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The determination of RWJ-38705 (tramadol N-oxide) and its metabolites in preclinical pharmacokinetic studies using LC–MS/MS

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Abstract

A rapid and reliable analytical method is described for the simultaneous determination of RWJ-38705 (tramadol N-oxide) and several of its major metabolites in the plasma of Sprague-Dawley rats and Beagle dogs. Sample preparation using solid phase extraction was followed by reversed phase liquid chromatography (LC) coupled with tandem mass spectrometric (MS/MS) detection in the positive ionization mode. The assay was linear for all analytes over concentrations ranging from approximately 6 to 2000 ng/ml. The inter-assay reproducibility was generally less than 15% while accuracy values were within 13% of theoretical. The overall recovery of the analytes ranged from approximately 40 to 64% in rat plasma and 53–75% in dog plasma. This assay has proven to be sensitive, specific and reproducible, and it has been readily implemented in preclinical PK studies. Representative plasma concentration versus time profiles resulting from administration of TNO to rats and dogs are presented in this communication.

Keywords: Tramadol N-oxide; Obsessive–compulsive disorder; Liquid chromatography/mass spectrometric

1. Introduction

RWJ-38705 (TNO), the N-oxide analog of the centrally acting analgesic drug tramadol, is a novel therapeutic agent under investigation by the R.W. Johnson Pharmaceutical Research Institute (RWJPRI), Raritan, NJ for the treatment of compulsive–obsessive disorder (OCD). Current pharmacotherapy for OCD is beset with problems such as poor efficacy, high incidence of side effects, relatively high dose requirements and relatively long response times. Although the N-oxides of centrally acting analgesics have generally demonstrated minimal analgesic activity, initial investigations of TNO indicated that it is antinociceptive, and is rapidly absorbed and metabolized in several mammalian species in similar fashion to tramadol but with a longer duration of action [1].

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The support of preclinical pharmacokinetic investigations requires reliable bioanalytical methodology for the measurement of potential drugs and their metabolites. This communication describes the validation and implementation of an efficient LC/MS/MS assay for the simultaneous determination of TNO and several of its major metabolites in plasma collected from Sprague-Dawley rats and Beagle dogs.

2. Experimental

2.1. Materials

(I) RWJ-38705, TNO, (dl)-*cis*-2-[(dimethylamino) methyl]-1(3-methoxyphenyl) cyclohexanol-N-oxide, its major metabolites; (II) tramadol (dl)-*cis*-2-[(dimethylamino) methyl]-1(3-methoxyphenyl) cyclohexanol monohydrochloride; (III) metabolite M1, (dl)-1-(hydroxyphenyl)-2 (dimethylaminomethyl)-cyclohexan-1-ol monohydrochloride hemihydrate; (IV) metabolite M2, 1(3-methoxyphenyl-2-[(methylamino) methyl] cyclohexanol; (V) metabolite M5, 3(′1-hydroxy-2-[(methylamino) methyl] cyclohexyl) phenol hydrochloride; (VI) metabolite M6, 1(3-methoxyphenyl)-2-(dimethylaminomethyl) cyclohexan-1,4-diol and the internal standard; (VII) IS, (dl)-*cis*-2-[(dimethylamino) methyl]-1(3-ethoxyphenyl) cyclohexanol monohydrochloride were synthesized and purified ($\geq 99\%$) by the R.W. Johnson P.R.I. Medicinal Chemistry Department. The chemical structure of compounds I–VII are shown in Fig. 1.

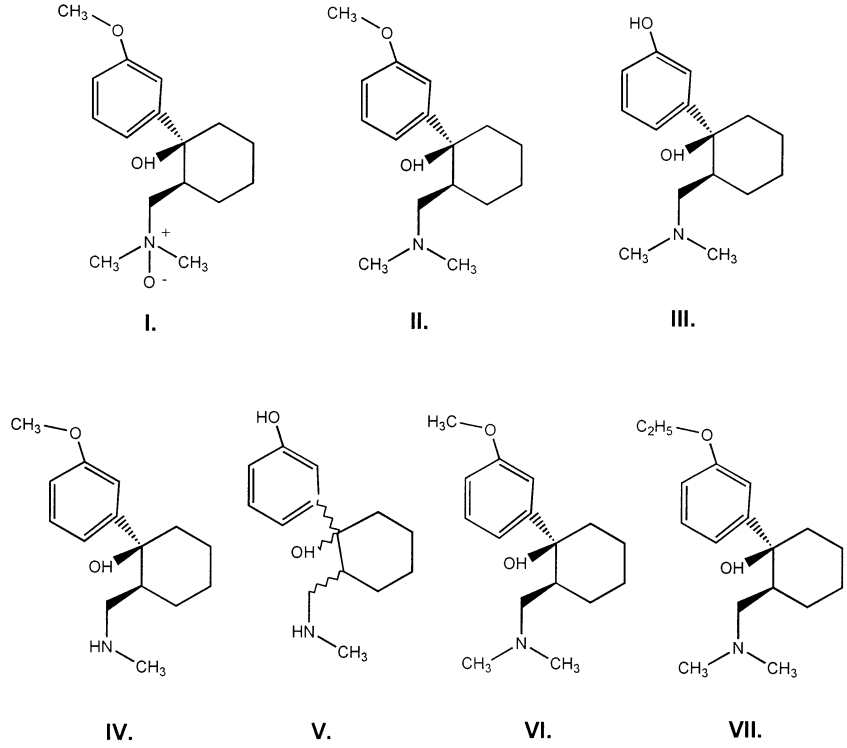

Fig. 1. Chemical structures of, (I) RWJ-38705 (TNO); (II) tramadol; (III) M1; (IV) M2; (V) M5; (VI) M6 and (VII) the internal standard IS.

Fig. 2. Mass fragmentation patterns (product ion spectra) of, (I) RWJ-38705 (TNO); (II) tramadol; (III) M1; (IV) M2; (V) M5; (VI) M6 and (VII) IS.

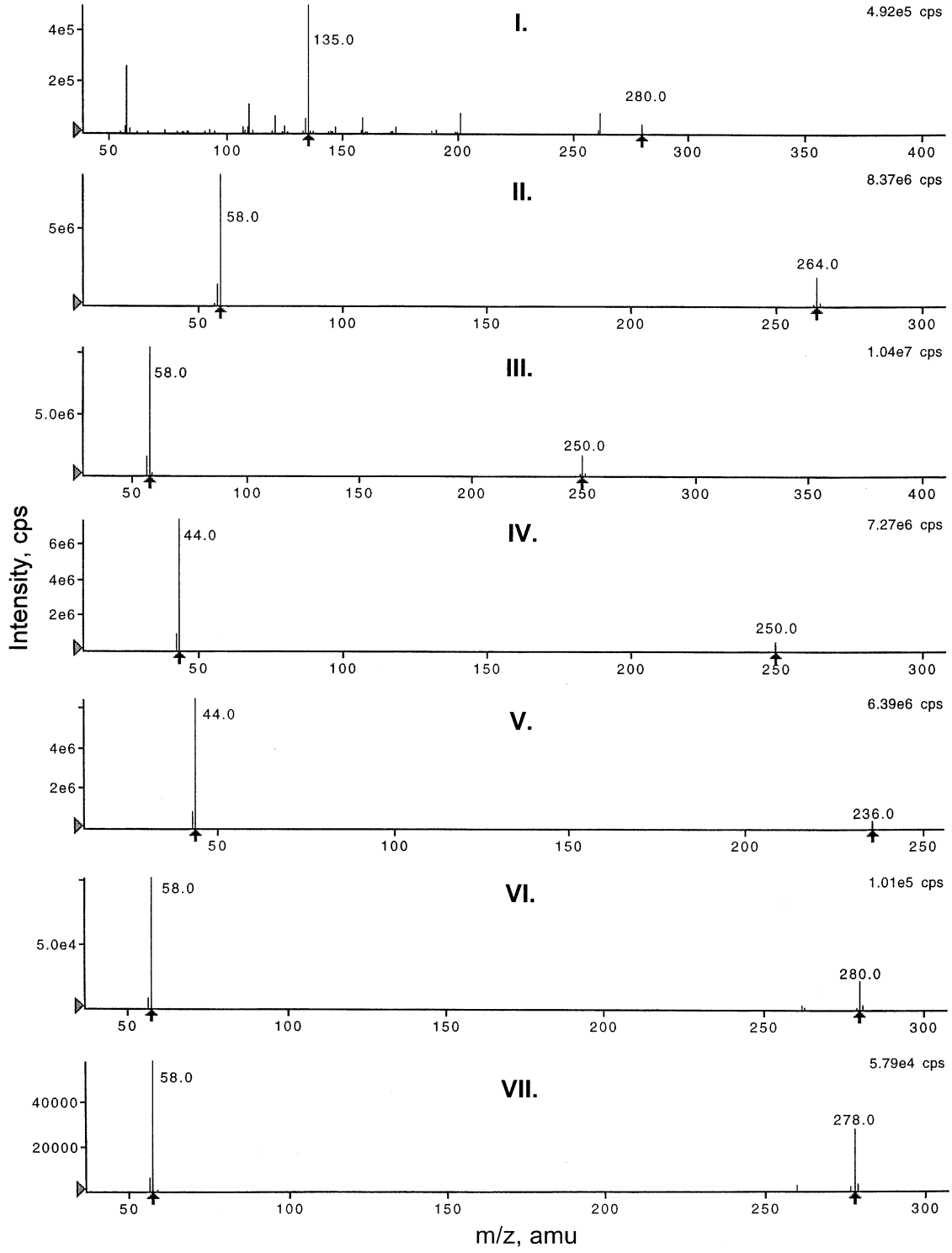
I.
4.92e5 cps
4e5
2e5
135.0
280.0
II.
8.37e6 cps
5e6
58.0
264.0
III.
1.04e7 cps
5.0e6
58.0
250.0
IV.
7.27e6 cps
6e6
4e6
2e6
44.0
250.0
V.
6.39e6 cps
4e6
2e6
44.0
236.0
VI.
1.01e5 cps
5.0e4
58.0
280.0
VII.
5.79e4 cps
40000
20000
58.0
278.0
50
100
150
200
250
300
350
400
Intensity, cps
m/z, amu

Fig. 2.

Methanol and acetonitrile (Burdick and Jackson, Muskegon, MI) were HPLC grade. Ammonium hydroxide, phosphoric acid (Mallinckrodt Inc., Paris, KY) and ammonium acetate (Aldrich Chemical Co., Milwaukee, WI) were of the highest grade commercially available. Samples of control plasma from Sprague-Dawley rats and Beagle dogs were obtained from the Vivarium of the RWJPRI, Raritan, NJ. All stock and working solutions were prepared in methanol. Calibration standards in both rat and dog plasma were prepared freshly for each assay via serial dilution of plasma spiked with aliquots (100 μl) of a stock solution containing each analyte at a concentration of about 200 μg/ml, affording concentrations of approximately 6, 12, 25, 100, 250, 1000 and 2000 ng/ml. In rat plasma, quality control samples were prepared at concentrations of 27.5, 275, and 1100 and in dog plasma, the quality control concentrations were 25, 250 and 1000 ng/ml. All stock solutions used in the preparation of plasma standards and quality control samples were stored refrigerated at 4 and 5°C.

2.2. Instrumentation and experimental conditions

Sample preparation was accomplished using solid phase extraction (SPE) on Isolute HCX™ 1 ml SPE columns (130 mg column bed size; Jones Chromatography, Lakewood, CO) and a Rapid Trace™ SPE apparatus (Zymark Inc., Hopkinton, MA).

2.2.1. Chromatographic conditions

The instrumentation employed in the HPLC portion of the assay included a Model L6200 pump (Hitachi, Danbury, CT) and a Model SIL-10A autosampler (Shimadzu, Columbia, MD).

A 5 μm protein-RP™ wide pore butyl reversed phase column (10 cm × 2.0 mm i.d.; YMC Inc., Wilmington, NC) was maintained at ambient temperature to separate TNO, its metabolites and the internal standard from endogenous coextractants. Isocratic elution of the analytes from the column was achieved using a mobile phase consisting of acetonitrile–ammonium acetate (pH 4, 0.005 M) (50:50, v/v) at a flow rate of 0.2 ml/min.

2.2.2. Mass spectral conditions

Mass spectral analyses were accomplished on a model API 300A triple quadrupole mass spectrometer (PE Sciex, Norwalk, CT) fitted with an API Turbo Ion spray source and operated in the positive ionization mode. The Turbo Ion spray temperature was maintained at 300°C. The orifice voltage was 14 V. The nebulizer and curtain gases were ultra high purity nitrogen (99.999%; ~80 psi) delivered at settings of 12 and 9, respectively, on the PE Sciex Sample Control software. The nitrogen flow was produced by a gas generation system (Nitrogen Generator model 75-72 (Whatman Inc., Haverhill, MA). Nitrogen was also used as the collision gas at a setting of six (6) in the PE Sciex Sample Control software, which produced an overall analyzer pressure of approximately 3.0×10^{-5} Torr. The collision energy was 20 V.

The mass spectrometer was operated in MS/MS mode using multiple reaction monitoring (MRM) to detect specific precursor ion to product ion transitions for each analyte as follows.

Following chromatographic separation, the protonated pseudomolecular ion (MH+) for each analyte was selected by the first quadrupole (Q1) and focused into the collision cell (Q2) where it fragmented into product ions. For each MH+ precursor ion, a distinct product ion was selected by the third quadrupole (Q3). The precursor ion intensity was monitored and subsequently stored by the mass spectrometer's computer system. These transitions are listed below.

Mass spectral Q1–Q3 transitions monitored

	TNO	T	M1	M2	M5	M6	IS
Q1 (m/z)	280	264	250	250	236	280	278
Q3 (m/z)	135	58	58	44	44	58	58

Instrument tuning parameters were optimized using a 10 ug/ml solution of each analyte prepared in acetonitrile–water (50:50, v/v). Each so-

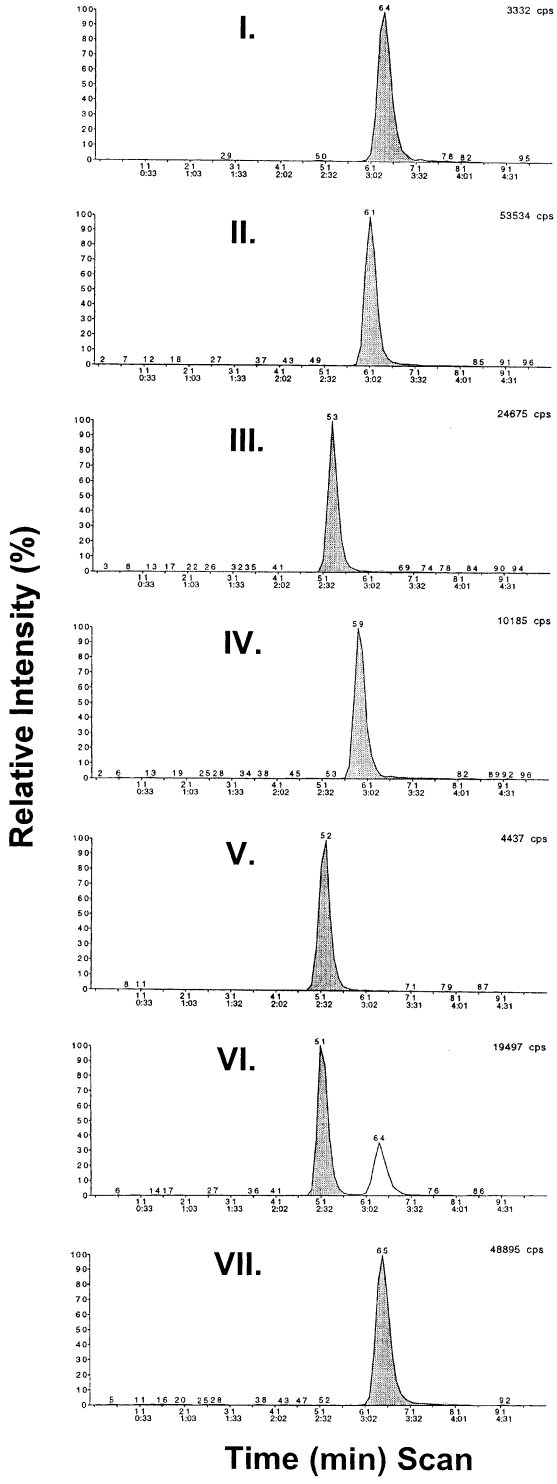

Fig. 3.

lution was infused at 10 ul/min into a standard PEEK line tee cell where it was mixed with a coaxial stream of acetonitrile–water (50:50, v/v) at a rate of 1.0 ml/min by an HPLC pump. The effluent stream of 0.2 ml/min was directed into the LCMS interface.

2.2.3. Data acquisition, processing and quantification

Data acquisition and processing were accomplished using Macintosh Power Mac 8500 and 120 computers and the proprietary PE Sciex software programs, Sample Control (version 1.2) and MacQuan (version 1.4). For data acquisition, the dwell time was set at 400 ms with a pause of 25 ms, permitting at least 10 scans across each chromatographic peak. Calibration standard curve calculations were based upon chromatographic peak area ratios of each analyte to the internal standard. Plasma concentrations of TNO and its metabolites were determined by using the slope and intercept of the standard curve obtained from a linear least squares regression (weighted as $1/x^2$, where x = concentration) for the analyte/internal standard peak area ratio versus the calibration standard concentration.

2.3. Extraction and assay procedures

To each sample (100 μl) of rat or dog plasma was added 50 μl of internal standard solution (1120 ng/ml) and 400 μl of phosphoric acid (0.15 N). The samples were applied to SPE columns preconditioned with successive column volumes of methanol, water and phosphoric acid solution. The columns were then washed with an additional volume of phosphoric acid solution. Sample elution was subsequently accomplished using one column volume of methanol–ammonium hydroxide (98:2, v/v), followed by evaporation under nitrogen at approximately 40°C and reconstitution of the extract residue in 100 μl water. An aliquot (10 μl) of the reconstituted residue was then injected onto the system.

Fig. 3. Representative mass spectral chromatograms of a rat plasma calibration standard containing approximately 250 ng/ml of, (I) RWJ-38705 (TNO); (II) tramadol; (III) M1; (IV) M2; (V) M5; (VI) M6 and (VII) IS.

Inter-assay precision and accuracy, expressed as percent coefficient of variation (% CV) and percent deviation from theoretical (target) value, respectively, were assessed in quality control samples prepared in triplicate on 3 separate days. For both matrices, assay specificity and analyte stability (freeze–thaw and frozen storage conditions) were assessed in plasma quality control samples in order to mimic the conditions to which authentic in-life samples might be subjected.

These were periodically assayed concurrently with freshly prepared standards. Recovery was assessed also using the plasma quality control samples.

Triplicate samples for each quality control concentration were spiked with the internal standard and were analyzed using the procedure outlined above. To determine the recovery, the mean chromatographic peak area responses for each analyte were compared to the peak area responses from analyses of equivalent amounts of reference standard injected from methanol solutions.

3. Results and discussion

The Q1 mass spectrum of each analyte was dominated by the intense, protonated molecular ion species (MH+). The MS/MS product ion spectra resulting from collision activated dissociation (CAD) of each molecular ion are depicted in Fig. 2. The instrument tuning parameters and collision energy were optimized for each analyte to give the most sensitive precursor to product ion transition without regard to the residual intensity of the precursor ion.

Representative chromatograms of a rat plasma calibration standard spiked with TNO and its metabolites at concentrations of approximately 250 ng/ml each, and an authentic in-life sample collected from rats dosed with TNO are shown in

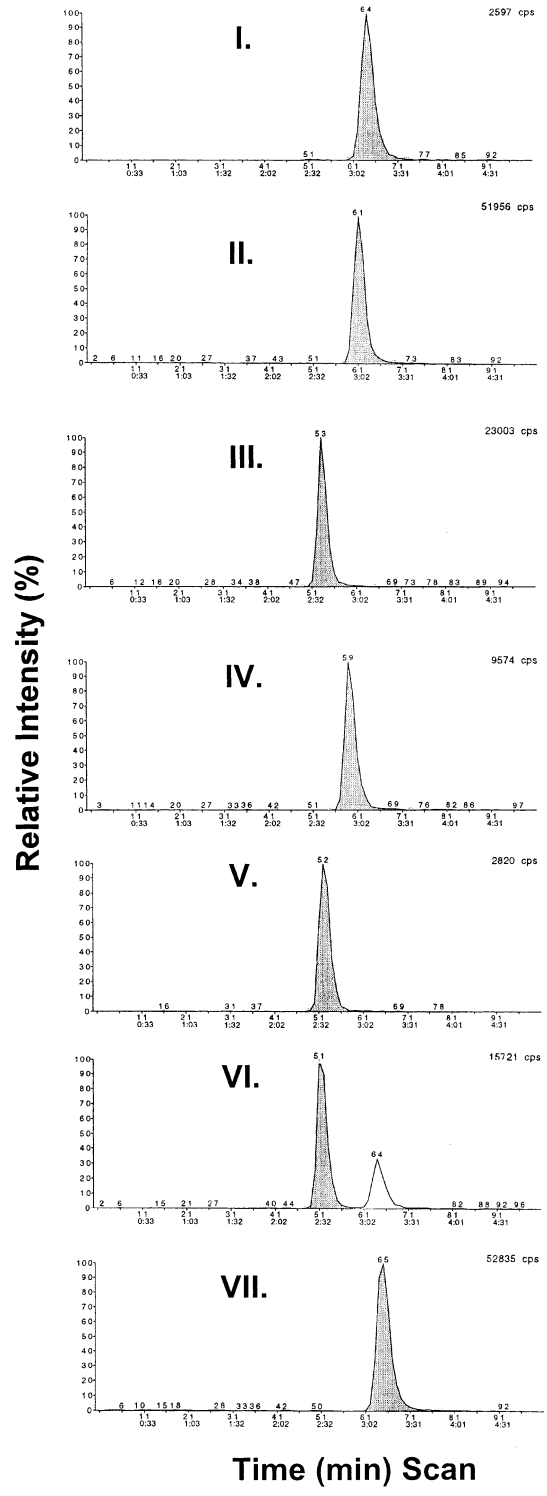

Fig. 4. Representative mass chromatograms of extracted plasma collected from a rat 4 h after administration of 10 mg/kg IV doses of RWJ-38705 (TNO). Estimated concentrations, (I) TNO = 549 ng/ml; (II) tramadol = 198 ng/ml; (III) M1 = 157 ng/ml; (IV) M2 = 1472 ng/ml; (V) M5 = 249 ng/ml and (VI) M6 = 28 ng/ml.

Figs. 3 and 4, respectively. The corresponding chromatograms in dog plasma are shown in Figs. 5 and 6, respectively. Compound VII, a structural analog of tramadol was chosen as the internal standard as it eluted without interference either from TNO, its metabolites or co-extracted endogenous plasma components. Using the chromatographic conditions described in the Section 2, the observed retention times were approximately, 3.1 min (TNO); 3 min (tramadol); 2.3 min (M1); 2.6 min (M2); 2.3 min (M5); 2.3 (M6) and 3.1 min (IS).

The assay demonstrated excellent linearity for all analytes over a plasma concentration range of approximately 6–2000 ng/ml as linear regression analyses of analyte, internal standard peak area ratios versus concentration consistently afforded mean coefficient of determination (r^2) values of 0.99 or greater. Regression results from calibration standard curves obtained in both rat and dog plasma on three successive days are shown in Table 1. Assay precision and accuracy were assessed in spiked quality control samples (triplicate replicates) which were analyzed on 3 separate days. As shown in Tables 2 and 3, overall inter-assay precision (% CV) was generally 15% or less, while accuracy was consistently within $\pm$ 13% of the target (theoretical) value.

The recovery of TNO and its metabolites from plasma was assessed using spiked quality control samples at three levels encompassing the general concentration range of the assay. The samples were subjected to identical extraction and assay procedures as described in the Section 2. The overall mean extraction recoveries of TNO and its metabolites ranged from approximately 40 to 64% in rat plasma and 53–75% in dog plasma.

There was no observable interference upon assay performance by co-extracted endogenous components of rat or dog plasma. Additionally, the stability of TNO and its metabolites was assessed under freeze–thaw and frozen storage conditions using replicates of QC samples. In

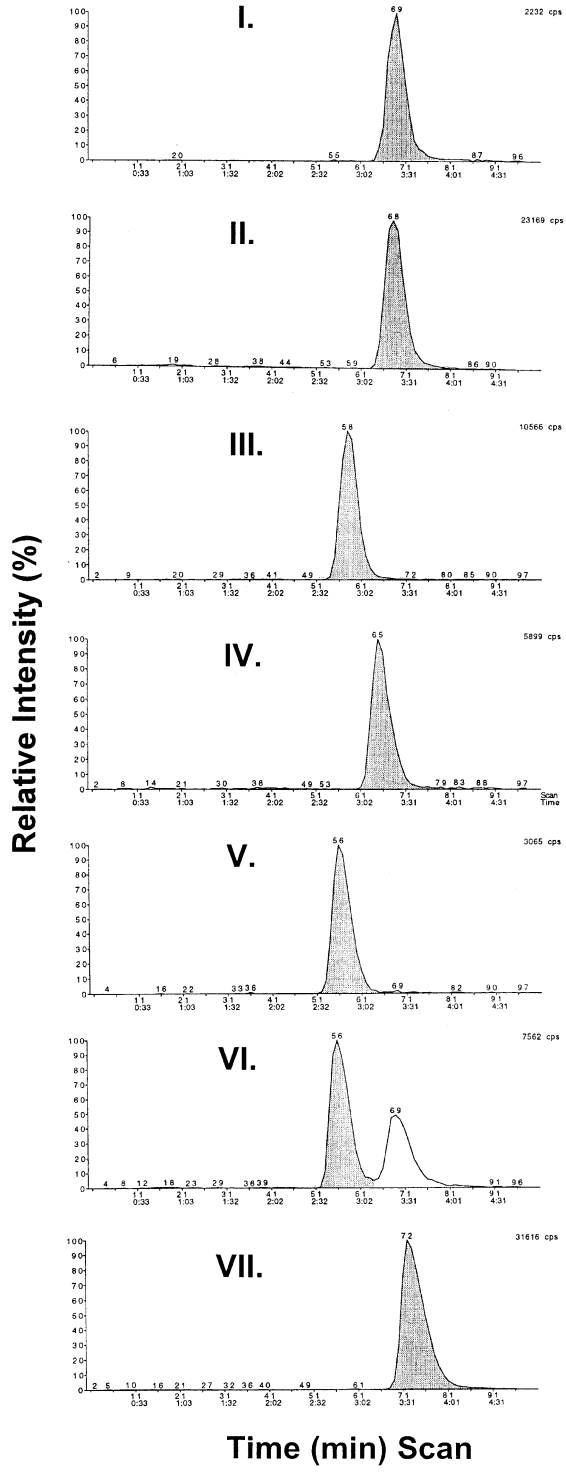

Fig. 5. Representative mass spectral chromatograms of a dog plasma calibration standard containing approximately 250 ng/ml of, (I) RWJ-38705 (TNO); (II) tramadol; (III) M1; (IV) M2; (V) M5; (VI) M6 and (VII) IS.

Fig. 5.

both matrices, the concentration of each analyte in stored QC samples was within 10% of the corresponding value observed in freshly prepared QC samples. That the stored samples consistently demonstrated little or no observable difference from the freshly prepared samples, confirmed that all analytes were stable in plasma for at least three freeze–thaw cycles and under frozen storage at −20°C for at least 2.5 months.

Although the SPE column conditioning and elution steps used in the sample preparation phase appeared to entail somewhat incongruous conditions, it was determined that washing the SPE column bed to remove the phosphoric acid prior to alkaline methanol elution was not necessary as the overall analyte recoveries and assay performance were consistently acceptable. Additionally, during assay development, it was observed that the responses obtained from both the standards and QCs were sufficient to allow for a lowering of assay quantification limits (LOQ) well below those reported in this communication. Indeed, the data obtained at the current LOQ of 6 ng/ml appeared to demonstrate signal to noise (S/N) ratios of at least 20:1 for both TNO and tramadol. However, further decreases in the assay LOQ were deemed unnecessary in view of subsequent results from in-life investigations.

The assay method described in this communication was applied to the measurement of TNO and its metabolites in support of preclinical drug metabolism studies in which Sprague-Dawley rats and Beagle dogs received both intravenous and oral doses of TNO. Following drug administration, plasma concentrations of parent TNO were consistently and significantly higher than those of, tramadol, M1, M2, M5 or M6. Fig. 7 shows overall plasma concentration–time

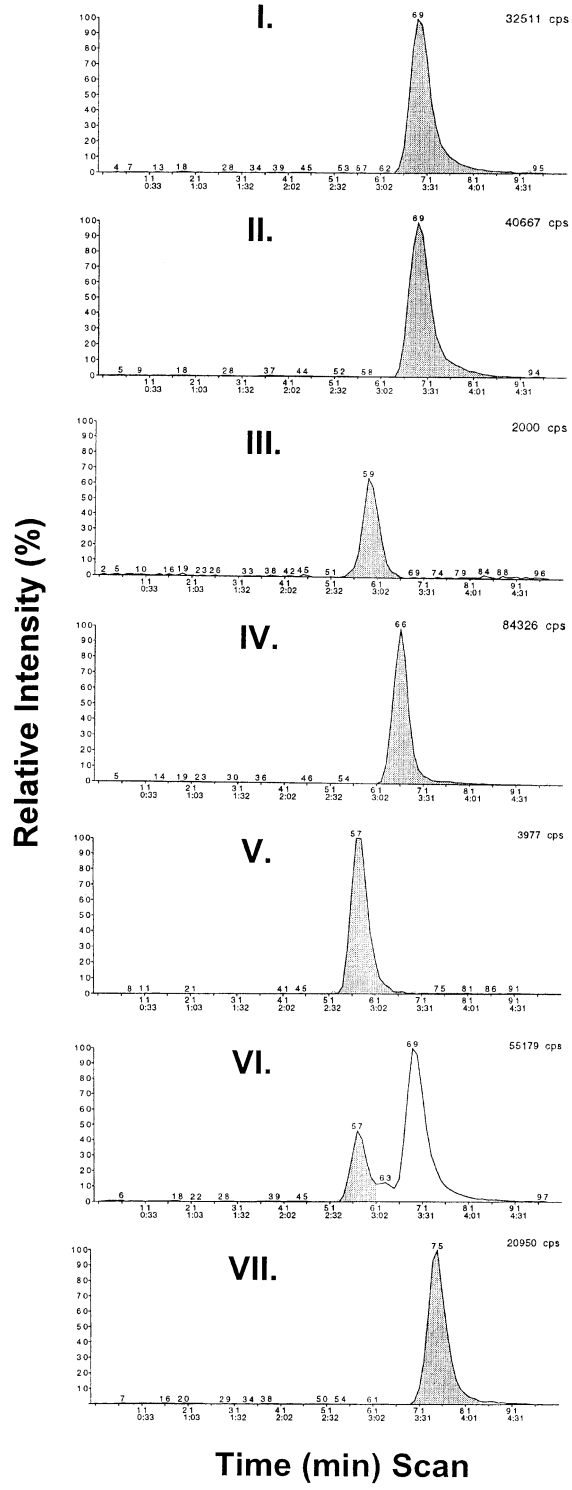

Fig. 6. Representative mass chromatograms of extracted plasma collected from a dog 2 h after administration of 10 mg/kg IV doses of RWJ-38705 (TNO). Estimated concentrations, (I) TNO = 13155 ng/ml; (II) tramadol = 1098 ng/ml; (III) M1 = 47 ng/ml; (IV) M2 = 5273 ng/ml; (V) M5 = 65 ng/ml and (VI) M6 = 1021 ng/ml.

Fig. 6.

Table 1
Linearity of the determination of RWJ-38705 and its metabolites obtained from calibration standard curves[a] (mean days 1–3, $n = 6$) in rat and dog plasma

Matrix	Analyte	R^2	Slope	y-Intercept
Rat plasma	TNO	0.9923	0.000	0.0033
	Tramadol	0.9943	0.002	0.0800
	M1	0.9950	0.001	0.0023
	M2	0.9970	0.001	0.0030
	M5	0.9970	0.000	0.0000
	M6	0.9957	0.001	0.0023
Dog plasma	TNO	0.9937	0.000	0.0033
	Tramadol	0.9900	0.003	0.0170
	M1	0.9953	0.001	0.0007
	M2	0.9987	0.001	0.0033
	M5	0.9943	0.000	−0.0010
	M6	0.9960	0.001	−0.0010

[a] Calibration standards bracketed the assay concentration range of approximately 6–2000 ng/ml.

profiles of TNO and its metabolites following administration of intravenous 10 mg/kg doses of TNO to rats and dogs.

Analyses of the analgesic drug tramadol in biological fluids by both enantioselective and non-enantioselective assay methods have been well documented in the literature. The predominance of chromatography [2–16] as the favored mode of biofluid analysis has been due primarily to the ability of techniques such as TLC, HPLC or GC to efficiently resolve, (1) the enantiomers of tramadol or (2) racemic tramadol from its major metabolites. More recently, electrophoretic techniques have been effectively applied to analyze enantiomers of both tramadol and its major metabolite M2 in solutions of relatively high concentration [17–20]. According to a comprehensive review of applications of capillary electrophoresis (CE) to the analysis of drugs and metabolites in body fluids by CE by Lloyd, analytical strategies have entailed the use of either extensive preliminary sample preparation steps (similar to those used with chromatographic techniques) or direct sample injection [21]. Although CE appeared to demonstrate great promise given its speed, resolving power and inherent nature as a microanalytical technique, problems concerning relatively poor assay sensitivity (i.e. quantitation limits significantly below the 100–1000 ng/ml range have not been routinely achieved) and electroosmotic variability and resultant peak broadening arising from endogenous matrix interferences (e.g. urinary elec-trolytes and plasma proteins) have remained. Given these current limitations, CE and related electrophoretic techniques would generally appear to be better suited to specific chiral resolution or formulation analysis applications than to rigorous quantitative pharmacokinetic investigations. In contrast, gas chromatography has been successfully combined with mass spectrometric detection to achieve fairly sensitive, robust assays of tramadol [22–27]. The overall performance of the GCMS technique has rivaled and even exceeded that of the previously established chromatographic assays. Carrying this overall analytical progression a step further, the more recent coupling of high performance liquid chromatography with mass spectrometry has been cited heretofore for the measurement of tramadol alone [28] or with its major metabolite [29] in human plasma. The LC–MS/MS procedure described in this report has been applied to the simultaneous determination of up to six tramadol-related analytes. During numerous preclinical drug metabolism investigations, this assay has demonstrated requisite specificity while maintaining excellent sensitivity. Additionally, as the overall procedure has proven to be rapid and simple, high sample throughput was consistently achieved. Thus, the methodology described in this communication should be generally useful for future support of the pharmacokinetics of TNO and/or tramadol in a variety of biological matrices.

Acknowledgements

The authors wish to thank Dr Rick Edom for both advice and technical assistance. We also wish to thank Dr R. John Stubbs for his support.

Table 2
Inter-assay precision and accuracy of the determination of RWJ-38705 and its metabolites in rat plasma quality controls as estimated on 3 separate days

Analyte	Target value (ng/ml)	Observed value (ng/ml)	CV (%)	Accuracy (%)
	Mean days 1–3			
TNO	27.5	30.7	9.1	11.8
	275	322	8.1	13.4
	1100	1136	7.4	3.3
Tramadol	26	30.8	7.3	12.5
	260	289	5.9	11.3
	1040	1046	7.7	6.3
M1	25	26.6	6.5	6.5
	250	244	14.2	−2.2
	1000	972	15.6	2.8
M2	28	29.1	8.2	4.1
	280	312	6.6	11.4
	1120	1155	6.7	3.2
M5	25	27.2	10.5	8.9
	250	267	8.9	6.6
	1000	1045	8.7	4.5
M6	25	27.9	11.4	11.6
	250	269	8.9	7.9
	1000	1079	8.8	7.9

Table 3
Inter-assay precision and accuracy of the determination of RWJ-38705 and its metabolites in dog plasma quality controls as estimated on 3 separate days

Analyte	Target value (ng/ml)	Observed value (ng/ml)	CV (%)	Accuracy (%)
	Mean days 1–3			
TNO	25	27	9.8	8.1
	250	249	13.5	−0.4
	1000	925	8.8	−7.4
Tramadol	25	26.2	13.0	4.7
	250	276	6.4	10.4
	1000	906	8.5	−9.4
M1	25	24	13.2	−4.0
	250	223	15.6	−10.9
	1000	936	14.4	−6.4
M2	25	24.7	14.6	−1.1
	250	245	11.3	−2.0
	1000	925	11.8	−7.5
M5	25	24.6	14.9	−3.5
	250	255	16.2	0.2
	1000	1101	14.9	9.1
M6	24.5	23.2	18.9	−5.4
	245	219	9.9	−10.2
	980	954	8.1	−2.7

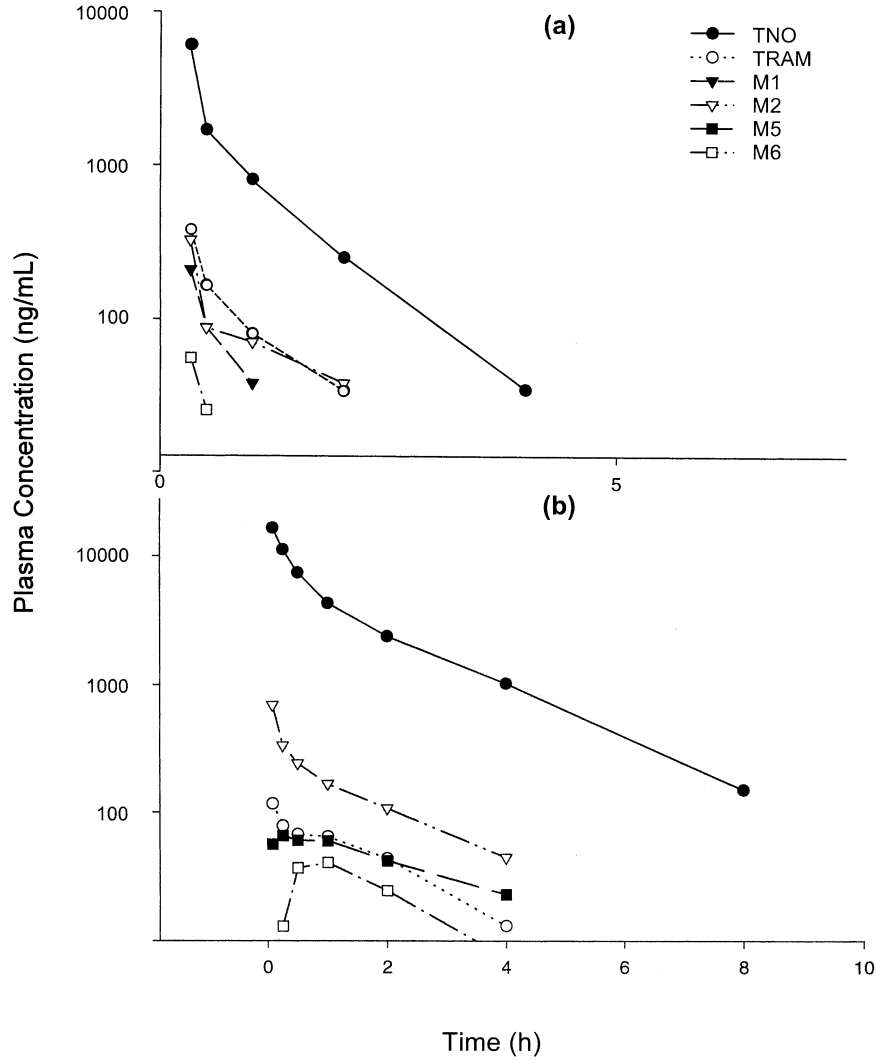

Fig. 7. Representative plasma concentration vs. time profiles of RWJ-38705 (TNO) and its metabolites in (a) rats and (b) dogs following administration of intravenous 10 mg/kg doses of TNO.

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