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DETERMINATION OF THE ANALGESIC 3-PHENOXY-N-METHYLMORPHINAN IN PLASMA BY GAS CHROMATOGRAPHY USING EITHER POSITIVE CHEMICAL IONIZATION MASS SPECTROMETRIC OR NITROGEN—PHOSPHORUS-SPECIFIC DETECTION ANALYSIS

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SUMMARY

The compound 3-phenoxy-N-methylmorphinan (I) is under development as an analgesic agent. Studies on the biotransformation of the drug in the rat and in the dog have shown that I is extensively metabolized by N-demethylation to yield the nor-analogue (I-A), by *para*-hydroxylation of the 3-phenoxy ring to yield the phenolic analogue (I-B), cleavage of the ether linkage to yield levorphanol (I-C), and its N-demethylation to yield nor-levorphanol (I-D). The presence of these four metabolites (two of which, I-B and I-C, have analgesic potential) in addition to the parent drug, necessitated the development of sensitive and specific assays for their quantitation in plasma. This was accomplished by the development of (a) a high-performance liquid chromatographic assay using UV detection to obtain a qualitative/semi-quantitative profile of the metabolites present in plasma; (b) a gas chromatographic—nitrogen—phosphorus-specific detection method for the determination of the parent drug (I) for pre-clinical drug evaluation; and (c) a sensitive and specific gas chromatographic—positive chemical ionization mass spectrometric assay for eventual clinical evaluation for the determination of I and a key metabolite levorphanol (I-C). This report presents some preliminary pharmacokinetic data on I and I-C in the dog obtained during pre-clinical development.

INTRODUCTION

The benzomorphinan class of compounds has yielded several clinically effective drugs currently marketed as antitussive (dextromethorphan) and analgesic (levorphanol, levallorphan, dextrorphan) agents [1] (Table I). Clinical interest in this class of compounds continues in the search for a non-narcotic analgesic agent without addiction potential [2]. The analogue 3-phen-

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oxy-N-methylmorphinan [I] is a member of a series of phenoxymorphinan analogues synthesized by Mohacsi [3, 4] and is under development as a non-narcotic analgesic agent. Studies on the biotransformation of I in the rat [5] and in the dog [6,7] have shown that the drug is extensively metabolized not only by N-demethylation to form the nor-analogue (I-A), but also by *para*-hydroxylation of the 3-phenoxy ring to yield I-B and cleavage of the ether linkage to yield levorphanol (I-C) and its subsequent N-demethylation to yield nor levorphanol (I-D) (Fig. 1). The presence of four metabolites in addition to the parent drug necessitated the development of separate assays for their quantitation.

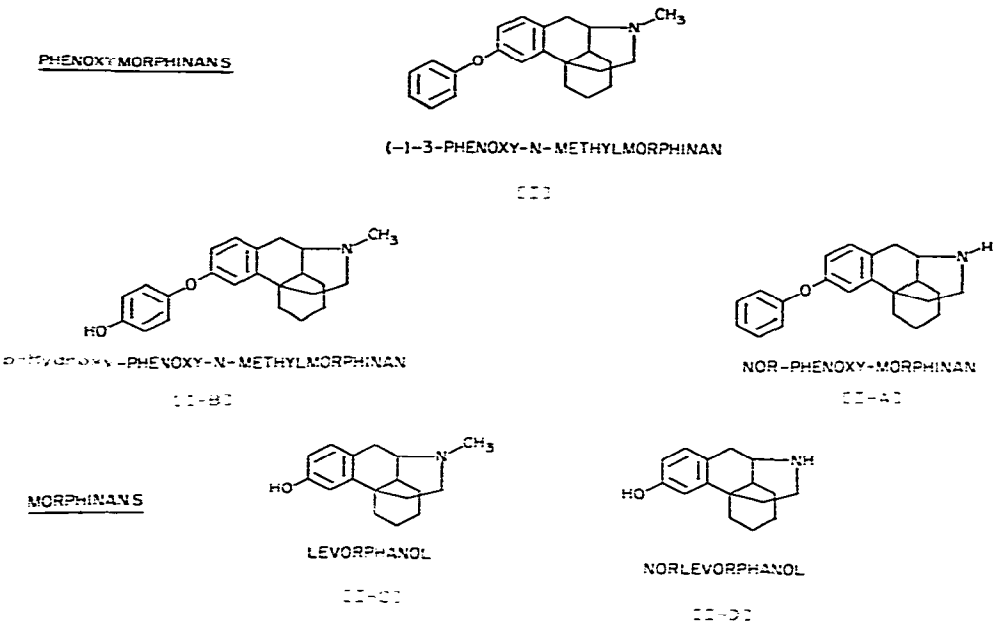

Fig. 1. Chemical structures of phenoxy-N-methylmorphinan and its major metabolites.

The morphinans are amenable to a variety of analytical techniques. Spectrofluorometry was initially used for dextromethorphan (V) [8] and dextrorphan (VI) [9] but was not sufficiently sensitive or specific for pharmacokinetic studies. Gas chromatography (GC) was used for the determination of dextromethorphan [10], 2-hydroxy-N-cyclopropylmethyl morphinan [11] and levorphanol (I-C) [12] by flame ionization detection, dextromethorphan [13] and levorphanol [14] by nitrogen—phosphorus-specific detection (NPD), and dextromethorphan by electron-capture detection [15]. Radioimmunoassay was successfully used for the determination of dextromethorphan [16], 2-hydroxy-N-cyclopropylmethyl morphinan [17], butorphanol [18] and levorphanol [19,20]. Recently, gas chromatographic—mass spectrometric (GC—MS) analysis [21—23], and high-performance liquid chromatographic (HPLC) analysis using either UV [24,25] or electrochemical detection [26] for morphines and morphinans, was also reported, attesting to the intensive analytical endeavor in this class of compounds.

Due to the extensive biotransformation of [I] in the rat [5] and in the dog [6,7], developing a suitable assay in plasma led to the investigation of: (a) an HPLC assay using UV detection to obtain a qualitative/semi-quantitative estimate of [I] and its metabolites detectable in plasma; (b) a GC—NPD method of adequate sensitivity (ca. 10 ng/ml of plasma) for pre-clinical drug evaluation; and (c) a sensitive and specific GC—positive chemical ionization mass spectrometric (GC—PC-MS) assay for I and a key metabolite (I-C) (sensitivity 2.5 ng/ml) for eventual clinical evaluation.

This report presents preliminary pharmacokinetic data on 3-phenoxy-N-methylmorphinan and its metabolite levorphanol in dog plasma using the aforementioned methods obtained during pre-clinical drug development.

EXPERIMENTAL

Reagents

The 1 *M* phosphate buffer, pH 11 was prepared by mixing 530 ml of 1 *M* K_2HPO_4 and 470 ml of saturated Na_3PO_4. Titrate to pH 11.0 with 1 *M* KH_2PO_4 or saturated Na_3PO_4 as needed. The 0.1 *N* hydrochloric acid, and 1.0 *N* sodium hydroxide solutions were made up in distilled water. The *n*-heptane was pesticide grade obtained from Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.). Methanol (Nanograde) was from Mallinckrodt (St. Louis, MO, U.S.A.) or from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methylene chloride (Nanograde) from Mallinckrodt (St. Louis, MO, U.S.A.). Pentafluorobenzyl bromide (PFBB) and N,O-bistrimethylsilyltrifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL, U.S.A.) and tetrabutylammonium hydrogen sulfate (TBAHS) was from Aldrich (Milwaukee, WI, U.S.A.).

GC—NPD of I in plasma

The sensitivity and specificity of GC—NPD for the determination of morphinans [13,14] prompted this investigation. The primary goal was the quantitation of the parent drug only, since metabolic studies in the dog [6,7] showed low concentrations of the metabolites in plasma which would be impractical to quantitate without derivatization and extensive clean-up.

Analytical standards

Authentic standards of I and II (Table I) of pharmaceutical grade purity (> 99%) were used as the analyte and internal standard, respectively, in the assay. Stock solutions of I containing 10 mg in 10 ml of methanol, and of II containing 1.0 mg in 10 ml of methanol were prepared. Six working standards of solutions containing 5, 10, 25, 50, 75 or 100 ng of I each containing 100 ng of II (internal standard) per 100 μl of solution were prepared using suitable aliquots of serial 1:10 dilutions of the stock solution of I in methanol. Aliquots (5 μl) equivalent to 0.25, 0.5, 1.25, 2.5, 3.75 or 5.0 ng of I and 5.0 ng of II of the working standard solutions were injected into the chromatograph to establish an external calibration curve to determine the linearity and stability of the system.

TABLE I

CHEMICAL NOMENCLATURE FOR PHENOXY-N-METHYLMORPHINAN, ITS MAJOR METABOLITES AND RELATED ANALOGUES

Compound	Chemical name	Molecular weight
Phenoxymorphinans		
I	(—)-3-Phenoxy-N-methylmorphinan	333
I-A	(—)-3-Phenoxymorphinan	319
I-B	(—)-3-(*p*-Hydroxy)phenoxy-N-methylmorphinan	349
II	(—)-3-(*p*-Methyl)phenoxy-N-methylmorphinan	347
III	(—)-3-Pentadeuterophenoxy-N-methylmorphinan	338
Morphinans		
I-C	(—)-3-Hydroxy-N-methylmorphinans; (levorphanol)	257
I-D	(—)-3-Hydroxymorphinan; (norlevorphanol)	243
IV	(—)-3-Hydroxy-N-allylmorphinan; (levallorphan)	283
V	(+)-3-Methoxy-N-methylmorphinan; (dextromethorphan)	271
VI	(+)-3-Hydroxy-N-methylmorphinan; (dextrorphan)	257
Compounds used as internal standards		
II	GC—NPD assay for I.	
III	GC—PCI-MS assay for I.	
IV	GC—PCI-MS assay for I-C.	
V	HPLC assay and carrier substance for GC—PCI-MS assay.	

Instrumental parameters

A Hewlett-Packard Model 5170A gas chromatograph equipped with NPD was used in conjection with a 1.22 m × 2 mm I.D. borosilicate glass column containing 2% OV-17 on 100—120 mesh Gas—Chrom Q. The flow-rates of helium carrier, hydrogen and air, were 1.4 2.5 and 4.0 ml/min, respectively and the operating temperatures for the column oven, injection port and detector were 230°C, 250°C and 300°C, respectively. The silicate bead of the detector was operated at 16 V d.c., the electrometer attenuation was 1 × 8.

Sample preparation

Into a 50-ml PTFE stoppered-centrifuge tube, add 100 μl of stock solution of internal standard (100 ng of II), 1 ml of plasma, 2 ml of phosphate buffer, pH 11 and 2 ml of distilled water and mix well. Extract the sample with 10-ml portions of *n*-heptane twice by shaking at moderate speed for 15 min on a reciprocating shaker (Eberbach). Centrifuge the samples in a refrigerated centrifuge (Damon/IEC, Model PR-J, Rotor No. 253, Needham, MA, U.S.A.), at 5°C for 5 min at approximately 2500 rpm (1500 *g*). Combine each 10-ml extract in another 50-ml PTFE stoppered centrifuge tube. Add 5 ml of 0.1 *N* hydrochloric acid and extract into the acid phase as described above. After centrifugation, remove the heptane phase by aspiration, wash the aqueous phase with another 10-ml portion of heptane and remove the heptane wash by aspiration. Add 0.5 ml of 1 *N* sodium hydroxide, and 2 ml of 1 *M* phosphate buffer, pH 11 (adjust pH to 11.0 ± 0.2). Extract with 10-ml portions of heptane twice as described above and combine these extracts in a tapered 15-ml PTFE-stoppered centrifuge tube. (The first extract is evaporated to near dryness, (ca. 100 μl), before addition of the second). Evaporate the combined

extract to dryness, redissolve in 100 μl of methanol, and inject a 5-μl aliquot for GC—NPD analysis. Typical chromatograms of I and II recovered from plasma, having retention times of 5.9 and 8.2 min, respectively, are shown in Fig. 2A.

Along with the samples, a 1-ml specimen of control plasma and six 1-ml specimens of control plasma containing 100 μl of the working standard solutions equivalent to 5, 10, 25, 50, 75 or 100 ng of I each containing 100 ng of II (internal standard) per ml of plasma, respectively, are run to establish a calibration curve for the quantitation of the unknowns.

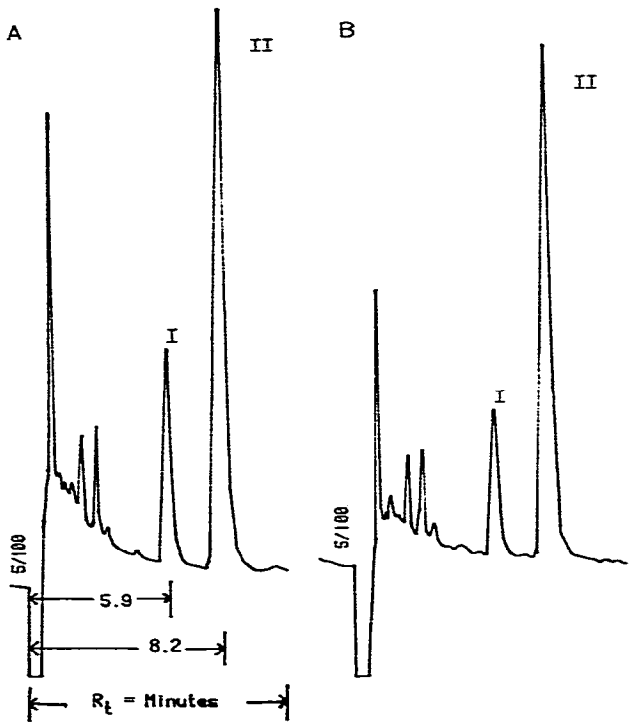

Fig. 2. Chromatograms of GC—NPD analysis of (A) parent drug (I) using the analogue II as the internal standard (equivalent to 75 ng of I and 300 ng of II) recovered from control plasma; (B) dog plasma 1-h post oral dose 10 mg/kg (57 ng of I per ml). R_t = Retention time. Conditions: column, 1.22 m × 2 mm I.D., 2% OV-17 on 100—120 mesh Gas-Chrom Q; temperature, injector 250°C, column 230°C, detector 300°C; flow-rates, helium 1.4 ml/min, hydrogen 2.5 ml/min, air 4.0 ml/min; detector parameters, applied voltage 16 V d.c., attenuation 1 × 8.

Percent recovery and sensitivity limits

The overall recovery of I and II is approximately 65 ± 5% (S.D.) over the concentration range of 10—225 ng/ml and the sensitivity limit is 10.0 ng of I per ml of plasma.

GC—PCI-MS of I in plasma

Metabolic studies in the dog [6,7] showed that the parent drug was the major component in plasma. The presence of levorphanol, (Fig. 1), a metab-

olite with known analgesic activity [27], necessitated the development of a sufficiently sensitive and specific method for the quantitation of the intact drug I, per se in the presence of I-C.

Analysis for intact drug [I] in plasma

Analytical standards. 3-Phenoxy-N-methylmorphinan (I) of pharmaceutical grade purity (> 99%) and 3-pentadeuterophenoxy-N-methylmorphinan (III) (Table I) of purity (> 98.6%) (synthesized by W. Burger and A. Liebman, Radiochemical Synthesis Group Chemical Research Division, Hoffmann-La Roche) were the analytical standards used. Compound IV (levallorphan) (Table I) was used as the carrier substance to minimize adsorption losses in the assay.

Stock solutions of each compound containing 1 mg/ml were prepared in methanol, and serial 1:10 dilutions of each were used to prepare working standard solutions containing 1.0, 2.5, 5.0, 7.5, 10, 25 or 50 ng of I, 25 ng of III (internal standard), and 200 ng of IV (carrier) per 100 μl of methylene chloride—methanol (9:1).

Instrumental parameters. A Finnigan Model 9500 gas chromatograph, equipped with a splitless injector (SGE, Austin, TX, U.S.A.), was coupled via a glass capillary restrictor to a Finnigan Model 3200 mass spectrometer, which was modified in house for chemical ionization in both positive [28] and negative [29] ion modes. Selected ion monitoring (SIM) was performed using a Finnigan Promim® peak monitor with the ion chromatograms recorded by a Rikadenki Model KA-41 four-channel recorder. The GC column was a 1.22 m × 2 mm I.D. pre-siliconized borosilicate glass column packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.), which was conditioned at 300°C with no flow of carrier gas for 4 h followed by overnight conditioning at 280°C with methane carrier gas flow-rate at 10 p.s.i.g. head pressure. The conditioned column was further primed with several (1 μl) injections of BSTFA followed by 10-μl injections of control plasma extract in order to obtain optimal sensitivity and chromatographic peak symmetry for I and III. The operation temperatures for column oven, injector port and interface oven were 265°C, 275°C and 215°C, respectively. Ammonia was introduced through the direct insertion probe inlet as the reagent gas and methane was the carrier gas at a head pressure of 10 p.s.i.g. The methane ion source pressure was 0.5 Torr with a total ion source pressure of 0.6 Torr with ammonia added.

The mass spectrometer was typically operated as follows: ion energy source, repeller and collector at +10 eV, lens at —40 V, electron energy source at —200 V, filament emission at 1.1 mA, the electron-multiplier was set at —2000 V and the continuous dynode electron multiplier at —2500 V. The ion source voltages were optimized daily to give the maximum signal response consistent with Gaussian peak shape and unit mass resolution.

The divert valve was open to vent the first 30 sec of column effluent after which it was closed to permit the effluent to enter the ion source and the filament current turned on. The Promim peak monitor channels were operated at a gain of 10^8 V/A, 100 msec dwell time and a filter setting of 0.5 Hz. The recorder chart speed was 2 cm/min.

The $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III) (I-D_5) were used for quantitative SIM based on the methane—ammonia-PCI-mass spectra of the respective compounds, (Fig. 3), and were eluted at 1.83 min, (Fig. 4A).

Sample preparation. Into a 50-ml PTFE stoppered centrifuge tube add 25 ng of III (internal standard) and 200 ng of IV (carrier substance) (Table I), in methylene chloride—methanol (9:1) solution, (total volume 100 µl). Add 1 ml of plasma, 2 ml of distilled water and 2 ml of 1 *M* phosphate buffer, pH 11, and mix well at very slow speed on a Vortex mixer.

Add 10 ml of *n*-heptane and extract for 15 min on a Eberbach reciprocating shaker at 80—100 strokes per min. Centrifuge the samples for 10 min at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Damon/IEC-Model PR-J, rotor No. 253) at 5°C. Transfer the heptane layer (ca. 9.5 ml) into a 15-ml conical centrifuge tube, evaporate to dryness, reconstitute the residue in 100 µl of chloroform and inject a 5—10 µl aliquot for GC—MS analysis. Along with the unknowns process seven 1-ml specimens of control plasma, one as the blank specimen and the remaining six samples containing 1.0, 2.5, 5.0, 10, 25, or 50 ng of I, each containing 25 ng of III (internal standard) and 200 ng of IV (carrier), [i.e., 100 µl of each of the working standard solutions], to establish the calibration curve for the quantitation of the unknowns by direct interpolation of the peak height ratio response of I/III vs. concentration of I in ng/ml of plasma.

Statistical validation of the assay for I in plasma. The inter-assay precision and reproducibility of added standards of I over the concentration range of 1.0—50.0 ng/ml of plasma are summarized in Table II. Although 1.0 ng/ml

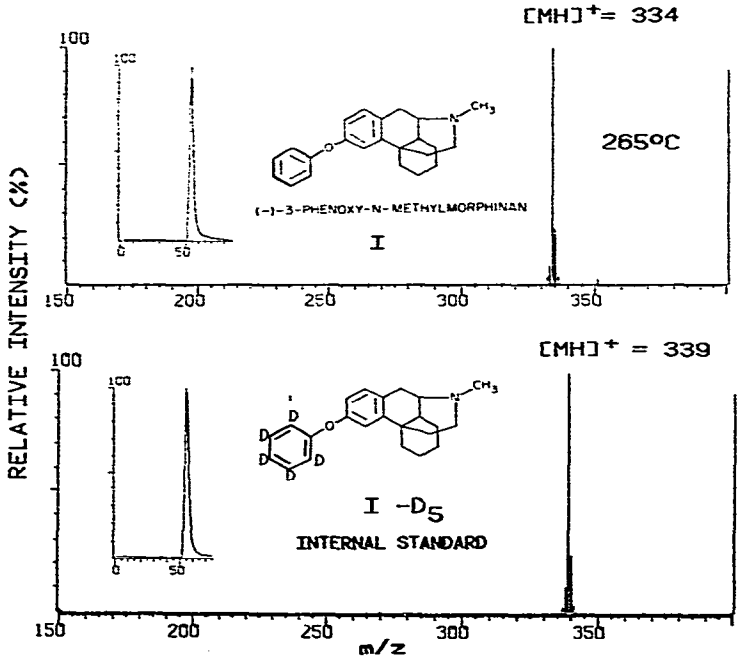

Fig. 3. Methane—ammonia PCI-mass spectra of I and its pentadeuteroanalogue (III) (I-D_5) showing the $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III), respectively. The inserts represent the total ion chromatogram for each compound.

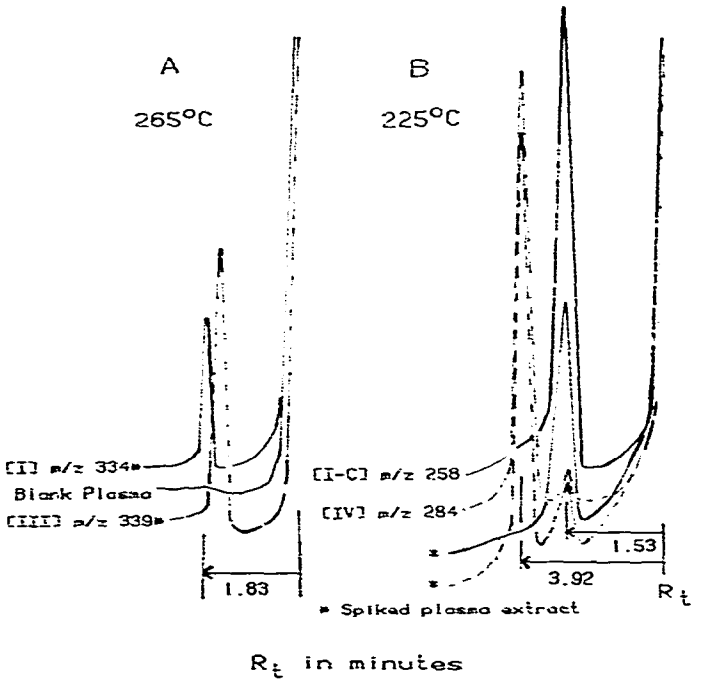

Fig. 4. Ion chromatograms of GC—PCI-MS analysis of: (A) parent drug (I) and its internal standard (III); (B) metabolite, levorphanol (I-C) and its internal standard, levallorphan (IV) determined as underivatized compound. ——, $[MH]^+$ of I and I-C; — — —, $[MH]^+$ of internal standards, III and IV.

TABLE II

INTER-ASSAY PRECISION OF RECOVERED CALIBRATION STANDARDS OF I FROM PLASMA DETERMINED BY GC—PCI-MS ANALYSIS

Sample concn. (ng/ml)	n*	Mean concn. found (ng/ml)	Standard deviation	Relative standard deviation (%)
1.0	9	1.34	± 0.55	40.6
2.5	11	2.42	± 0.43	17.8
5.0	9	4.91	± 0.67	13.6
7.5	10	7.45	± 0.55	7.3
10.0	9	9.45	± 0.63	6.6
25.0	8	25.69	± 1.44	5.6
50.0	8	49.79	± 2.63	5.3

*Out of five replicate sets, some standards were injected two to three times during the assay.

of I is measurable its precision is unsatisfactory (40% deviation). Concentrations from 2.5—50.0 ng/ml show good precision especially above 5.0 ng/ml, the mean recovery in this range being 65—70%. The sensitivity limit of the assay was established at 2.5—5.0 ng/ml with a sample/blank response ratio of > 2:1 as the limit of detection.

RESULTS AND DISCUSSION

The extensive biotransformation of 3-Phenoxy-N-methylmorphinian (I) in the rat [5] and in the dog [6,7] resulted in at least four metabolites (Fig. 1), two of which (I-B and I-C) have known analgesic activity. Of these two, I-C (levorphanol) is a marketed analgesic agent (L-Dromoran®), [27], hence the quantitation of this metabolite is relevant.

High-performance liquid chromatographic (HPLC) analysis with UV absorbance at 254 nm was used to obtain a qualitative profile of the plasma concentrations of I as its active metabolites I-B and I-C in animal studies following chronic dosing at 100 mg/kg.

Plasma (0.5—1.0 ml) was extracted with 10 ml of *n*-heptane twice, the combined extracts were evaporated to dryness, the residue was dissolved in 100 μl of methylene chloride—methanol (9:1) and a 50-μl aliquot was injected for HPLC analysis.

Normal-phase chromatography was performed on a Whatman Partisil PXS, 10-μm column 25 cm × 4.6 cm I.D. generating 39,000 plates/m. The mobile phase was a mixture of methylene chloride—methanol—concentrated ammonia (90 : 9.4 : 0.6), the column head pressure was 4.8 MPa (ca. 700 p.s.i.) with a flow-rate of 2 ml/min. 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.), operated at a sensitivity of $5 \cdot 10^{-3}$ a.u.f.s., connected to a Hewlett-Packard 5-mV recorder (equivalent to $2.5 \cdot 10^{-3}$ a.u.f.s.) operated at 0.5 in./min. The retention times of I, I-A, I-B and I-C, were 1.6, 13.5, 5.9 and 11.2 min, respectively, using dextromethorphan (V) as the internal standard which had a retention time of 3.2 min. The corresponding capacity factors (k') were 1.0, 8.4, 2.7, 6.0 and 1.6, respectively (Fig. 5). The detector sensitivity yielded full scale peaks for 80 ng of I, 200 ng of I-A, 120 ng of I-B, 200 ng of I-C and 1200 ng of V (internal standard), resulting in sensitivity limits of approximately 10, 30, 20, and 500 ng/ml for I, I-A, I-B and I-C, respectively.

The analysis of plasma specimens from dogs dosed chronically for 90 days at 100 mg/kg showed the presence of measurable amounts of I and I-B (Fig. 5C). Due to the poor UV absorbance of the morphinan analogues I-C and I-D at 254 nm, these two pharmacologically active metabolites could not be determined with adequate sensitivity by HPLC analysis. Even fluorometric detection of these compounds [8,9], (with excitation at 280 nm and fluorescence emission at 315 nm), did not significantly increase the sensitivity limits of their detection. The HPLC method was suitable only for the determination of I and its metabolites I-A and I-B in plasma profiles in the rat and in the dog dosed chronically at high doses (> 100 mg/kg). It was not sufficiently sensitive for single-dose pharmacokinetic studies which necessitated the investigation of the inherently more sensitive GC—NPD analysis [13,14].

Preliminary studies with GC—NPD showed that I, I-A and I-C could be analyzed in a single run using II as the internal standard. The peak for I-C showed tailing indicating the need for derivatization of the phenolic OH group to improve its chromatographic behavior and detector sensitivity, when analyzed at 210°C on a 1.22 m × 2 mm I.D. borosilicate glass column containing 3% OV-17 on 100—120 mesh Gas-Chrom Q.

The retention times for I, I-A, I-C and II (internal standard) were 11.2, 13.3, 3.1 and 15.7 min, respectively, indicating the feasibility of a specific assay. Metabolic studies in a dog administered a single 10 mg/kg oral dose of ^{14}C-labeled I [6] indicated however that I-A, I-B and I-C were minor metabolites in plasma, therefore the assay was modified for the specific determination

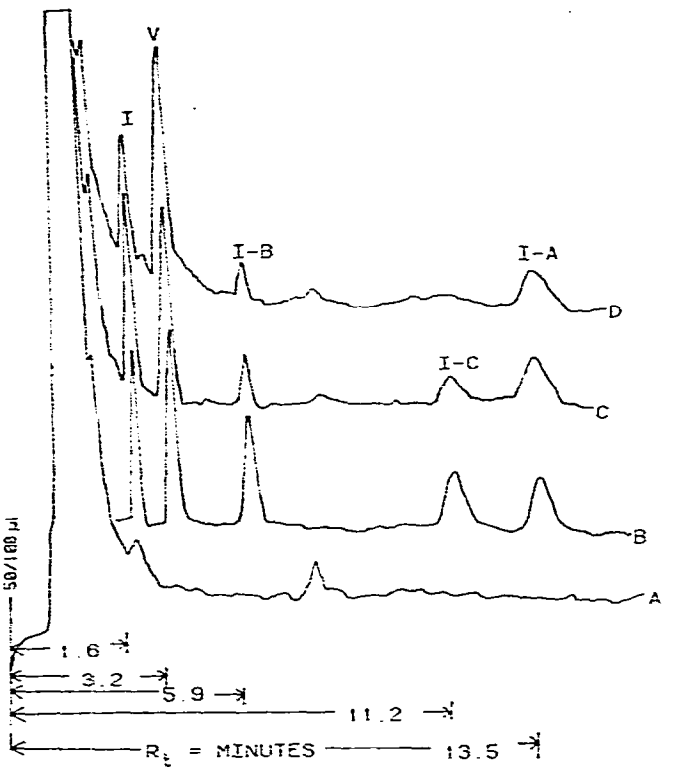

Fig. 5. Chromatograms of the HPLC analysis of (A) control dog plasma extract; (B) authentic standards; (C) authentic standards recovered from plasma equivalent to 25 ng of I, 25 ng of I-A and I-B, 250 ng of I-C and 400 ng of V (internal standard); (D) dog plasma drug—metabolite profile 6-h post chronic oral dosing at 100 mg/kg/day. Conditions: column, Whatman Partisil; mobile phase, chloroform—methanol—concentrated ammonia (90:9.4:0.6); detection, UV at 254 nm, 0.005 a.u.f.s.; recorder, 5 mV; flow-rate 2 ml/min.

of I using II as the internal standard in the assay for preclinical biopharmaceutic—pharmacokinetic studies in the dog (Fig. 2A and B).

The GC—NPD assay was only sufficiently sensitive for monitoring plasma concentrations in the dog at doses of 10 mg/kg or higher. Thus, the need for a more sensitive and specific assay for I capable of also determining I-C (a pharmacologically active metabolite), for eventual clinical pharmacokinetic evaluation of the drug led to the investigation of GC—MS analysis as the method of choice since it has found extensive application in the analysis of narcotics and related analogues [21,22].

Methane—ammonia PCI mass spectra of I using the analogue II as the internal standard (during the early phases of method development), gave intense $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 348 (II), respectively with potential low nanogram sensitivity. This lead was developed into a validated method once the 3-pentadeuterophenoxy-N-methylmorphinan analogue (III) was synthesized, monitoring the $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III), respectively, for quantitation (Fig. 3).

The methane—ammonia full-scan GC—PCI-mass spectra were determined

using a Finnigan 9500 gas chromatograph coupled to a Finnigan 1015 mass spectrometer [modified in house for PCI analysis [28]] equipped with a Finnigan 6000 computer for spectral analysis. Methane (carrier gas) was introduced at a head pressure of 5 p.s.i.g., and ammonia (reagent gas) was introduced into the ion source at a total source pressure of 0.45 Torr. The operating temperatures ranged as follows: column (225—265°C), injection port (275—300°C) and the interface oven was at 250°C, depending on the individual compound analyzed. The GC effluent was vented for 30—45 sec before the sample was introduced into the ion source of mass spectrometer.

The analysis of the phenolic metabolites I-B and I-C was attempted as the intact compound, and following derivatization either via silylation (O-trimethylsilyl, O-TMS) or extractive alkylation with PFBB using TBAHS in 0.2 *N* sodium hydroxide as the counter ion to yield the corresponding O-PFB derivatives [23], amenable to GC—PCI-MS analysis.

GC—PCI-MS analysis of the phenolic metabolites I-B and I-C

Intact levorphanol (I-C). The analgesic activity of this compound [27] focused attention on its quantitation to demonstrate its presence at pharmacologically significant concentrations in dog plasma. The determination of levorphanol was accomplished using compound IV (levallorphan) as the internal standard (originally added as the carrier substance in the assay for I). The sample remaining after the analysis of I was re-analyzed, at a lower temperature (225°C) and the $[MH]^+$ ions at *m/z* 258 (I-C) (Fig. 6A) and *m/z* 284 (IV) (internal standard) were monitored for quantitation as shown in the

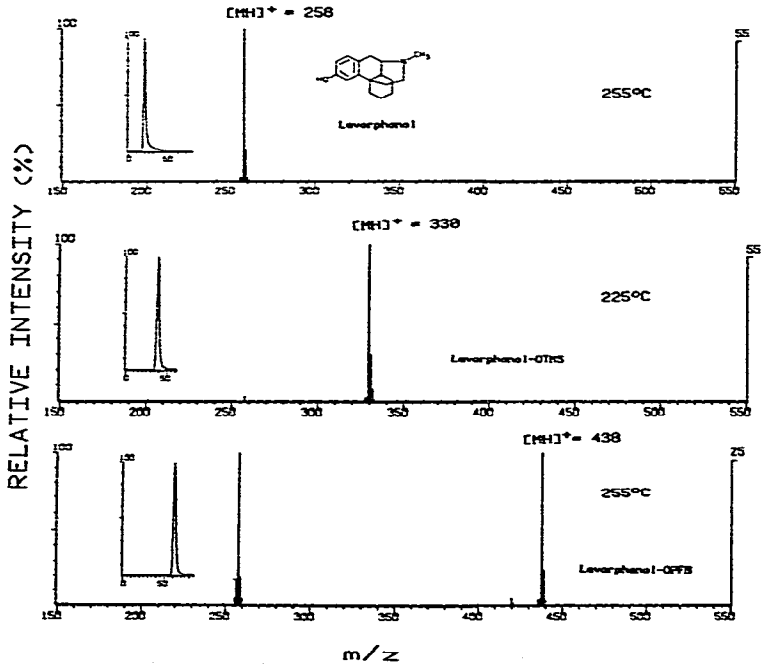

Fig. 6. Methane—ammonia-PCI-mass spectra of levorphanol, levorphanol—O-TMS and levorphanol—O-PFB showing the $[MH]^+$ ions at *m/z* 258, *m/z* 330 and *m/z* 438, respectively. The inserts represent the total ion chromatogram for each compound or derivative.

ion chromatograms (Fig. 4B), at their respective retention times of 1.53 and 3.92 min. The sensitivity limit of quantitation was about 2.5 to 5.0 ng of levorphanol per ml of plasma with an overall recovery of about 50%.

The tailing chromatographic peak for levorphanol indicated the need for derivatization of the phenolic OH group to improve its chromatographic behavior and sensitivity to detection. This was attempted via either silylation or extractive alkylation with PFBB. The former derivative would only improve the chromatographic behavior of the compound by reducing the polarity of the phenolic group and its adsorption on the column. The latter derivative should also increase its sensitivity to GC—PCI-MS due to the introduction of the electron capturing PFB-electrophore.

Trimethylsilyl ethers of I-B and I-C. Varying concentrations of I-B and I-C (2.5—50.0 ng/ml) each containing 50 ng of IV (internal standard) were vacuum dried and treated with 100 μl of BSTFA in glass stoppered 15-ml conical centrifuge tubes, by heating in a water bath at 60—70°C for 1 h. The excess reagent was evaporated to dryness under nitrogen, the residue was reconstituted in 100 μl of chloroform, 10-μl aliquots of which were injected for GC—PCI-MS using the previously described parameters. The $[MH]^+$ ions monitored were: *m/z* 422 (I-B—O-TMS), *m/z* 330 (I-C—O-TMS) and *m/z* 356 (IV—O-TMS) (internal standard), respectively.

The column and injection port temperatures were lowered to 225°C and 250°C respectively, for the analysis of the TMS ethers. Although the formation of I-C—O-TMS in vitro was demonstrated; *m/z* 330 (Fig. 6B), the ion chromatograms of the O-TMS derivatives of I-B, I-C and IV following extraction from plasma were very weak indicating either poor reaction yield and/or chemical instability of the derivatives. However, I-C and IV can be analyzed without derivatization by sequentially monitoring the $[MH]^+$ ions at *m/z* 258 (I-C) and *m/z* 284 (IV) (internal standard) at 225°C, followed by raising the column temperature to 265°C for monitoring *m/z* 334 (I) and *m/z* 348 (II) (internal standard), respectively (Fig. 4B).

Pentafluorobenzyl ethers of I-B, I-C and IV. Direct reaction of I-B, I-C and IV with PFBB using diisopropylethylamine as the catalyst resulted in very low yields of the derivatives. However, extractive alkylation of these compounds dissolved in methylene chloride using PFBB as the alkylating agent and a solution containing 0.1 *M* TBAHS in 0.2 *N* sodium hydroxide as the counter ion for extractive alkylation [23], yielded useable derivatives for levorphanol (I-C) and levallorphan (IV), but not for I-B which apparently did not react.

Varying amounts of I-C (2.5—100 ng/ml) each containing 50 ng of [IV] (as internal standard) were dissolved in 6 ml of methylene chloride to which 10 μl of PFBB reagent was then added and extracted with 2 ml of a solution containing 0.1 *M* TBAHS in 0.2 *N* sodium hydroxide as the counter ion for extractive alkylation. The methylene chloride layer was washed with dilute acid (0.05 *N* sulfuric acid), followed by water to remove the acid, and evaporated to dryness. The residue was dissolved in 100 μl of methylene chloride and a 10-μl aliquot injected for analysis. The $[MH]^+$ ions at *m/z* 438 (I-C—O-PFB) and *m/z* 464 (IV—O-PFB) (internal standard) were monitored for the quantitation of I-C.

Methane—ammonia-PCI-mass spectra of I-C, I-C—O-TMS and levorphanol—O-PFB derivatives showing the $[MH]^+$ ions at *m/z* 258, *m/z* 330 and *m/z* 438, respectively, substantiated the formation of the derivatives (Fig. 6). The second major fragment at *m/z* 258 in the levorphanol—O-PFB mass spectrum is most likely due to rearrangement in the ion source resulting in the loss of the PFB fragment (*m/z* 179) and protonation to levorphanol (*m/z* 258). This competing reaction may reduce the overall potential sensitivity of the derivative.

Typical ion chromatograms (determined on a 1.83 m × 2 mm I.D. column containing 3% OV-1 on 100—120 mesh Gas-Chrom Q at 255°C) of authentic standards of I and II (internal standard) and of I-C and IV (internal standard) recovered from plasma and derivatized by extractive alkylation are shown in Fig. 7 demonstrating the feasibility of the technique for the simultaneous quantitation of phenoxymorphinan and levorphanol.

The overall data for I and I-C—O-PFB were reproducible and linear over the concentration range studied (r=0.9971). The procedure was recently modified

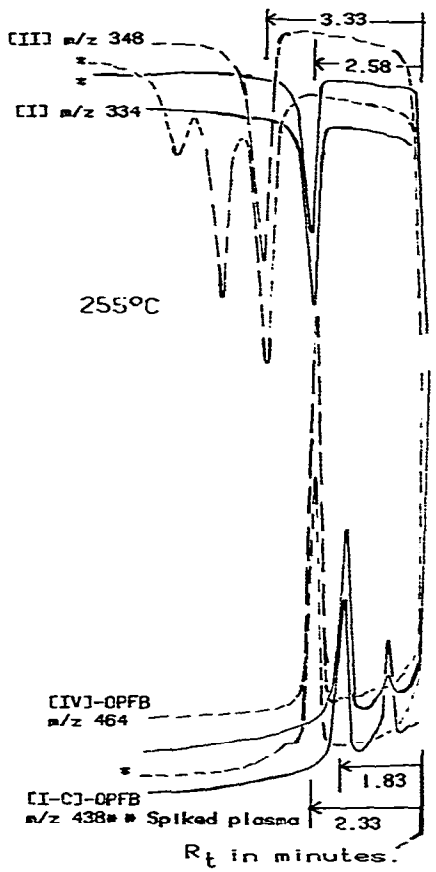

Fig. 7. Ion chromatograms of GC—PCI-MS analysis by selected ion monitoring, of I at *m/z* 334, internal standard (II) at *m/z* 348 and of the metabolite levorphanol (I-C) at *m/z* 438 and its internal standard levallorphan (IV) at *m/z* 464 as their respective pentafluorobenzyl ethers.

to yield a GC—negative chemical ionization (NCI)-MS assay specific for I-C per se as its PFB ester derivative using the trideuteromethyl analogue as the internal standard in the asssay [30].

Application of the methods to biological samples

Plasma samples (0—48 h) from a dog administered a single oral 10 mg/kg dose of I as the tartrate salt (in a hand packed gelatin capsule) were first analyzed by the GC—NPD method, plasma concentrations however, were below the sensitivity limit of the assay (≤10ng/ml).

They were reanalyzed by GC—PCI-MS, and the plasma concentration—time profile of I showed an absorption peak of 9.6 ng/ml at 30 min post dose which decline biexponentially to the limit of detectability of the assay (i.e., 2 ng/ml) at 10 h, with an apparent half-life of elimination ($t_{1/2\beta}$) of 4.5 h. In order to evaluate plasma concentrations of the metabolite I-C, plasma samples (0—48 h) from a dog administered a higher oral dose of I·tartrate (three 100-mg tablets) equivalent to 24 mg (free base)/kg were analyzed by GC—PCI-MS for I and I-C using the direct extraction (underivatized) procedure. Plasma concentrations of I were initially seen at 12 min (5.3 ng/ml), reaching a maximum between 30—60 min (47.6 ng/ml) and declined to non-detectable amounts (<2.0 ng/ml) by 12 h (Fig. 8). I-C was detectable at 30 min (3.5 ng/ml), the concentrations gradually increasing to a peak (12.4 ng/ml) at 10 h and declined to 2.4 ng/ml by 73 h (Fig. 8). The concentration of I-C present in this dog was in the same therapeutic concentration range reported for levorphanol in cancer patients treated with this analgesic drug (L-Dromoran) by different routes of administration at doses ranging from 1 mg t.i.d. (i.m.) to 28 mg q.i.d. (oral) determined by radioimmunoassay [19,20], therefore its quantitation would be clinically relevant to the overall analgesic effect of the parent drug.

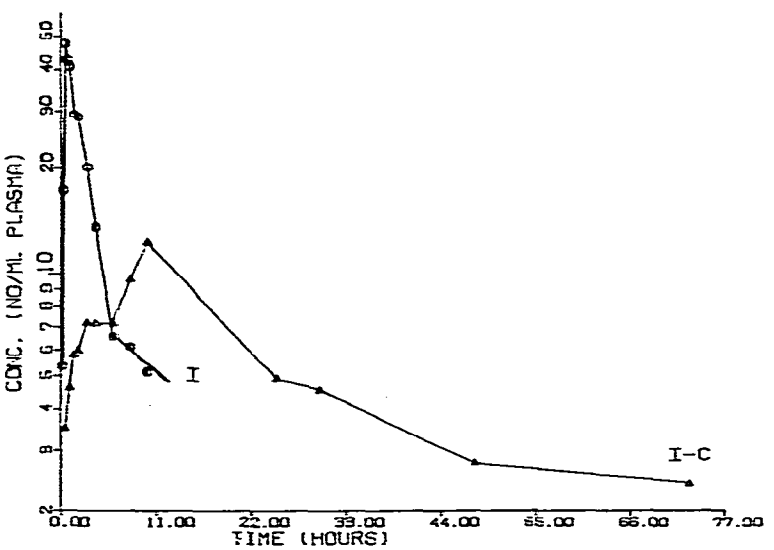

Fig. 8. Plasma concentration of I and metabolite I-C in a dog following a single oral dose of I·tartrate (equivalent to 24.1 mg (free base)/kg) determined by GC—PCI-MS analysis.

These data substantiate the fact that the GC—PCI-MS assay would be the method of choice for use in future clinical pharmacokinetic studies due to its ability to quantitate the parent drug (I) and a pharmacologically active metabolite levorphanol (I-C) with high sensitivity (ca. 2.0 ng/ml) and specificity.

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