible enough for pharmacokinetic studies. Application to a preliminary disposition study in the rat indicated that Am580 was characterized by a relatively large apparent volume of distribution (1.1–1.5 l kg^{-1}) and small clearance (8.8–9.7 ml min^{-1} kg^{-1}). Its pharmacokinetic behaviour was linear within the dose range considered (2 and 10 mg kg^{-1}, i.p.).

1. Introduction

4-[[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbonyl]amino]benzoic acid (Am580, I, Fig. 1) is a new synthetic retinobenzoic acid benzanilide derivative [1] and is one of the most selective ligands for the retinoic acid α subtype receptors (RARα). Compound I has significant in vitro cyto-differentiating activity on various cell lines including HL-60 human myelogenous leukemia and F9 mouse teratocarcinomas [2–6]. Its physico-chemical properties differ from retinoic acids and conventional synthetic retinoids [2,7]. In view of its receptor selectivity, high stability to light, heat and oxidation, compound I may prove to be a significant addition to the family of retinoids, which are already used pre-clinically and clinically. It is also definitely more polar than conventional retinoids and might thus offer more favourable pharmacokinetic–pharmacodynamic properties, particularly in view of the high toxicity and teratogenicity of these derivatives, partly related to their high hydrophobicity [2,8]. However, little is known about the pharmacokinetic–pharmacodynamic relationships of this retinobenzoic acid derivative compared to the conventional retinoids in humans and animals.

For a comprehensive investigation of its pharmacokinetics and pharmacodynamics, I must be

* Corresponding author.

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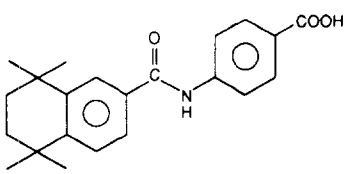

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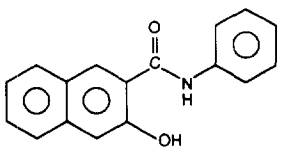

Naphthol AS (I.S.)

Fig. 1. Chemical structures of I and the internal standard (I.S.) naphthol AS.

determined in body fluids and tissues and a validated analytical method is a prerequisite for such studies. We have therefore set up a relatively simple, yet highly selective high-performance liquid chromatographic method for the rapid detection of this compound in rat plasma. The method relies on the one-step extraction procedure previously developed for all-*trans*-retinoic acid [9] and resolution by an isocratic reversed-phase system. Naphthol AS (Fig. 1) was used as an internal standard (I.S.) because of its structural similarity and adequate absorbance at the wavelength selected for I (280 nm). The method was successfully applied in preliminary kinetic studies of intraperitoneally injected I in rats.

2. Experimental

2.1. Chemicals and reagents

Compound I was kindly supplied by GIRD Galderma (Valbonne, France). Naphthol AS (3-hydroxy-2-naphthoic acid anilide) was provided by Fluka (Milan, Italy). Acetonitrile, methanol and *n*-butanol were HPLC grade and were obtained from C. Erba (Milan, Italy). Other chemicals, acetic acid and ammonium acetate (E. Merck, Darmstadt, Germany), were of analytical-reagent grade and were used without further purification.

2.2. Chromatographic apparatus and conditions

HPLC analysis was done on a Waters system equipped with a Wisp-712 sample processor, a Model 510 solvent delivery system and a Model 440 UV detector set at 280 nm, with a range of 0.01 AUFS (Waters, Milford, MA, USA), coupled to a Model C-R6A Chromatopac Shimadzu integrator (Shimadzu, Kyoto, Yapan). A reversed-phase analytical column Supelcosil LC18-DB, 5 μm, 150 × 4.6 mm I.D. (Supelchem, Milan, Italy) was used, fitted with a MPLC New Guard RP-18 precolumn, 7 μm, (Brownlee Labs., Dupont, Santa Clara, CA, USA), maintained at room temperature. The mobile phases were filtered through a 0.45-μm filter, degassed before use, and delivered isocratically at a flow-rate of 1.2 ml/min.

2.3. Preparation of standard solutions

Stock solutions of I and the I.S. were prepared by dissolving the compounds in methanol at a concentration of 1 mg ml^{-1} and were stable over six months when stored at −20°C. The standard solutions, containing 1–10 $\mu g\ ml^{-1}$ of I and 10 $\mu g\ ml^{-1}$ of I.S. were prepared from stock solutions by dilution with methanol and kept at −20°C.

2.4. Sample preparation

The sample preparation for analysis of I in rat plasma involved a one-step extraction procedure using direct protein precipitation with acetonitrile. After addition of the I.S. (250 ng) to the plasma (0.2 ml) and shaking with 0.8 ml of acetonitrile for 60 s in a vortex-mixer, the samples were centrifuged for 2 min at 12 000 *g* in an Eppendorf centrifuge and the supernatant was evaporated to dryness under a nitrogen stream. The residue was reconstituted with 0.2 ml of the mobile phase, samples were vortex-

mixed, transferred to autosampler vials and 170 μl were injected onto the HPLC system.

2.5. Determination of stability

To verify the stability of I in rat plasma before extraction, portions of the standard solution were spiked (250 and 2500 ng ml^{-1}) into extraction tubes containing fresh rat plasma (heparinized). The tubes were stored at room temperature or at −20°C before analysis. The tubes stored at room temperature were analyzed at various intervals up to 24 h, and those at −20°C were analyzed two months later to check the long-term stability of I. The analytical responses of the stored samples were compared with those of samples prepared on the day of analysis.

2.6. Validation

The precision and reproducibility of the method was determined by replicate analysis of quality control samples (QC) containing known small, medium and large amounts of I (25–2500 ng ml^{-1}), stored at −20°C. With each day's analysis, these QC were assayed with standard samples, and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by replicate analysis of QC samples (six at each concentration) on the same day. Daily standard curves with seven concentrations over the working range (the lowest concentration was equal to the limit of quantitation, determined in separate studies) were plotted in duplicate with QC samples injected between the two sets of standards. Standard calibration curves were constructed by linear least squares regression analysis of the plot of the peak-height ratios between the compound and the I.S., against their concentration in biological samples. The concentrations of I in the plasma samples and in QC were determined by interpolation from the calibration curves using peak-height ratios obtained from the samples. The specificity of the assay was checked by running blank rat plasma.

2.7. In vivo studies

Male CD-COBS rats (Charles River, Italy), weighing 300–325 g, were used. The procedures involving animals and their care were conducted in conformity with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No 85-23, 1985).

A chronic jugular cannula was implanted in each rat under chloral hydrate anesthesia 24 h before testing. Kinetic parameters were then determined after intraperitoneal (i.p.) injection of I (2 and 10 mg kg^{-1}) dissolved in saline.

During the study, rats were housed in individual cages with free access to standard laboratory chow and water. Serial 0.35-ml blood samples were drawn in heparinized tubes before (time 0) and at various times after dosing. Samples were wrapped in aluminium foil, centrifuged in an Eppendorf centrifuge and the plasma was stored at −20°C until assayed.

Individual plasma curves of concentrations of I versus time were analyzed following a one-open model system after oral administration, fitting the experimental points by a nonlinear regression iterative program [10] on a personal computer. Details of pharmacokinetic calculations are described in classical texts [11].

3. Results and discussion

3.1. Sample preparation and chromatography

Various procedures were tested for extracting I from body fluids [12]. Direct protein precipitation with acetonitrile developed for all-*trans*-retinoic acid [9] was found to recover I and the I.S. efficiently, extracting only a few impurities and no interfering compounds from plasma, except for a chemically unknown compound which, depending on the chromatographic conditions, interfered with either I or the I.S.

The chromatographic behaviour of I, the I.S. and endogenous constituents was examined on a reversed-phase column Supelcosil LC18-DB

(150 × 4 mm I.D.) initially using common aqueous acetonitrile- (40:60, v/v) and methanol-based (30:70, v/v) eluents, containing either 10 m*M* ammonium acetate or 5 m*M* sodium dihydrogen phosphate. The eluents were adjusted to pH 7.0 or pH 4.0 with acetic acid or phosphoric acid. Elution of the acidic derivative I was strongly influenced by the eluent pH, the retention time being approximately three times longer at pH 4.0; the interfering endogenous substance showed a similar behaviour, causing insufficient resolution of the analytes in biological fluids. Different compositions of aqueous-acetonitrile–methanol eluents were then sequentially examined to improve the separation, varying the acidity as well. The resolution of the solutes was acceptable with water–acetonitrile–methanol (45:40:15, v/v) containing 65 m*M* ammonium acetate. Modification of this mobile phase with a small amount of *n*-butanol, to give water–acetonitrile–methanol–*n*-butanol (45:40:14:1, v/v) and 65 m*M* ammonium acetate, improved peak separation and also resulted in better elution of the endogenous impurity. Under these conditions the approximate retention times were 5.7 min for I and 8.3 min for the I.S. and no interference was observed from drug-free plasma.

Typical chromatograms of an extract from drug-free rat plasma, plasma spiked with I (500 ng ml^{-1}) and I.S. (1250 ng ml^{-1}), and plasma of a rat given 2 mg kg^{-1} of I i.p. and containing 418 ng ml^{-1} of unchanged compound are illustrated in Fig. 2.

The overall mean recovery established by comparing the peak-height ratios for the extracts of plasma spiked with I with that of non-extracted acetonitrile-diluted standard solutions was 96 ± 6%, without any significant dependence on concentration over the range studied (25–5000 ng ml^{-1}). Mean recovery of the I.S. averaged 91 ± 5%.

3.2. Validation studies

Compound I was stable in plasma for at least 24 h at room temperature and up to two months at −20°C.

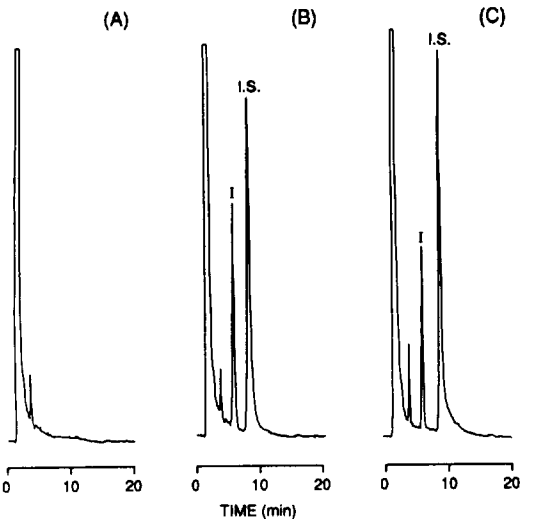

Fig. 2. Chromatograms of extracts from (A) drug-free plasma, (B) plasma sample spiked with 500 ng ml^{-1} of I and (C) plasma from an intraperitoneally treated rat (2 mg kg^{-1} of I) (0.125 h after dosing) containing 418 ng ml^{-1} of unchanged compound. I.S. = internal standard (1250 ng ml^{-1}). Column: Supelcosil LC18-DB 5 μm (150 × 4.6 mm I.D.). Mobile phase: water–acetonitrile–methanol–*n*-butanol (45:40:14:1, v/v) containing 65 m*M* ammonium acetate. Flow-rate: 1.2 ml/min. UV detection at 280 nm.

The relationships between the peak-height ratios of I to the I.S. and the amount of the compound added to plasma (25–5000 ng ml^{-1}) were always linear, with a correlation coefficient invariably exceeding 0.99. The slopes of the three curves prepared over a period of two weeks had a coefficient of variation (C.V.) of 7.8% and average regression equation $y = 0.0064x + 0.0199$. The lower limit of quantitation (the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision) was about 25 ng ml^{-1}, using approximately 0.2 ml of plasma. At this concentration the mean C.V. was 7.7% ($n = 6$).

Rat plasma QC samples (0.2 ml) containing the equivalent of 50, 500 and 2500 ng ml^{-1} of I were assayed ($n = 6$) with each of the HPLC runs in support of the study. The mean C.V. of intra-assay precision ranged from 4.4% to 7.8%. The mean C.V. of inter-assay precision indicated that the method had good reproducibility, the C.V. being less than 9% at all concentrations tested.

The mean accuracy of the method (relative error, R.E.) calculated from the deviation of the mean concentrations found from the nominal value, indicated intra-assay variation from −0.5% to 11.8%. Therefore, the precision and accuracy of the method were acceptable over the concentration range studied.

3.3. Application

Currently the method is being used successfully in pharmacokinetics and concentration–response studies in rats. The mean plasma concentration–time curves of I in male rats given intraperitoneal doses of the compound (2 and 10 mg kg^{-1}) are shown in Fig. 3. Some kinetic parameters derived from these figures are summarized in Table 1. Compound I rapidly appeared in rat plasma reaching peak concentrations (C_{max}) within 1 h regardless of the dose. The mean plasma C_{max} and area under the plasma concentration–time curve (AUC) increased approximately in proportion to the dose, although there was some inter-animal variability at each dose. Under the assumption of complete absorption of I from the i.p. site of injection into the systemic circulation, the mean apparent volume of distribution (Vd) was 1.1 l kg^{-1} at the lower dose and 1.5 l kg^{-1} at the higher dose, suggesting linearity in the uptake of the compound into rat tissue. Mean apparent total clearance (Cl), 8.8 and 9.7 ml min^{-1} kg^{-1} at the low and higher doses respectively, was relatively low compared to liver blood flow. This low Cl in conjunction with the relatively high Vd resulted in a mean elimination $t_{1/2}$ of 1.4–1.7 h over the 2–10 mg kg^{-1} dose range. Similar elimination $t_{1/2}$ have been reported for conventional retinoids after comparable (parenteral) doses [13,14], taking however into account that the kinetics of some of these derivatives appear to be non-linear in rats and that distribution and clearance parameters both change with the dose [14].

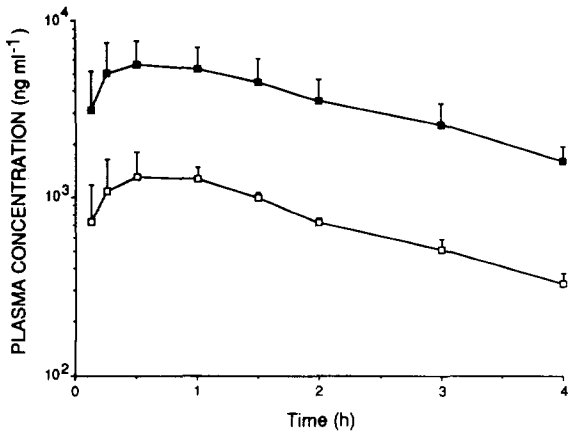

Fig. 3. Mean plasma concentration–time curve of I after a single intraperitoneal dose in rats. Each value is the mean ± S.D. of three rats for the lower (2 mg kg^{-1}) (□) and higher dose (10 mg kg^{-1}) (■).

Table 1
Pharmacokinetic parameters of I in rats after 2 and 10 mg kg^{-1} intraperitoneal doses

Parameter	Value	
	2 mg kg^{-1}	10 mg kg^{-1}
t_{max} (h)	0.8 ± 0.3	0.6 ± 0.4
C_{max} (μg ml^{-1})	1.4 ± 0.4	5.8 ± 2.1
AUC (μg h ml^{-1})	3.8 ± 0.3	18.4 ± 5.4
Vd (l kg^{-1})	1.1 ± 0.3	1.5 ± 0.7
Cl (ml min^{-1} kg^{-1})	8.8 ± 0.8	9.7 ± 3.2
$t_{1/2}$ (h)	1.4 ± 0.3	1.7 ± 0.3

t_{max} = time to maximum concentration (C_{max}); $t_{1/2}$ = elimination half-life; AUC = area under the concentration–time curve; Vd = apparent volume of distribution; Cl = apparent total body clearance.

4. Conclusions

Interest in retinoids has been increasing in recent years in the light of their potential pharmaceutical use and utility in the elucidation of retinoidal action [15].

The development of analytical methods for determining these compounds in body fluids and tissues may help clarify their pharmacokinetic behaviour and any relationships with the pharmacological response [12,16]. The method proposed here for quantitation of the retinobenzoic

acid derivative I in plasma is simple, selective and requires only a small volume of plasma. Its usefulness in pharmacokinetic studies in small animals has been demonstrated. The method can be applied to measure I in other biological specimens and has been employed on cell preparations such as the human promyelocytic leukemia cell line HL-60 (data not shown). It may thus have potential for other pharmacologically important applications.

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