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Simultaneous determination of the two components of picrotoxin in serum by reversed-phase high-performance liquid chromatography with application to a pharmacokinetic study in rats*

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Abstract A reversed-phase HPLC method is reported which allows the quantification of picrotin and picrotoxinin in serum A linear response was obtained for both drugs in the range 0 2–20 μg ml^{-1} The within-day and between-day precisions were 0 8–3 7% and 1 3–4 9%, respectively The mean recoveries were greater than 94 2% The method was applied to a pharmacokinetic study following intraperitoneal (i p) administration of 3 mg kg^{-1} of picrotoxin in rats The obtained data suggest a relatively slow absorption after i p administration followed by a rapid elimination from the central compartment according to a one-compartment open model The elimination half-lives were 0 340 ± 0 0308 h for picrotin and 0 312 ± 0 0241 h for picrotoxinin

Keywords *Reversed-phase, HPLC, picrotoxin, picrotin, serum, picrotoxinin, pharmacokinetics, rat, pharmaceutical analysis*

Introduction

Picrotoxin (PCX) is a stimulant of the central nervous system produced by *Anamerta cocculus*, a plant of the Menispermaceae family Its convulsive effects were first described in 1875 by Browne [1] At present, it is generally agreed that PCX exerts its neurophysiological action by blockage of the GABA-mediated inhibitory transmission [2] Since it blocks the GABA-induced increase in chloride flux [3] and does not inhibit the binding of GABA to its receptor [4], it has been suggested that PCX inhibits the GABA receptor–ionophore system at a different site to the GABA recognition site [5] Chemically, PCX is on equimolar mixture of two sesquiterpenes, picrotin (PCN, hexahydro-2a-hydroxy-9-(1-hydroxy-1-methylethyl)-8b-methyl-3,6-methano-8H-1,5, 7-trioxacyclopenta[ij]cycloprop[a]azulene-4,8-(3H)dione) and picrotoxinin (PCXN, hexahydro-2a-hydroxy-8b-methyl-9-(1-methylethenyl)-3,6-methano-8H-1,5,7-tri-

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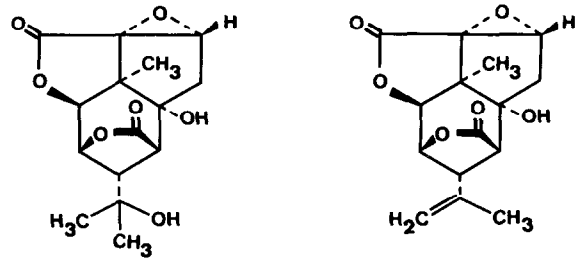

Figure 1
Structures of PCN and PCNX

oxacyclopenta[1j]cycloprop[a]azulene-4,8(3H)-dione) (Fig 1) Pharmacologically, PCXN is almost 50 times more active than PCN [6]

Although PCX has been used in the treatment of barbiturate poisoning, at present it is exclusively used as a chemoconvulsant to create experimental models of epilepsy [7] These models are used to investigate the biochemical basis of epilepsy and also in the screening of new antiepileptic drugs [8] However, despite the widespread use of PCX in studying and treating epilepsy, there is as yet no reported method for separating and quantifying the two components of the drug, and there is very little information on its pharmacokinetic behaviour The aim of this study was to develop a sensitive and reproducible method for the simultaneous determination of PCXN and PCN in serum that could be used in basic biomedical research In this paper, a reversed-phase HPLC procedure is reported for the quantification of PCXN and PCN in serum, together with its successful application to evaluate the pharmacokinetics of these two compounds after the administration of a convulsive dose of PCX to rats

Materials and Methods

Chemicals

PCX, PCXN and PCN were obtained from Sigma Chemical Co (St Louis, MO, USA) Dihydrocarbamazepine (internal standard) was purchased from Aldrich-Chemie (Steinheim, FRG) HPLC grade acetonitrile was obtained from Romil Chemicals Ltd (Shepshed, Leicester, UK) Chloroform and *n*-hexane were LiChrosolv® grade from Merck (Darmstadt, FRG) HPLC grade water was prepared with the Milli-RO/Q water purification system (Millipore Corp , Bedford, MA, USA) All other chemicals were of analytical reagent grade from Merck

Apparatus

The liquid chromatograph consisted of a Model 620 solvent delivery system (Kontron AG, Zurich, Switzerland) equipped with a Model 7125 Rheodyne injector, a Uvikon Model 720LC variable-wavelength detector (Kontron AG), a Model 3390A integrator (Hewlett–Packard, Avondale, PA, USA), and a Model 200 programmer (Kontron AG) The separation was carried out on a Spherisorb ODS, 5 μm, 250 × 4 6 mm i d column (Kontron AG) linked to a pre-column (50 × 4 6 mm i d) filled with Co Pell ODS (Whatman, Clifton, NJ, USA)

Standards

Stock solutions containing 1 mg ml^{-1} of PCX, PCXN, PCN and dihydrocarbamazepine were prepared in methanol These solutions were stable for at least 2 weeks at 4°C in vials with Teflon-faced rubber liners (Alltech Associates, Deerfield, IL, USA) The vials were wrapped with aluminium foil to exclude light

Calibration standards containing PCXN and PCN (both 0 2–20 μg ml^{-1}) were prepared in drug-free sera from Wistar rats

A working solution of the internal standard was prepared daily in water to give a concentration of 2 μg ml^{-1} dihydrocarbamazepine

Extraction procedure

To 1 ml of serum or calibration standard, 100 μl of the working internal standard solution was added, followed by 3 ml of *n*-hexane After vortex-mixing for 1 min the centrifuging at 3000 ***g*** for 5 min, the upper *n*-hexane layer was discarded, and the aqueous layer was immediately extracted with 5 ml of chloroform After centrifugation (3000 ***g*** for 5 min), the aqueous layer was discarded and the chloroform layer evaporated to dryness at room temperature (20°C) in a Speed Vac Concentrator (Savant Instruments Inc , Farmigdale, NY, USA) The residue was reconstituted in 50 μl of the mobile phase and 20 μl injected into the chromatograph

Chromatographic conditions

The mobile phase consisted of acetonitrile–1 mM ammonium acetate buffer (pH 6 4) 34.66, v/v) Prior to use, this mixture was filtered and degassed by passing it through a 0 5 μm filter type HVLP 04700 (Millipore Corp) under reduced pressure The chromatography was performed at room temperature with a flow rate of 1 5 ml min^{-1} The effluent was monitored at 200 nm, and quantification was based on peak-height ratio of analyte to the internal standard

Pharmacokinetic analysis

The pharmacokinetics of PCN and PCNX were analysed according to a one-compartment open model based on the monophasic decay of the serum concentration–time curves [9] The absorption rate constant, elimination half-life, and the area under the serum concentration–time curve (*AUC*) were calculated using the interactive program IGPHARM [10], implemented on a model 50 IBM PS/2 computer Total body clearances were also calculated using the formula CL/F = dose/AUC, where F represents the bioavailability

For this study 35 male Wistar rats weighing 250–350 g were used After i p administration of a single 3 mg kg^{-1} dose of PCX dissolved in a saline solution, groups of five animals were sacrificed by decapitation at 5, 10, 15, 30, 60, 90 and 120 min Blood was collected from the severed great vessels of the neck and serum was later separated by centrifugation, and then frozen at −20°C for subsequent analysis

Results and Discussion

Method evaluation

Figure 2 shows typical chromatograms of (A) a blank serum extract and (B) a serum extract from a rat treated intraperitoneally with a dose of 3 mg kg^{-1} of PCX and it can be seen that PCN and PCNX eluted as symmetrical peaks free of interference from

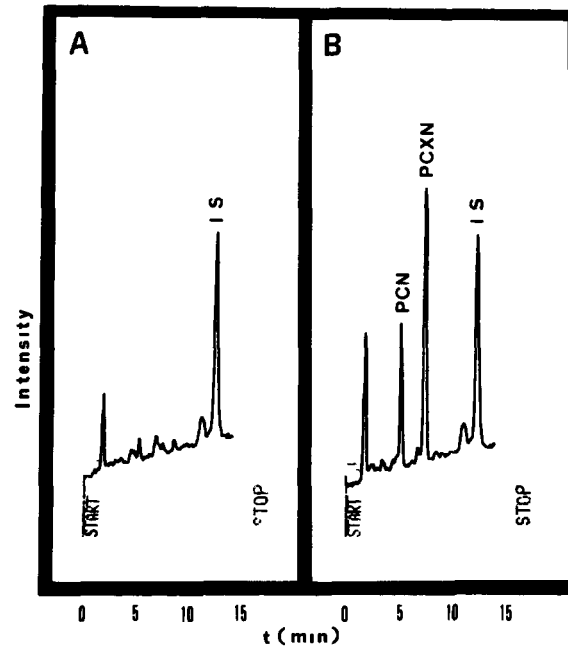

Figure 2
Chromatograms of (A) a drug-free serum extract, and (B) an extract of a serum sample obtained 30 min after i p administration of 3 mg kg^{-1} PNX I S = internal standard Attenuation was 2^3

endogenous components The retention times were 5 08 (PCN), 7 31 (PCXN) and 12 44 min (internal standard)

Ammonium acetate buffer was preferred to other buffers (citrate, phosphate) in the mobile phase since it gave the minimum background signal Concentrations of ammonium acetate buffer higher than 1 mM were accompanied by an increase in the background signal

Calibration curves for PCN and PCXN were linear within the range examined (0 2–20 μg ml^{-1}) The correlation coefficients for the regression lines were 0 997 for PCN and 0 999 for PCXN

The within-day and between-day precisions were established at three different concentrations (0 2, 5 and 20 μg ml^{-1}) for PCN and PCXN by adding these two compounds to serum The coefficients of variation 0 8–3 7% for the within-day and 1 3–4 9% for the between-day precision (Table 1) From these results it was concluded that calibration curves can be used for at least 1 week

It was found that the pre-extraction with *n*-hexane to eliminate neutral lipids, appreciably extended the useful life of the analytical column The mean recoveries of the drugs from serum were greater than 94 2 and 97 3% for PCN and PCXN, respectively These results were obtained for three concentrations (0 2, 5 and 20 μg ml^{-1}) of each compound

To determine the specificity of the method, the relative retention times of some antiepileptic drugs, and their major metabolites, that might be co-administered with PCX in basic biomedical research were determined None of the compounds tested showed any interference with the present assay method Other drugs tested, but

Table 1
Precision in the simultaneous determination of PCN and PCXN in spiked serum

Drug	Added ($\mu g\ ml^{-1}$)	Within-day (n = 10)* Found ($\mu g\ ml^{-1}$)	C V ‡ (%)	Between-day (n = 10)† Found ($\mu g\ ml^{-1}$)	C V ‡ (%)
PCN	0 2	0 21	3 7	0 23	4 9
	5	5 3	1 7	5 4	3 3
	20	20 5	0 9	20 8	2 0
PCXN	0 2	0 19	2 6	0 21	4 6
	5	5 1	1 4	5 2	2 5
	20	20 3	0 8	20 5	1 3

*Number of analyses in 1 day
†Number of days with one analysis/day
‡Coefficient of variation

Table 2
Relative retention times of assayed drugs and some potentially interfering substances

Drug	RRT
Phenylethylmalonamide	0 25
p-Hydroxyphenobarbital	0 27
Ethosuximide	0 31
Primidone	0 31
Trans-10,11-dihydro-10,11-dihydroxycarbamazepine	0 31
PCN	0 43
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	0 46
Phenobarbital	0 47
Carbamazepine-10,11-epoxide	0 52
PCNX	0 59
Phenytoin	0 75
Pentobarbital	0 89
10,11-Dihydrocarbamazepine (int stand)	1 00
Carbamazepine	1 07

RRT = relative retention time

undetected using the reported chromatographic conditions, were clonazepan, diazepam, oxazepam, *N*-desmethyldiazepam, temazepam, and flunitrazepam

Pharmacokinetic study

Figure 3 shows the mean serum concentration–time profiles for PCN and PCXN after i p administration of a single dose (3 mg kg^{-1}) of PCX The peak concentrations were reached after 30 min and the serum levels then followed a monoexponential decay with time From these results, it was assumed that a one-compartment open model approximates the pattern of PCN and PCXN distribution in rats after i p administration of PCX Table 3 summarizes the mean pharmacokinetic parameters estimated for PCN and PCXN In an attempt to correlate the PCX-induced seizure activity and the serum levels of its components, the convulsant behaviour of the animals was examined throughout the experiments About 15 min after administration of PCX, an increasing muscular twitching was observed, followed after some minutes by clonic convulsions, accompanied by intermittent tonic–clonic seizures Convulsant activity started to decline about 50 min after administration of the drug These observations are consistent with

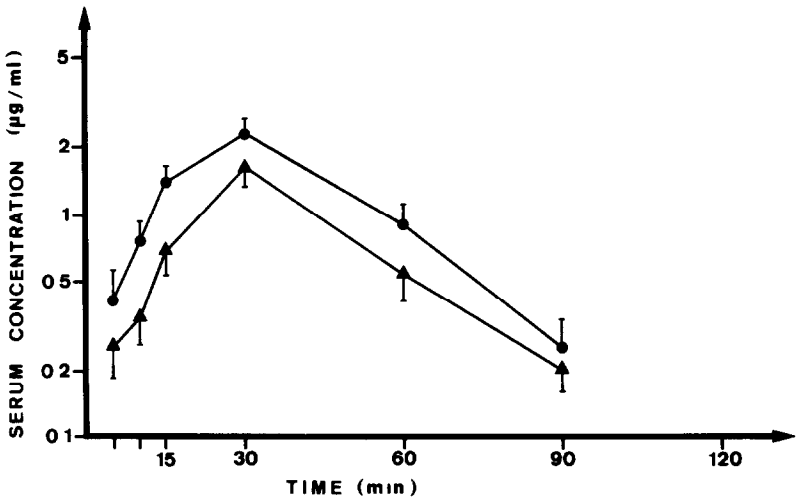

Figure 3
Mean (SE) serum concentration–time profiles of PCN and PCX after i p administration of a single dose of 3 mg kg^{-1} of PCX (n = 5) ▲, PCN, ●, PCNX

Table 3
Pharmacokinetic parameters after i p administration of a single dose of 3 mg kg^{-1} of PCX

	PCN	PCNX
Absorption rate constant (h^{-1})	2 76 ± 0 182	4 26 ± 0 240
Elimination half-life (h)	0 340 ± 0 0308	0 312 ± 0 0241
AUC (µg ml^{-1} h)	1 18 ± 0 112	1 44 ± 0 0985
Clearance bioavailability (ml h^{-1} kg)	2542 ± 97 5	2083 ± 62 8

Data are mean ±SE values, 35 animals were used

those reported previously [11, 12] These data suggest a close correlation between the convulsant activity produced by PCX and the serum levels of PCN and PCXN However, in view of the low activity reported for PCN [6], the observed pharmacologic effects of PCX may be related to the serum levels of PCXN alone During the course of these experiments, six rats died approximately 30 min after administration of PCX

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