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Short Communication

High-performance liquid chromatographic separation of caffeine, theophylline, theobromine and paraxanthine in rat brain and serum

PILAR PARRA* and ANNA LIMON

Departament de Farmacologia y Psquiatria, Universitat Autònoma de Barcelona, Hospital de Sant Pau, Avda Sant Antoni Mª Claret 167, 08025 Barcelona (Spain)

SERGI FERRE and TERESA GUIX

Laboratori de Neuropsicofarmacologia, Fundació d'Investigació Sant Pau, Programa Sant Pau-Citran, Hospital de Sant Pau, Barcelona (Spain)

and

FRANCESC JANE

Departament de Farmacologia y Psquiatria, Universitat Autònoma de Barcelona, Hospital de Sant Pau, Barcelona (Spain)

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ABSTRACT

Caffeine and its metabolites theophylline, theobromine and paraxanthine have been determined in rat brain and serum samples by high-performance liquid chromatography with ultraviolet detection. The recovery, 85–103%, allowed quantification by external standard methods. The variability was found to be less than 3 and 7% for intra-day and inter-day assays, respectively. The detection limit, 1.57 ng of methylxanthines on column, allowed the determination of 62.5 ng/g or ml in biological material. Rats treated with 30 mg/kg caffeine (subcutaneously) were sacrificed at different times (1, 6, 12 and 24 h). Higher concentrations of methylxanthines (specially paraxanthine) were observed in the striatum than in the rest of the brain, and it was also observed that the clearance of methylxanthines was faster in serum than in brain structures.

INTRODUCTION

Caffeine (CF), theophylline (TH) and theobromine (TB) are widely consumed substances because they are present in coffee, tea, chocolate and cola drinks. Moreover, they are present in some pharmaceutical preparations (most of which can be freely purchased). CF, in particular, is probably the most widely consumed drug in the world (*e.g.* its mean daily consumption in the adult population of the

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USA is *ca.* 3 mg/kg) [1]. TH is the most frequently used in therapy, especially as a bronchodilator in lung diseases [2]. The fetes and the breast-feeding child are exposed to these substances because they easily cross the placental barrier and they also appear in the maternal milk [3].

Chemically, these alkaloids are methylxanthines: CF is a trimethylxanthine (1,3,7-trimethylxanthine) and TH and TB are dimethylxanthines (1,3- and 3,7-dimethylxanthine, respectively). In humans and other mammals, the primary metabolism of CF is by N-demethylation to the dimethylxanthines TH, TB and paraxanthine (PX, 1,7-dimethylxanthine) [4]. It has recently been shown that PX is involved in the behavioural stimulation produced by CF in rodents [5,6]. In addition, appreciable concentrations of PX are detected in the plasma of coffee and tea drinkers, suggesting that normal consumers are exposed continuously to PX [7]. Consequently, PX has to be considered when measuring CF plasma or tissue levels in animals or humans.

As various environmental and polymorphic genetic factors affect the clearance of methylxanthines from plasma [4,8], and these substances have a narrow therapeutic range [2,3], monitoring of their plasma levels is frequently performed when they are administered in high concentrations. A large variety of techniques and analytical procedures for determining methylxanthines in biological fluids have been reported: radioimmunoassay (RIA) [8,9], fluorescence polarization immunoassay (FPIA) [8,10], enzyme immunoassay (EIA) [9,12–15] (the most frequently used in TH monitoring), gas chromatography (GC) [13,15] (a technique rarely used because it needs derivatization and has a low sensitivity) and high-performance liquid chromatography (HPLC) [16–20].

Many reported studies stress the advantages of HPLC in the measurement of methylxanthines, because it shows a high sensitivity, requires small samples and allows the automation of the chromatographic procedure by direct injection [18,21–23]. This paper describes a new HPLC method for the simultaneous determination of CF, TH, TB and PX in rat serum and brain tissue.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 280–320 g were mantained at constant temperature, humidity and a light–dark cycle of 12 h.

Reagents and standards

CF, TH, TB and PX were supplied by Sigma (St. Louis, MO, USA). Dichloromethane, 2-propanol, tetrahydrofuran, sodium dihydrogenphosphate, disodium hydrogenphosphate, phosphoric acid and sodium hydroxide were all analytical grade from Sharlau (Barcelona, Spain).

Xanthine solutions were prepared in distilled water at a concentration of 1 mg/ml (TB and TH required some drops of sodium hydroxide), which remained

stable for at least six months at 4°C. Stock solutions were diluted with mobile phase to the following concentrations: 62.5, 124, 250, 500, 1000, 2000 and 4000 ng/nl, of which 25 μl were daily injected into the chromatograph to obtain the calibration curve.

The phosphate buffer solution used for extraction was 0.5 *M* sodium dihydrogenphosphate and 1 *M* disodium hydrogenphosphate (pH 7.2).

Equipment and chromatography

A Waters (Milford, MA, USA) HPLC system, consisting of a U6K injector, a solvent-delivery system M510 and a UV detector Model 480 was used. A wavelength of 273 nm (maximum wavelength for CF) was used, with a detector sensitivity of 0.005 a.u.f.s. The chromatograms were recorded using a Data Module.

A C_{18} μBondapak reversed-phase column (25 cm × 0.46 cm I.D., 10 μm particle size) was used. The mobile phase was tetrahydrofuran–aqueous 10 m*M* disodium phosphate (3:97, v/v), and the pH was adjusted to 6.5 using phosphoric acid. It was filtered through a Millipore 0.45-μm filter under reduced pressure before use. The solvent flow-rate was 2.5 ml/min. A guard column with the same reversed phase was used, and the chromatography was performed at room temperature.

Procedure

The animals received CF subcutaneously at a dose of 30 mg/kg, and at 1, 6, 12 or 24 h they were decapitated. Blood was collected from the severed neck and rapidly centrifuged at 1500 *g* for 10 min. Brains were quickly removed, and the corpus striatum and cerebellum were dissected. Samples of serum, striatum and brain (minus striatum and cerebellum) were stored at −20°C. Before the assay, striatum and brain were homogenized in ten and twenty volumes of distilled water, respectively, centrifuged at 1500 *g* for 10 min, refrozen at −20°C for 2 h and centrifuged once more. The supernatants of striatum and brain homogenates and serum were then processed in the same way: 0.2-ml samples were mixed first with 0.1 ml of phosphate buffer (pH 7.2) and then with 6 ml of dichloromethane–propanol (95:5), mixed for 2 min and centrifuged at 1500 *g* for 10 min. The resulting upper aqueous layer was discarded by vacuum suction, and the organic solvent was evaporated to dryness under a stream of nitrogen at 35°C, the residue being reconstituted in 0.2 ml of the mobile phase. The reconstitution was achieved by vortex-mixing the tube contents for 1 min. An aliquot (25 μl) was injected into the column.

Recoveries were determined by spiking serum and brain homogenate samples from untreated rats with a solution of CF, TH, TB and PX. Intra-day and inter-day variabilities were determined in the same way with six different serum samples or with brain homogenate samples during four days, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic saparation of CF and its metabolites in biological samples after the subcutaneous administration of CF (30 mg/kg) to rats. There was no interfering peaks in serum and brain blank samples. The recoveries of these compounds in brain and serum, and the intra-day and inter-day variabilities, are shown in Table I. The detection limit and linearity of the method are shown in Table II. The absolute detection limit was estabished at 1.57 ng for CF, PX, TH and TB on the column, at a signal-to-noise ratio of 2; in practice, this would allow the detection of 62.5 ng per gram of tissue or per millilitre of serum.

The high recoveries and sufficient reproducibilities obtained during the extraction procedure make possible the determination of CF and its principal metabolites in biological samples by the external standard method.

It has been reported that, in the rat, after *ca.* ten days of treatment with CF, this drug is 1.5 times more concentrated in the brain than in the serum [24]. In agreement with this, we have found that CF maintained a higher concentration in the brain than in the serum during the period of analysis (24 h). Among the metabolites of CF, TH had a similar pharmacokinetic pattern to CF, but TB and PX were less concentrated in the brain than in the serum. In addition, we found that CF and its metabolites were more concentrated in the striatum than in the rest of the brain. These last results have already been published [5].

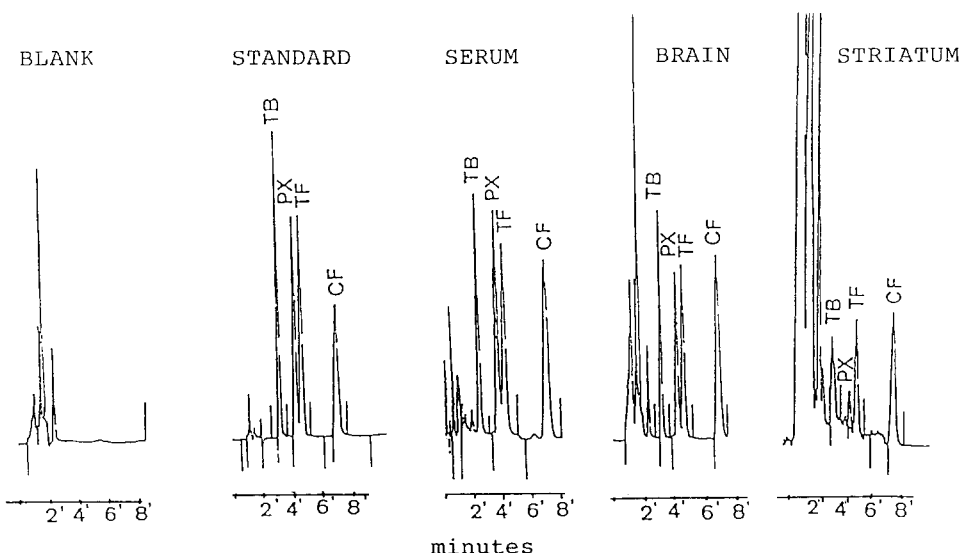

Fig. 1. Chromatograms of caffeine (CF) and its metabolites theophylline (TF), theobromine (TB) and paraxanthine (PX). Column: μBondapak C_{18}, particle size 10 μm; eluent, 10 m*M* disodium phosphate buffer (pH 6.5) and 3% tetrahydrofuran; flow-rate, 2.5 ml/min; UV detection wavelength, 273 nm.

TABLE I

RECOVERIES AND VARIABILITY OF THE METHOD

Compound	Concentration (μg/ml)	Recovery (mean ± S.D.) (%)		Variability (C.V., %)	
		Serum	Brain	Intra-day	Inter-day
Caffeine	0.1	101.36 ± 4.56 ($n=5$)	103.43 ± 8.41 ($n=4$)	2.55 ($n=4$)	6.08 ($n=4$)
	1	100.22 ± 3.25 ($n=5$)	99.95 ± 4.56 ($n=5$)	2.00 ($n=4$)	6.00 ($n=6$)
	10	100.02 ± 3.24 ($n=5$)	102.31 ± 3.28 ($n=5$)	1.84 ($n=6$)	5.24 ($n=4$)
Theophylline	0.1	97.98 ± 3.60 ($n=5$)	92.87 ± 3.87 ($n=4$)	2.00 ($n=6$)	6.77 ($n=4$)
	1	98.32 ± 2.52 ($n=5$)	94.55 ± 3.57 ($n=5$)	1.99 ($n=4$)	5.38 ($n=6$)
	10	100.02 ± 3.24 ($n=5$)	98.31 ± 2.08 ($n=4$)	2.12 ($n=4$)	5.58 ($n=4$)
Theobromine	0.1	85.52 ± 2.80 ($n=5$)	93.33 ± 5.34 ($n=4$)	2.73 ($n=6$)	6.02 ($n=4$)
	1	90.35 + 3.00 ($n=4$)	92.22 ± 3.32 ($n=5$)	2.71 ($n=4$)	6.35 ($n=6$)
	10	97.23 ± 2.82 ($n=5$)	93.12 ± 2.82 ($n=5$)	3.04 ($n=4$)	5.50 ($n=4$)
Paraxanthine	0.1	91.74 ± 3.00 ($n=5$)	88.50 ± 4.21 ($n=4$)	2.35 ($n=6$)	3.95 ($n=4$)
	1	96.87 + 2.75 ($n=5$)	92.03 ± 3.12 ($n=4$)	1.89 ($n=6$)	5.34 ($n=4$)
	10	99.80 ± 3.02 ($n=5$)	96.16 ± 4.16 ($n=5$)	2.55 ($n=4$)	6.06 ($n=6$)

TABLE II

DETECTION LIMIT AND LINEARITY OF THE METHOD

Compound	Retention time (min)	Detection limit (ng)	Linearity
Caffeine	7.35	1.57	$-0.2208x + 10.0674$ ($r=0.9997$)
Theophylline	4.93	1.57	$-0.3777x + 6.2186$ ($r=0.9999$)
Theobromine	3.35	1.57	$-0.0172x + 4.3960$ ($r=0.9999$)
Paraxanthine	4.48	1.57	$-0.7904x + 6.2603$ ($r=0.9999$)

Our results show that the HPLC method used, which is a modification of one previously described by Grgurinovich [20], permits quick and simple extraction and the simultaneous determination of CF and its first-stage metabolites (TH, TB and PX) in rat serum and brain tissue, with suitable resolution, sensitivity, recovery and reproducibility.

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