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DETERMINATION OF PIQUINDONE IN CANINE PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This report describes a rapid, sensitive and selective method for the determination of piquindone in canine plasma and piquindone and the N-demethyl metabolite of piquindone in canine urine, utilizing normal-phase high-performance liquid chromatography (HPLC) with isocratic elution at ambient temperature and monitoring the ultraviolet absorbance of the eluent at 254 nm The trimethyl analogue of piquindone is used as the internal standard in the HPLC assay of plasma The assay was applied to the measurement of concentrations of piquindone in the plasma and urine of a dog following single intravenous and oral administration of 5 mg/kg doses of piquindone hydrochloride dihydrate

INTRODUCTION

Piquindone, *rac* -3-ethyl-2,6-dimethyl-4,4a,5,6,7,8,8a,9-octahydro-4a,8a-*trans*-1H-pyrrolo[2,3-*g*]isoquinolin-4-one (compound I, Fig 1), is a member of series of pyrroloisoquinolines synthesized by Olson et al [1] which has demonstrated neuroleptic activity and which is being presently investigated as an antipsychotic agent [2] A gas chromatographic—chemical-ionization mass spectrometric assay has recently been described and used to measure piquindone concentrations following single, oral 5-mg doses of I · HCl · $2H_2O$ to normal human volunteers [3]

This report describes a rapid, sensitive and selective method for the determination of piquindone in canine plasma and piquindone and the N-demethyl metabolite [4] of piquindone (compound II, Fig. 1) in canine urine, utilizing

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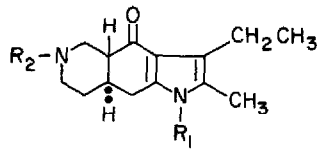

COMPOUND	R_1	R_2
I	H	CH_3
II	H	H
III	CH_3	CH_3

Fig 1 Chemical structures of compounds I, II and III

normal-phase high-performance liquid chromatography (HPLC) with isocratic elution at ambient temperature and monitoring the ultraviolet (UV) absorbance of the eluent at 254 nm. The trimethyl analogue of piquindone (compound III, Fig. 1) is used as the internal standard in the HPLC assay of plasma The assay was used to measure concentrations of I in the plasma and urine of a dog following single intravenous and oral administration of 5 mg/kg doses of $I \cdot HCl \cdot 2H_2O$.

EXPERIMENTAL

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV detector with a 254-nm wavelength kit (Waters Assoc , Milford, MA, U.S A.) The column was a 30 cm × 4 mm I D , stainless-steel column containing 10-μm μPorasil silica gel (Waters Assoc) The mobile phases used for isocratic normal-phase chromatography were mixtures of methylene chloride—methanol—ammonium hydroxide (90 10 0.1 for system 1 and 80 20 0 1 for system 2) for the plasma and urine assays, respectively The chromatographic systems were operated at ambient temperature with a flow-rate of 2 5 ml/min and at column head pressures of 6.9 and 8 3 MPa for system 1 and system 2, respectively The capacity factors (k') of compounds I and III in system 1 were 4 6 and 2 13, respectively, and for I and II in system 2 were 1.31 and 5.81, respectively. A typical chromatogram for 50 ng of I and 25 ng of III injected using system 1 with a detector sensitivity of $5 \cdot 10^{-3}$ a.u.f s. is shown in Fig. 2D. Compound I (100 ng) and compound II (300 ng) injected using system 2 yielded peaks of nearly full scale pen response, with the detector sensitivity set at $1 \cdot 10^{-2}$ a u f s The chart speed on the 10-mV recorder was 1.27 cm/min (Speedomax, XL, 625 Series, Leeds and Northup, North Wales, PA, U S.A.)

Upon completion of a day's analysis, the ammonium hydroxide and the endogenous material were flushed from the column with methanol and the column was stored in methylene chloride Although it is reported that high alkalinity reduces the useable life of HPLC columns, the column was used as described for several months without any noticeable deterioration in performance

Standard solutions

Plasma assay Weigh out 12.94 mg of compound I · HCl · $2H_2O$ ($C_{15}H_{22}N_2O$ · HCl · $2H_2O$, mol wt = 318.85, m p = 124°C, equivalent to 10 00 mg of the free base of compound I) into a 10-ml volumetric flask and dissolve the powder in 10 ml of methanol This stock solution (solution A) contains 1 0 mg/ml I Weigh out 11 40 mg of compound III · HCl (internal standard, $C_{16}H_{16}H_{24}N_2O$ · HCl, mol wt = 296.84, m.p = 241—243°C, equivalent to 10 00 mg of the free base of compound III) into a 10-ml volumetric flask and dissolve the powder in 10 ml of methanol. This stock solution (solution B) contains 1.0 mg of III.

These stock solutions are used to prepare 10-ml working solutions containing 0, 0 025, 0.5, 0 10, 0 25, 0 50, 1 0 or 2 0 μg of I and 0 5 μg of III per 100 μl of methylene chloride—methanol (90 10).

Aliquots (10 μl) of these standards (equivalent to 0, 2 5, 5, 10, 25, 50, 100 or 200 ng of I and 50 ng of III) are injected to establish the external standard curve. Aliquots (100 μl) of the same working solutions are added to drug-free plasma to construct the recovered standard curve for the direct quantitation of the concentrations in the experimental samples and for the determination of the percentage recovery Aliquots (100 μl) of the working solution containing 0 μg of I and 0 5 μg of III are added to each experimental sample as the internal standard The recovered standard curve is prepared for each day of analysis to establish the reproducibility of the assay

Urine assay Weigh out 11 57 mg of compound II · HCl ($C_{14}H_{20}N_2O$ · HCl, mol wt = 268.79, m.p > 300°C, equivalent to 10 00 mg of the free base of compound II) into a 10-ml volumetric flask and dissolve the powder in 10 ml of methanol This stock solution (solution C) contains 1 0 mg/ml II

Stock solutions A and C are used to prepare working solutions in 10-ml volumetric flasks by suitable dilution with methylene chloride—methanol (80 20) containing 0 5, 1 0, 2 0, 3 0, 4 0 or 6 0 μg of I and 0 5, 1.0, 2 0, 4 0, 6 0 or 8 0 μg of II per 200 μl

Aliquots (10 μl) of these working solutions (equivalent to 25, 50, 100, 150, 200 or 300 ng of I and 25, 50, 100, 200, 300 or 400 ng of II) are injected to establish the external standard curve Aliquots (200 μl) of the same working solutions are added to drug-free urine to construct the recovered standard curves of I and II, respectively, and for the determination of the percentage recovery.

The internal standard (III) cannot be used in the assay of dog urine owing to endogenous interferences which co-elute with III Analysis of the parent compound (I) and the N-demethyl metabolite (II) in dog urine samples are performed by direct interpolation against the peak height of I or II obtained from the recovered standard curve.

Reagents

All reagents are of analytical-grade purity and are prepared in deionized, distilled water Phosphate buffer (1.0 *M*, pH 11) is prepared by mixing 530 ml of 1 *M* K_2HPO_4 · $3H_2O$ (228 23 g/l) and 470 ml of a saturated solution of Na_3PO_4 After thoroughly mixing the pH of the mixture is adjusted to 11 0 with 1 *M* KH_2PO_4 · $3H_2O$ or saturated solution Na_3PO_4 as needed. Other

reagents include ammonium hydroxide (ACS grade, J T. Baker, Phillipsburg, NJ, U.S.A), chloroform, methylene chloride and Na_2SO_4-dried anhydrous methanol (Burdick & Jackson Labs. Muskegon, MI, U.S A.). The mobile phases are deaerated in an ultrasonic batch prior to use

Plasma assay. Into a 50-ml centrifuge tube (equipped with a PTFE No. 16 stopper) add 100 μl of the solution containing 0 μg of I and 0.5 μg of internal standard (compound III), 1 0 ml experimental plasma and 2 5 ml of 1 *M* phosphate buffer (pH 11) and mix well. Extract the samples with 12 ml of chloroform by slowly shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S A.) and centrifuge the samples in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S A.) at 0—5°C for 10 min at approx. 1207 *g* (2200 rpm). Aspirate off as much of the aqueous phase as possible Transfer a 9-ml aliquot of this extract into a tapered 15-ml centrifuge tube (equipped with a PTFE No. 13 stopper) Evaporate the chloroform extract to dryness at 60°C in an N-EVAP evaporator (Organomation Assoc., Worcester, MA, U S.A.) under a stream of clean, dry nitrogen Dissolve the residue in 200 μl of methylene chloride—methanol (90.10) and inject a 20-μl aliquot using system 1 as the solvent system for HPLC analysis. A 1.0-ml specimen of drug-free plasma and seven 1.0-ml specimens of drug-free plasma containing 100 μl of the working solutions (equivalent to 0 025, 0.05, 0.1, 0.25, 0 5, 1 or 2 μg of compound I and 0 5 μg of compound III per ml of plasma) are processed along with experimental samples as recovered standards. These standards are used to establish the power regression calibration curve for the quantitation of I in the experimental samples

Urine assay A procedure identical to the plasma assay is followed, except that no internal standard (III) is added The extraction residues are dissolved in methylene chloride—methanol (80·20), a 5—20 μl aliquot is injected into the HPLC system and system 2 is used as the solvent system for HPLC analysis. Six 1.0-ml specimens of drug-free urine containing 200 μl each of working solution (equivalent to 0.5, 1 0, 2.0, 3 0, 4.0 or 6.0 μg of compound I and 0.50, 1.0, 2.0, 4 0, 6.0 or 8.0 μg of compound II per ml of urine) are analyzed as the recovered standards These standards are used to establish a linear regression calibration curve for the quantitation I and II in the experimental samples

RESULTS AND DISCUSSION

Statistical evaluation of the method

The method was evaluated over a concentration range of 0 025—2.00 μg/ml piquindone in plasma (Table I) One sample was assayed at each of seven concentrations in six analytical experiments The mean inter-assay coefficient of variation for the six experiments was 5.8%. The recovery of piquindone from the plasma samples was 99.2 ± 6.1% (S.D.) and the limit of quantitation was 0.025 μg/ml.

The method was also evaluated in urine over concentration ranges of 0.5—06 0 μg/ml piquindone and 0.5—8.0 μg/ml N-demethyl metabolite (II). In one single experiment in which each calibration concentration was analyzed in duplicate mean intra-assay coefficients of variation of approx. 5% were obtained. The recoveries of I and II from urine samples were 90.4 ± 4.8%

TABLE I

INTER-ASSAY PRECISION OF THE HPLC ASSAY OF COMPOUND I IN PLASMA

Concentration added (μg/ml)	Concentration found (mean ± S D)* (μg/ml)	Coefficient of variation (%)
0 025	0 025 ± 0 002	8 2
0 050	0 050 ± 0 002	3 5
0 100	0 099 ± 0 005	4 6
0 250	0 250 ± 0 015	6 1
0 500	0 511 ± 0 038	7 3
1 00	1 00 ± 0 04	3 5
2 00	1 99 ± 0 14	7 4
Mean		5 8

*Data accumulated from single analysis of standards in six analytical experiments

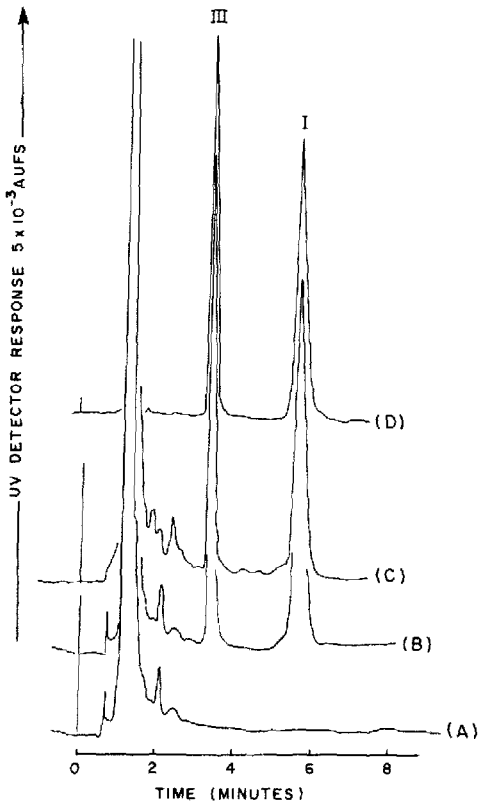

Fig. 2 HPLC profiles of (A) drug-free dog plasma, (B) drug-free dog plasma containing 1 0 μg/ml I and 0 5 μg/ml III, (C) plasma from a dog 5 min after a 5 mg/kg intravenous dose of I · HCl · $2H_2O$ (concentration 1 04 μg/ml), (D) authentic standards of 50 ng of I and 25 ng of III (10 μl injected)

(S.D.) and 70.7 ± 5 5% (S.D.), respectively, with a limit of quantitation at 0 5 μg/ml.

Application of the method to biological specimens

The assay was applied to the analysis of plasma (Fig 2A—C) and urine (Fig 3) samples from a dog given intravenous and oral solution doses of 5 0 mg/kg of I · HCl · $2H_2O$ (Table II).

Following intravenous administration, a maximum level of 1.39 μg/ml of plasma was measured at 2.5 min and the concentration declined to non-detectable amounts (< 0 025 μg/ml) after 4 h. Following oral administration, a peak level of 0 92 μg/ml of plasma was measured at 45 min and the concentration declined with a half-life of approx 45 min to non-detectable amounts (< 0 025 μg/ml) after 4 h Plasma concentrations of II were below the limit of quantitation (< 0.05 μg/ml) at all sampling times

The urinary excretion of compounds I and II was also determined in the same dog Following intravenous and oral administration, 3 2 and 5 9% of the dose were recovered as I in the 0—24 h excretion period, respectively No measurable concentrations (< 0.5 μg/ml) were obtained in the 24—48 h urine sample, following either administration The N-demethyl metabolite (II),

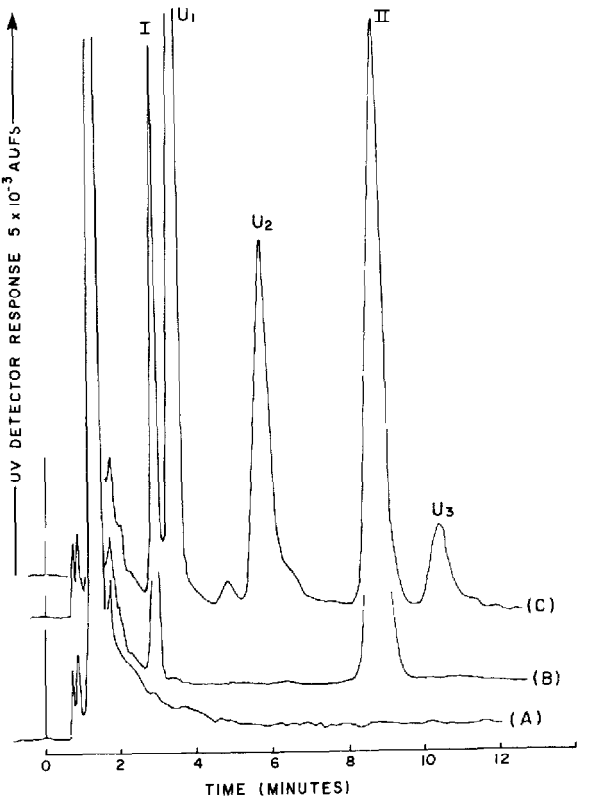

Fig 3 HPLC profiles of a chloroform extract of (A) drug-free dog urine, (B) drug-free dog urine containing 2 μg/ml I and II and (C) 0—24 h urine from a dog after a 5 mg/kg intravenous dose of I · HCl · $2H_2O$ (concentrations of I and II were 4 7 and 7 5 μg/ml, respectively)

TABLE II

PLASMA CONCENTRATION IN THE DOG FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF A SINGLE 5 0 mg/kg DOSE OF I · HCl · $2H_2O$

Time	Concentration (μg/ml)	
	Intravenous	Oral
0 min	N D *	N D
2 5 min	1 39	—**
5 min	1 04	–
10 min	0 93	0 04
20 min	0 79	0 69
30 min	0 78	0 91
45 min	0 64	0 92
1 h	0 70	0 73
1 5 h	0 40	0 53
2 h	0 23	0 34
3 h	0 12	0 20
4 h	0 06	0 10
6 h	N D	N D
7 h	N D	N D

*N D = Not detectable (< 0 025 μg/ml)
**No sample taken

calculated as I equivalents (molecular weight factor, F = 246.35/268.80) accounted for 5 1 and 5.3% of the intravenous and oral doses, respectively. A chromatogram of the extract of the 0—24 h urine following intravenous administration (Fig. 3) indicated the presence of three possible metabolites noted as U_1, U_2 and U_3 with capacity factors of 1.68, 3.50 and 7.31, respectively. Similar peaks were seen in the chromatograms of the extracts of the 0—24 h urine following oral administration. Quantitation of these components will have to await structural elucidation and synthesis of authentic metabolites.

Evaluation of analytical parameters

Determination of compound II was based on its UV absorption spectrum in methylene chloride—methanol—ammonium hydroxide (90 10 0 1) which showed a maximum for the compound at 250 nm. Molindone hydrochloride, a structural analogue which has UV absorption maxima at 255 and 299 nm, was evaluated as the internal standard for the assay of I but could not be used since it was not retained on the chromatographic column under the HPLC conditions used. The trimethyl analogue (III) of compound I has a UV absorption maximum at 257 nm and similar, but not identical, chromatographic properties as I, and could be used as the internal standard.

Reversed-phase chromatography with varied compositions of water, methanol and isopropanol as the mobile phase utilizing a stainless-steel μBondapak C_{18} column (10 μm particle size, 30 cm × 4 mm I D , Waters Assoc.) was initially investigated These studies involved extraction of I into methylene chloride—chloroform (80 20), hexane—methylene chloride (90 10) and hexane—isopropanol (95 5) from blood, plasma or a protein-free filtrate of plasma buffered to pH 9 0 and 11 0 with either 1 *M* phosphate or 1 *M* borate

buffer. The respective extracts were evaporated to dryness and the residue was reconstituted in the mobile phase. Problems of solubility were encountered in that lipids which were extracted from the samples would not dissolve in a mobile phase which had a high (> 50%) water content Injection of this material into the HPLC system caused a significant increase in column head pressure owing to precipitation of lipids which prevented injection of more than three to four samples for analysis on a routine basis. Reconstitution of the extract in methanol and injection of this solution also resulted in increases in pressure, most probably due to precipitation of lipids in the mobile phase

Increasing the solubility of the residue in the reversed-phase chromatographic system was attempted by increasing the methanol concentration and by the introduction of an ion-pair reagent [5] These reagents form ion-pair complexes with I and permitted an increase in the methanol concentration to as much as 80%. In general, it was noted that the larger the counter ion, the less polar the ion-pair complex, and the greater the percentage of methanol required to give an equivalent k' value The ion-pair reversed-phase chromatographic system worked quite well with a chloroform extract of a protein-free filtrate of plasma obtained with perchloric acid and buffered to pH 11, with little or no pressure build-up during a single day of operation However, equilibration time to obtain a stable baseline exceeded 1 h with the larger counter ions The highest recovery obtained was approx 75% and no suitable chemical analogue of I could be found for use as an internal standard.

The use of a mobile phase containing a small percentage of concentrated ammonia on a silica gel column has been described [6—8]. The mobile phase of methylene chloride—methanol—ammonium hydroxide (90 10·0 1) provides a system for the analysis of I in plasma in which the lipid materials are totally soluble. The assay is straightforward and precludes the need for either a protein-free filtrate of plasma and/or centrifugation of the reconstituted extract. The data indicated that increasing the methanol content from 10 to 20% in the methylene chloride—methanol—ammonium hydroxide system yielded k' values for compounds I and II which decreased from 4.60 and 21.1 to 1.31 and 5 81, respectively The increase in methanol concentration thus permitted the simultaneous measurement of I and II in the dog urine samples within 10 min. The more rapid system was not used in the plasma analysis because of the need to maintain good resolution between I (k' = 4.6) and III (k' = 2 13). The internal standard (III) could not be used in the assay of dog urine, owing to extracted endogenous materials which co-elute with III in the mobile phase used.

CONCLUSION

A normal-phase HPLC assay for the measurement of piquindone in dog plasma and piquindone and its N-demethyl metabolite in dog urine is described. The assay was applied to the measurement of piquindone and metabolite concentrations in dog plasma and urine following 5 mg/kg intravenous and oral doses of piquindone hydrochloride dihydrate. The assay does not possess sufficient sensitivity to measure concentrations of piquindone in human plasma following low single oral doses of piquindone to normal human volunteers.

However, it is expected that the assay will be useful in therapeutic drug monitoring in psychotic patients receiving relatively high doses of piquindone hydrochloride dihydrate

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