Journal of Chromatography, 579 (1992) 123–129
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6423

High-performance liquid chromatographic determination of pamaquine, primaquine and carboxy primaquine in calf plasma using electrochemical detection

Yuuko S. Endoh, Haruo Yoshimura, Nobuo Sasaki, Yoshihito Ishihara, Hideharu Sasaki, Shigeyuki Nakamura, Yoshimitsu Inoue and Michiko Nishikawa

National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185 (Japan)

(First received December 11th, 1991; revised manuscript received March 26th, 1992)

ABSTRACT

A high-performance liquid chromatographic method with electrochemical detection is described for quantification of pamaquine, primaquine and carboxy primaquine in calf plasma. After the proteins had been precipitated with acetonitrile, the drugs were separated on a 5-μm C_{18}-modified polymer gel column with an isocratic mobile phase. The detection limit was 0.01 μg/ml in plasma for all three compounds. The applicability of the method in pharmacokinetic studies was demonstrated by determining the plasma concentrations of the three substances in calves administered a single dose of pamaquine or primaquine.

INTRODUCTION

Primaquine (PRQ), N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, and pamaquine (PAQ), N^1,N^1-diethyl-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine, are 8-aminoquinoline derivatives used as antitheilerial agents in cattle [1–3].

PRQ and its major metabolite carboxy primaquine (CPRQ) have been quantified in human or rat plasma by gas chromatography (GC) [4,5], gas chromatography–mass spectrometry (GC–MS) [6], high-performance liquid chromatography with UV detection (HPLC–UV) [5,7–9] and HPLC with electrochemical detection (HPLC–ED) [10]. Zubrod *et al.* [11], and Hughes and Schmidt [12] determined plasma concentrations of PAQ in humans, dogs and monkeys by spectrophotometry, which is not sufficiently specific or sensitive for studying the pharmacokinetics. More sensitive and selective methods to distinguish these 8-aminoquinolines have thus to be established prior to pharmacokinetic studies in cattle.

The purposes of the present work are to develop an HPLC method capable of quantifying PAQ, PRQ and CPRQ in calf plasma samples, to determine whether CPRQ occurs as a metabolite in calf plasma, and to ascertain the applicability of the method to the determination of these compounds in plasma from calves treated with PAQ or PRQ.

EXPERIMENTAL

Chemicals and standards

PRQ diphosphate was obtained from Aldrich

Correspondence to: Dr. Y. S. Endoh, National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185, Japan.

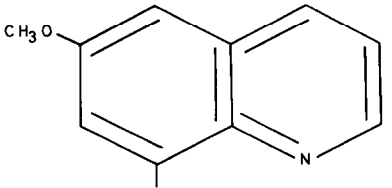
0378-4347/92/$05.00

(Milwaukee, WI, USA), and PAQ was a generous donation from Toshin Chemical Industry (Tokyo, Japan). CPRQ was provided by Dr. J. D. McChesney (University of Mississippi, USA). The molecular structures of PAQ, PRQ and CPRQ are shown in Fig. 1. The internal standard (I.S.), 2-methoxy-5-methylaniline (MMA), and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile, hydrated disodium hydrogenphosphate ($Na_2HPO_4 \cdot 12H_2O$) and potassium dihydrogenphosphate (KH_2PO_4) were obtained from Wako (Osaka, Japan).

Stock solutions of 100 μg/ml were prepared for the following compounds: PAQ and MMA in acetonitrile; PRQ in water; and CPRQ in acetonitrile–water (50:50, v/v). The solutions were stored in the dark at 4°C.

Instrumentation and HPLC conditions

The HPLC system comprised a Model 635A pump (Hitachi, Tokyo, Japan), a Model SIL-6A autoinjector (Shimadzu, Kyoto, Japan), a Model L-ECD-6A electrochemical detector (Shimadzu) with a glassy-carbon electrode set at +0.75 V *vs.* the Ag/AgCl reference electrode, and a Model C-R3A integrator (Shimadzu). A model SPD-M6A photodiode-array detector (Shimadzu) set at 195–380 nm was used instead of the electrochemical detector for the identification of the CPRQ peak. The column was a Asahipak ODP-50 stainless-steel column (15 cm × 4.6 mm I.D.) packed with 5-μm particles of C_{18}-modified polymer gel, obtained from Asahi Chemical Industry (Tokyo, Japan). The mobile phase was acetonitrile–70 m*M* phosphate buffer (pH 5.8) (23:77, v/v), containing 10 mg/l Na_2EDTA. The injection volume was 20 μl, and the flow-rate was 0.8 ml/min. The column temperature was 40°C. The chromatograms were recorded with a chart speed of 5 mm/min.



$R=CH_2NH_2$	primaquine (PRQ)
R=COOH	carboxy primaquine (CPRQ)
$R=CH_2N(C_2H_5)_2$	pamaquine (PAQ)

Fig. 1. Structures of primaquine (PRQ), carboxy primaquine (CPRQ) and pamaquine (PAQ).

Sample preparation

To a calf plasma sample (0.5 ml), in a 10-ml glass or polypropylene centrifugation tube, 0.5 ml of acetonitrile solution containing 0.2 μg/ml MMA was added and mixed with a mixer (Labomixer NS-8, Iuchi, Tokyo, Japan). The tube was shaken by hand for 2 min, allowed to stand for 15 min with occasional shaking and centrifuged at 1200 *g* for 15 min. The supernatant was filtered through a 0.45-μm membrane filter, Ekicrodisk 13-CR (Gelman Sciences Japan, Tokyo, Japan), and subsequently injected into the HPLC system. All procedures were done under protection from light.

Recovery

Recovery values were evaluated by comparing peak-height ratios of each compound extracted from spiked plasma samples with standard curves obtained from unextracted standard solutions.

Calibration curves and calculations

Calibration standards were prepared by adding known amounts of PAQ, PRQ and CPRQ to blank plasma samples. Solutions were analysed by the method described above. Peak-height ratios of the test compound to the I.S. were plotted against known concentrations for each compound. At least four to seven points were used to construct a calibration line. Values for unknowns were calculated from the slope and intercept by linear regression of the calibration curves.

Precision and accuracy of assays

Blank plasma samples were spiked with known amounts of PAQ, PRQ and CPRQ (0.02–1.0 μg/ml), and quantified by comparison of the peak-

height ratios with the calibration curves. The intra-day and inter-day precisions were determined. The coefficient of variation (C.V.) was determined by dividing the sample standard deviation by the mean and expressing the quotient as a percentage.

Application

Three Holstein calves weighing 130–300 kg body weight were used. They were kept indoors and provided with a hay and concentrate diet twice a day. Water was available ad libitum.

Calf 1 was injected with a single dose of 2 mg/kg PAQ base as an oily solution by the intramuscular route, calf 2 had a single dose of 1.0 mg/kg PRQ diphosphate (corresponding to 0.57 mg/kg as PRQ base) as an aqueous solution by the subcutaneous route, and calf 3 was given a single dose of 0.51 mg/kg PRQ diphosphate (corresponding to 0.29 mg/kg as PRQ base) by the intravenous route. Blood samples were collected from the cervical vein in heparinized tubes at appropriate intervals. Plasma was obtained by centrifugation for 15 min at 1200 *g*, and kept frozen at −20°C and protected from light until analysis.

RESULTS AND DISCUSSION

Sample treatment

Parkhurst *et al.* [9] used acetonitrile for deproteination because of the highest recovery values of the five deproteinizing agents tested for the extraction of PRQ and CPRQ from human plasma. It also gave good recoveries of PAQ, PRQ and CPRQ from fortified calf plasma in our experiments. Table I shows the recovery data for the three compounds. Mean recoveries from calf plasma fortified with 0.02–1.0 μg/ml of PAQ, PRQ and CPRQ ranged from 100.8 to 107.2%, 89.5 to 92.8% and 68.3 to 94.8%, respectively.

Chromatography

Reversed-phase liquid chromatography was used for the determination of PAQ, PRQ and CPRQ, on the basis of previous HPLC studies of PRQ and/or CPRQ [5,7–10]. The Asahipak ODP-50 column, containing an octadecyl polymer gel obtained by the introduction of C_{18} groups at the hydroxyl groups of vinyl alcohol copolymers, gave better peak shapes and separation of the three compounds from plasma components than did the Nucleosil-5 C_{18} column (Macherey-Nagel, Düren, Germany), the Nucleosil-5 CN column (Macherey-Nagel) and the LiChrosorb RP-select B column (E. Merck, Darmstadt, Germany), the C_8 column that is specially made for the separation of basic compounds. ED was used because of its better sensitivity and selectivity. Under the present HPLC conditions, the detection of the three compounds with the electrochemical detector set at +0.75 mV was two to nine times more sensitive than that with the UV detector set at 267 nm, which was shown to be the maximum absorption wavelength. Fig. 2 shows the hydrodynamic voltammograms for PAQ, PRQ and CPRQ. We used +0.75 mV for detection because the response of three compounds reached a plateau at this value.

Chromatograms of PAQ, PRQ and CPRQ standard solutions (0.025 μg/ml), and of extracts from blank, PAQ-administered and PRQ-administered calf plasma are shown in Fig. 3. Under the conditions used, PAQ, PRQ, CPRQ and MMA were well separated, and no interfering plasma components appeared in any chromatogram. A chromatogram of a plasma sample 3 h after administration of PAQ is shown in Fig. 3C. The

TABLE I

RECOVERY OF PAQ, PRQ AND CPRQ FROM CALF PLASMA (n = 3)

Compound	Concentration added (μg/ml)	Recovery, (mean ± S.D.) (%)	C.V. (%)
PAQ	0.02	107.2 ± 15.5	14.5
	0.10	100.8 ± 8.9	8.8
	1.00	103.0 ± 2.0	2.0
PRQ	0.02	89.5 ± 7.1	7.9
	0.10	92.8 ± 1.7	1.8
	1.00	91.6 ± 3.2	3.4
CPRQ	0.02	68.3 ± 16.0	23.4
	0.10	89.2 ± 8.5	9.5
	1.00	94.8 ± 1.7	1.8

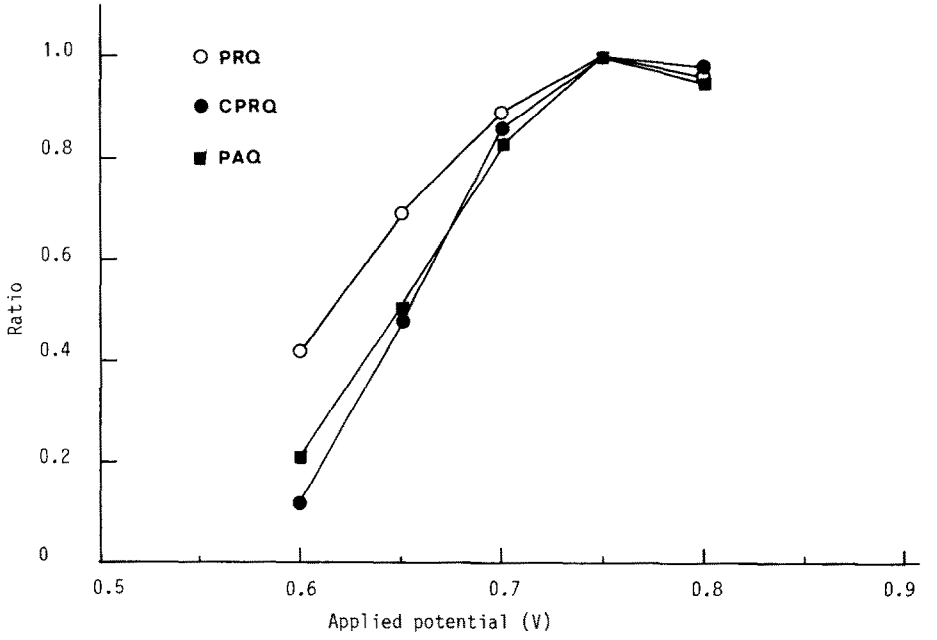


Fig. 2. Hydrodynamic voltammograms of PAQ (■), PRQ (○) and CPRQ (●). Abscissa, applied potential (V) *vs.* Ag/AgCl reference electrode; ordinate, ratio of response to maximal resposne at a given potential.

PAQ peak appeared at the retention time (t_R) of 17.3 min. A chromatogram of a plasma sample 3 min after intravenous administration of PRQ diphosphate is shown in Fig. 3D. The t_R of PRQ, CPRQ and MMA peaks were 8.3, 13.4 and 22.1 min, respectively.

Calibration curves and the detection limit

The calibration curves of PAQ, PRQ and CPRQ were linear and reproducible over the investigated concentration range of 0.01–1.0 μg/ml ($r \geqq 0.9996, n = 7$). They were constructed using linear regression, where x was the peak-height ra-

TABLE II

INTRA-DAY PRECISION AND ACCURACY OF THE ASSAY OF PAQ, PRQ AND CPRQ IN CALF PLASMA ($n = 5$)

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	M.D.[a] (%)
PAQ	0.02	0.022 ± 0.003	11.0	8.0
	0.10	0.101 ± 0.003	2.7	0.9
	1.00	0.943 ± 0.027	2.9	−5.7
PRQ	0.02	0.020 ± 0.001	5.2	1.0
	0.10	0.101 ± 0.004	4.0	0.7
	1.00	0.979 ± 0.028	2.9	−2.2
CPRQ	0.02	0.020 ± 0.003	14.0	−0.5
	0.10	0.098 ± 0.005	4.6	−1.6
	1.00	1.040 ± 0.021	2.0	4.0

[a] Mean percentage difference, M.D. (%) = (mean − added)/added × 100.

TABLE III

INTER-DAY PRECISION AND ACCURACY OF THE ASSAY OF PAQ, PRQ AND CPRQ IN CALF PLASMA

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	M.D.[a] (%)	n
PAQ	0.02	0.019 ± 0.004	19.9	−5.5	5
	0.10	0.100 ± 0.001	1.4	−0.3	5
	1.00	0.980 ± 0.019	1.9	−2.0	4
PRQ	0.02	0.021 ± 0.003	12.0	3.0	5
	0.10	0.099 ± 0.006	5.9	−0.8	5
	1.00	1.009 ± 0.023	2.3	0.9	4
CPRQ	0.02	0.020 ± 0.003	13.9	2.0	5
	0.10	0.099 ± 0.004	3.8	−1.0	5
	1.00	1.041 ± 0.051	4.9	4.1	4

[a] Mean percentage difference, M.D. (%) = (mean − added)/added × 100.

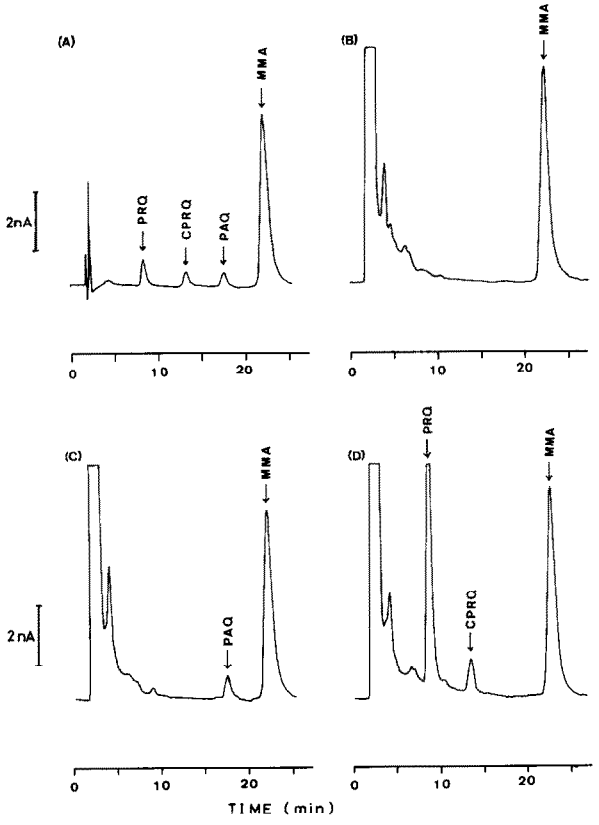


Fig. 3. Typical chromatograms of PAQ, PRQ, CPRQ and MMA (I.S.): (A) standard solution containing 0.025 μg/ml PAQ, PRQ and CPRQ; (B) extract of blank plasma; (C) extract of a plasma sample from calf 1 3 h after intramuscular administration of 2 mg/kg PAQ base; (D) extract of a plasma sample from calf 3 3 min after intravenous administration of 0.51 mg/kg PRQ diphosphate.

tio and y was the fortified concentration of the compounds (μg/ml). The calculated regression equations for PAQ, PRQ and CPRQ were $y = (0.312 \pm 0.006)x + (2.40 \pm 1.35) \cdot 10^{-3}$ ($r = 0.9999$), $y = (0.187 \pm 0.005)x + (2.93 \pm 6.38) \cdot 10^{-3}$ ($r = 0.9997$) and $y = (0.600 \pm 0.007)x - (5.08 \pm 3.70) \cdot 10^{-3}$ ($r = 0.9996$), respectively. The detection limit was 0.01 μg/ml for all compounds (signal-to-noise ratio of 2–3).

Precision and accuracy

Intra-assay and inter-assay precision and accuracy data are shown in Tables II and III. The method exhibited good accuracy and reproducibility. In one case (PAQ, 0.02 μg/ml) we could not obtain an inter-assay precision of less than 15%. We suppose that the presence of mechanical noise could interfere with accurate evaluation of this peak at very low concentrations.

Identification of CPRQ peak

Photodiode-array detection of plasma extracts was performed in order to identify the CPRQ peak and to confirm the peak purities. The extract of a plasma sample 6 h after subcutaneous administration of PRQ diphosphate was evaporated to dryness. The residue was dissolved in a volume of acetonitrile eight times less than the volume before evaporation, and analysed by photodiode-array HPLC. The concentration step

was necessary because the CPRQ concentration in plasma was too low for direct photodiode-array detection. The spectrum of the CPRQ peak from plasma of the PRQ-administered calf was identical with the standard spectrum (Fig. 4A and B). No difference was observed in the UV spectrum of CPRQ from the PRQ-administered calf for upslope (t_R = 13.24 min), apex (t_R = 13.38 min) and downslope (t_R = 13.56 min), demonstrating that there were no impurities in the peak (Fig. 4C). We conclude that CPRQ, the major PRQ metabolite in human and rat plasma, also occurs in calf plasma.

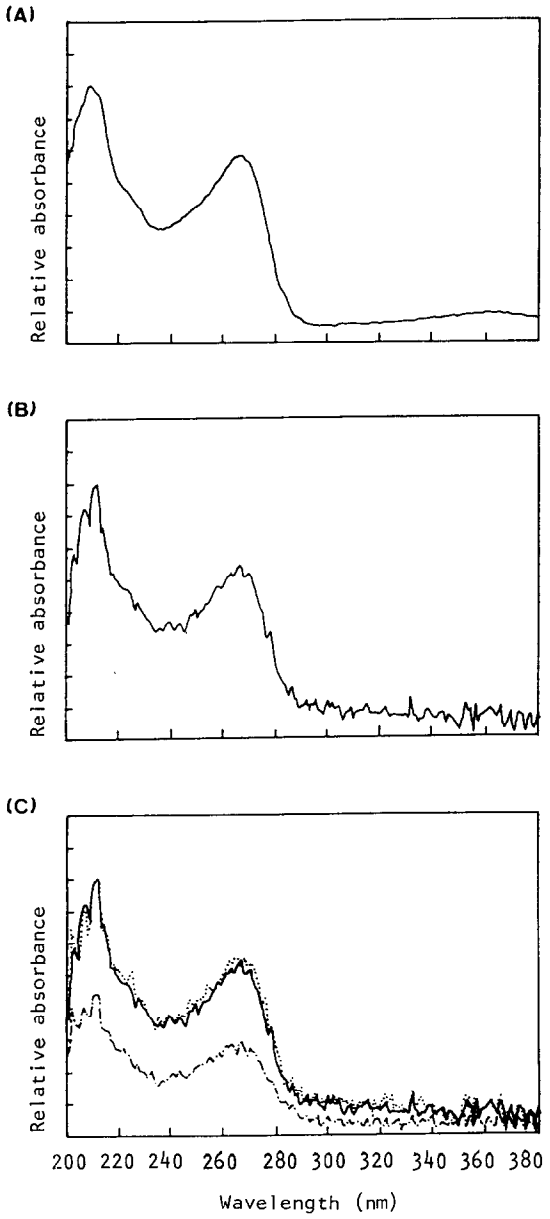


Fig. 4. UV spectra of CPRQ peaks. (A) UV spectrum of CPRQ peak from a standard solution containing 10 μg/ml CPRQ; (B) UV spectrum of CPRQ peak from the extract of a plasma sample from calf 2 6 h after a single dose of 1.0 mg/kg PRQ diphosphate (corresponding to 0.57 mg/kg as PRQ base) as aqueous solution by the subcutaneous route; (C) UV spectra of upslope (t_R = 13.24 min, -----), apex (t_R = 13.38 min, ——) and downslope (t_R = 13.56 min, – · –) of CPRQ peak from the same extract as (B).

Application

An attempt was made to determine whether the present method is applicable to the quantification of PAQ, PRQ and CPRQ in plasma of calves given the recommended therapeutic doses of PAQ or PRQ diphosphate.

A plasma concentration *versus* time curve for PAQ after intramuscular administration is shown in Fig. 5. PAQ was detected up to 8 h, with a maximum concentration of 0.067 μg/ml at 2 h.

A plasma PRQ and CPRQ concentration *versus* time curve after intravenous administration of PRQ diphosphate is shown in Fig. 6. The plas-

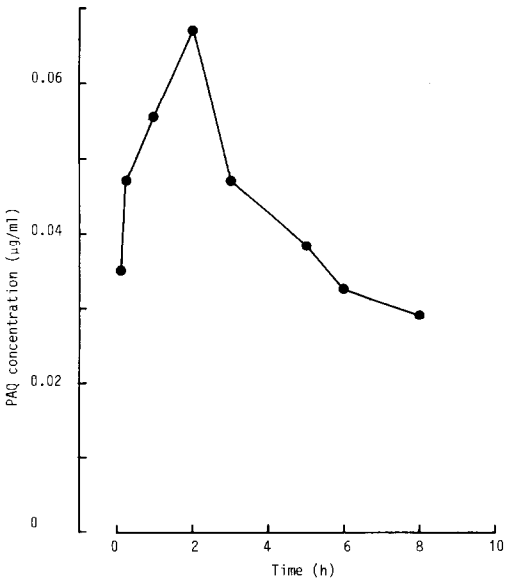


Fig. 5. Plasma concentration of PAQ *versus* time in calf 1 receiving a single intramuscular dose of 2 mg/kg PAQ base as oily solution.

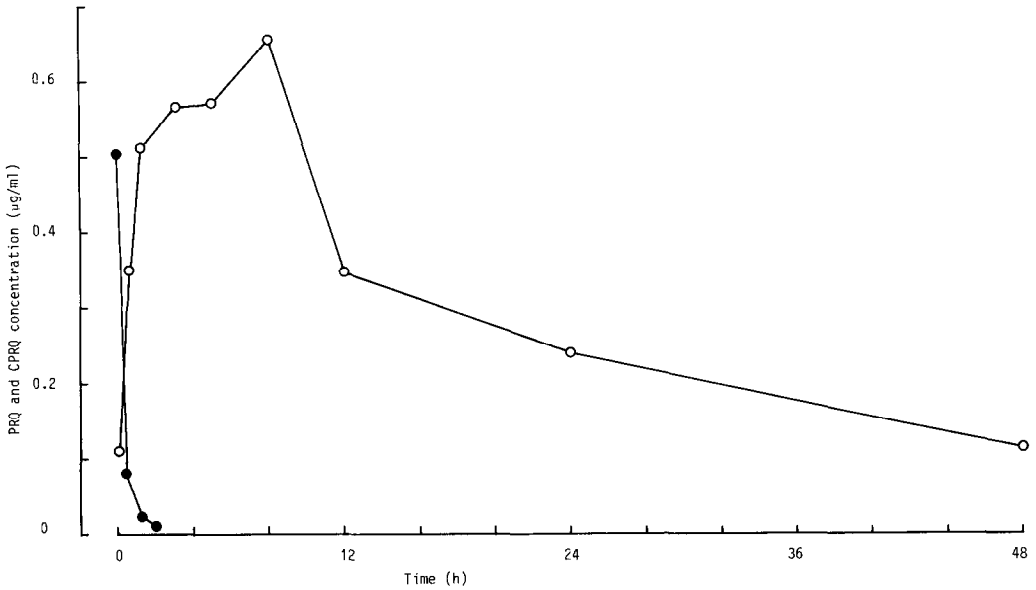


Fig. 6. Plasma concentrations of PRQ (●) and CPRQ (○) in calf 3 after an intravenous dose of 0.51 mg/kg PRQ diphosphate (corresponding to 0.29 mg/kg as PRQ base) as aqueous solution.

ma concentration of PRQ at 3 min was 0.506 μg/ml, which rapidly decreased to 0.011 μg/min at 2 h. No PRQ could be detected at 2.5 h. CPRQ reached a maximum concentration of 0.654 μg/ml at 8 h, after which it decreased gradually and fell below the detection limit at 96 h.

CONCLUSION

The present HPLC–ED method for the simultaneous determination of PAQ, PRQ and CPRQ is rapid and sensitive, and was shown to be applicable to pharmacokinetic studies in calves. Our preliminary studies revealed the presence of CPRQ, the major metabolite in human and rat plasma, in the plasma of PRQ-administered calves.

ACKNOWLEDGEMENT

The authors thank Dr. James D. McChesney, of the University of Mississippi, for providing the CPRQ.

REFERENCES

1 Z. H. Zhang, *Vet. Parasitol.*, 23 (1987) 11.
2 N. P. Stewart, A. J. de Vos and I. Shiels, *Trop. Anim. Health Prod.*, 22 (1990) 109.
3 N. P. Stewart, A. J. de Vos, N. McHardy and N. F. Standfast, *Trop. Anim. Health Prod.*, 22 (1990) 116.
4 T. G. Rajagopalan, B. Anjaneyulu, V. D. Shanbag and R. S. Grewal, *J. Chromatogr.*, 224 (1981) 265.
5 J. K. Baker, *J. Chromatogr.*, 230 (1982) 69.
6 J. Greaves, D. A. Price-Evans, H. M. Gilles and J. D. Baty, *Biomed. Mass Spectrom.*, 6 (1979) 109.
7 G. W. Mihaly, S. A. Ward, G. Edwards, M. L'E. Orme and A. M. Breckenridge, *Br. J. Clin. Pharmacol.*, 17 (1984) 411.
8 S. A. Ward, G. Edwards, M. L'E. Orme and A. M. Breckenridge, *J. Chromatogr.*, 305 (1984) 239.
9 G. W. Parkhurst, M. V. Nora, R. W. Thomas and P. E. Carson, *J. Pharm. Sci.*, 73 (1984) 1329.
10 M. V. Nora, G. W. Parkhurst, R. W. Thomas and P. E. Carson, *J. Chromatogr.*, 307 (1984) 451.
11 C. G. Zubrod, T. J. Kennedy and J. A. Shannon, *J. Clin. Invest.*, 27 (1948) 114.
12 H. B. Hughes and L. H. Schmidt, *Proc. Soc. Exp. Biol. Med.*, 73 (1950) 581.