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Gas chromatographic method using nitrogen–phosphorus detection for the measurement of tramadol and its *O*-desmethyl metabolite in plasma and brain tissue of mice and rats

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Abstract

A method that allows the measurement of plasma and brain levels of the centrally-acting analgesic tramadol and its major metabolite (*O*-desmethyl tramadol) in mice and rats was developed using gas chromatography equipped with nitrogen–phosphorus detection (GC–NPD). Plasma samples were extracted with methyl *tert.*-butyl ether (MTBE) and were injected directly into the GC system. Brain tissue homogenates were precipitated with methanol, the resulting supernatant was dried then acidified with hydrochloric acid. The aqueous solution was washed with MTBE twice, alkalinized, and extracted with MTBE. The MTBE layer was dried, reconstituted and injected into the GC system. The GC assay used a DB-1 capillary column with an oven temperature ramp (135 to 179°C at 4°C/min). Dextromethorphan was used as the internal standard. The calibration curves for tramadol and *O*-desmethyl tramadol in plasma and brain tissue were linear in the range of 10 to 10 000 ng/ml (plasma) and ng/g (brain). Assay accuracy and precision of back calculated standards were within ±15%.

Keywords: Tramadol; *O*-Desmethyltramadol

1. Introduction

Tramadol, (1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl) cyclohexanol (Fig. 1), is a centrally-acting analgesic [1] in widespread clinical use throughout the world. The mechanism of action of tramadol is multimodal, involving opioid and nonopioid components [2,3]. Based on relative binding affinity for opioid receptors, the *O*-desmethyl metabolite (M1; Fig. 1) of tramadol is the presumptive major contributor to the opioid component. However, the inability of the opioid antagonist naloxone to block tramadol-induced analgesia [4], and the low level of abuse of tramadol in the United

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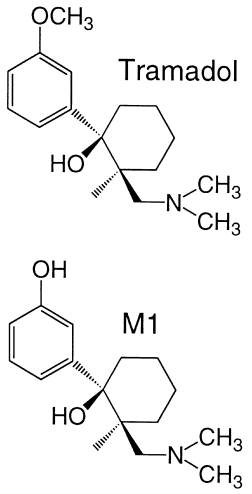

Fig. 1. Chemical structures of tramadol and its desmethyl metabolite (M1).

States [5] and other countries [6], suggest that a significant portion of tramadol-induced analgesia is attributable to the nonopioid component or to synergistic interaction between opioid and nonopioid components [7]. The in vitro binding data and the clinical experience could be reconciled if M1 does not readily enter the brain, particularly at therapeutic doses. Although several chromatographic methods have been used for quantification of tramadol and M1 in plasma or in other biological matrices, including high-performance liquid chromatography (HPLC), gas chromatography–nitrogen–phosphorus detection (GC–NPD), GC–mass spectrometry (MS) and LC–MS [8–18], we are unaware of any published analytical method for the quantitation of tramadol or M1 in brain tissue. The objective of the present study was to develop an assay with which to measure plasma and brain levels of tramadol and its *O*-desmethyl metabolite (M1) in mice and rats.

2. Experimental

2.1. Chemicals and reagents

Tramadol and *O*-desmethyltramadol (M1) were supplied by The R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA). Internal standard, D-3-methoxy-*N*-methylmorphinan hydrobromide monohydrate (dextromethorphan), and anhydrous methyl *tert*.-butyl ether (MTBE) (HPLC grade) were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA), respectively. Methanol (HPLC grade), toluene (HPLC grade), sodium hydroxide (analytical grade) and 36.5% hydrochloride acid (analytical grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Heparin sodium (10 000 USP u/ml) was obtained from Eli Lilly (Indianapolis, IN, USA).

2.2. Animals

Male virus-free Swiss-derived albino Crl:CD-1 (ICR) BR mice, purchased from Charles River Labs. (Kingston, NY, USA and Portage, ME, USA), weighing 18–35 g at the time of use, were housed 5–10 per box (with wood chip bedding) under controlled conditions of temperature, humidity, and a 12 h light–dark cycle (lights on at 06:00 h), with food and water available ad libitum. Male virus-free Swiss-derived albino Crl:CD (SD) BR rats, also purchased from Charles River Labs., weighing 250–450 g at the time of use, were housed seven per cage under controlled conditions as described above. All animals were treated in accordance with NIH (National Institutes of Health) guidelines for care and use.

2.3. Sample collection

Blood samples from anesthetized (CO_2) animals were collected into heparinized syringes via cardiac puncture, transferred to heparinized 1.5-ml polypropylene micro-centrifuge tubes (containing 45 USP units sodium heparin) and subjected to immediate centrifugation at 2600 *g* for 10 min. Plasma was then separated and immediately frozen and stored at −70°C in polypropylene tubes until analysis. Whole-brain (minus cerebellum) was removed from each animal, washed with 0.9% (w/v) saline, dried with tissue paper, weighed, and transferred to a 10-ml capped tube. Following addition of ice-cold Tris–HCl buffer (4 ml/g tissue), the brain was homogenized using a Polytron tissue homogenizer (homogenates thus contained 0.25 g/ml of brain tissue). The

homogenates were stored at −70°C in polypropylene tubes until analysis.

2.4. Extraction procedure: plasma

To 1.5-ml polypropylene micro-centrifuge tubes, 50 μl of a 1 μg/ml solution of internal standard (dextromethorphan) in methanol was added and evaporated to dryness in a 40°C water bath under nitrogen gas. Plasma samples were thawed at room temperature for approximately 30 min, then 0.25 ml of plasma was removed with pipettes and added to the tubes described above and mixed for 30 s, followed by addition of 1 drop of 0.1 *M* NaOH. The mixture was extracted with 150 μl of MTBE by vortex-mixing for an additional 1 min followed by centrifugation at 2600 *g* for 10 min. The supernatants were transferred to injection vials fitted with 100-μl glass inserts and 5 μl was injected into the GC system.

2.5. Extraction procedure: brain tissue

To 10-ml glass tubes (1 cm I.D.) 50 μl of a 1 μg/ml solution of the internal standard (dextromethorphan) in methanol was added. Samples of Tris buffer homogenates of brain tissue were thawed at room temperature for approximately 30 min then 1 ml of homogenates was pipetted to the tubes described above and mixed for 30 s. To the mixture, 4 ml of methanol was added and the mixture was vortex-mixed for 30 s followed by centrifugation at 2600 *g* for 10 min. The supernatants were transferred to 10-ml glass tubes and evaporated to dryness under nitrogen flow in a 40°C water bath. To the residues, 1 ml of 0.1 *M* HCl was added and vortex-mixed for 30 s. To the aqueous solution was added 6 ml of MTBE, the mixture was shaken for 1 min and centrifuged at 2600 *g* for 1 min. The MTBE layer was discarded. The process was repeated. The aqueous layer aliquots were alkalinized with 3 ml of 0.1 *M* NaOH and the mixture was extracted with 6 ml of MTBE by shaking for 3 min followed by centrifugation at 2600 *g* for 5 min. The MTBE layer was evaporated under nitrogen flow in a 30°C water bath. The residue was reconstituted with 100 μl of a mixture of toluene–methanol (9:1, v/v) and 5 μl of the sample was injected into the GC system.

2.6. GC–NPD apparatus and assay conditions

The analytical system consisted of a Hewlett-Packard gas chromatograph (Model 5890A) (Hewlett-Packard, Avondale, PA, USA) equipped with a nitrogen–phosphorus detector and a split–splitless injector. Injections were made using a Hewlett-Packard automatic injector (Model 7673) and separation was achieved on a DB-1 (dimethylpolysiloxane coated) fused-silica capillary GC column (15 m× 0.25 mm I.D., 0.25 μm film thickness) (J & W Scientific, Folsom, CA, USA). Helium (99.95%) (BOC Gases, Murray Hill, NJ, USA) was used as a carrier gas at a flow-rate of 3 ml/min. A wide bore liner (4 mm I.D.) and splitless injection was used with the purge valve set at 20 s. The injection port and detector temperatures were 275 and 300°C, respectively. The column temperature was a ramp function starting at 135°C and increased to 179°C at a rate of 4°C/min. Following analysis of each brain tissue sample, the oven temperature was increased from 179 to 299°C at a rate of 30°C/min in order to purge residual materials out of the column. The NPD bead power was set to maintain the baseline signal level at 50 pA. An HP 5895A GC workstation was used to process and store data.

2.7. Calibration curves and method validation

The validation method was based on the procedure described by Hartman et al. [19].

2.7.1. Extraction

Pooled rat plasma ''blank'' samples were examined to confirm the absence of interference. ''Spiked'' samples were prepared by mixing known amounts of tramadol and M1 with 0.25 ml of rat plasma or 1 ml of rat brain tissue homogenates. The extraction procedure and analytical protocol described above was then followed. Peak ratios of tramadol and M1 to that of internal standard were calculated. Peak ratios of the replicates of each concentration point vs. the concentrations of tramadol and M1 were used to create calibration

curves by applying least-squares linear regression analysis. Calibration curves were generated five times on different days in each 7-day period. Limit of quantitation (LOQ) was estimated in the process of calibration curve construction, and assay accuracy and precision were satisfied (% RSD and % bias $\leq \pm 20\%$ at the lower concentrations and $\leq \pm 15\%$ at the higher concentrations). The absolute extraction recovery of tramadol and M1 was estimated using spiked rat plasma and brain tissue samples (20, 100, 500, 2000, 10 000 ng/ml and ng/g). The spiked samples were extracted as described previously and internal standard was added, yielding actual concentrations (ACs). The measured concentrations (MCs) of the spiked samples were calculated from the curve and were compared to the actual concentration in order to determine the absolute extraction recovery (AER), according to the equation: AER (%)=100×MC/AC. The same approach was used for the determination of the recovery of the internal standard, except that tramadol was used as the ''internal standard''.

2.7.2. Assay quality controls

Rat plasma or brain tissue homogenate pool was spiked with a known amount of tramadol and M1 to make samples with three concentrations (20, 200, 2000 ng/ml or ng/g) for the purpose of quality controls. The samples were kept at −70°C and inserted into the routine analysis daily.

2.7.3. Stability testing of samples

The performance of the GC column and NPD bead, stability of the sample extracts, stability of tramadol and M1 in plasma and brain tissue samples which underwent freeze–thaw circles, and long-term storage were tested.

3. Results

Tramadol, M1 and internal standard were almost fully recovered from mouse and rat plasma over a 500-fold concentration range. The recovery was apparently concentration-independent, which was also reflected in the linearity of the calibration curves. The recovery of tramadol and M1 from brain tissue was also quite high, despite the additional extraction steps. The mean recovery ($\pm$SD) from mouse plasma for the concentrations tested was 97.6±1.04% for tramadol, 96.8±1.86% for M1 and 95.6±2.12% for internal standard. The mean recovery from mouse brain tissue was 84.7±1.25% for tramadol, 91.2±1.76% for M1 and 90.5±2.72% for internal standard. There were no significant differences in extraction recovery between mouse and rat plasma or between mouse and rat brain tissue.

The chromatographic conditions produced clear blank samples (Figs. 2 and 3) and sharp, symmetrical peaks for tramadol, M1 and the internal standard, with consistent and convenient retention times (6.2, 7.3 and 9.3 min, respectively) (Figs. 4 and 5). There was no interference in the detection of tramadol, M1 or internal standard from the chromatograms of rat plasma or brain tissue blanks. Although at least three other peaks were observed in the chromatograms of plasma and brain tissue samples of rats administered tramadol (which might be due to tramadol metabolites other than M1), the peaks corresponding to tramadol, M1 and internal standard were completely resolved from these other substances. The chromatograms remained reproducible for about 200 injec-

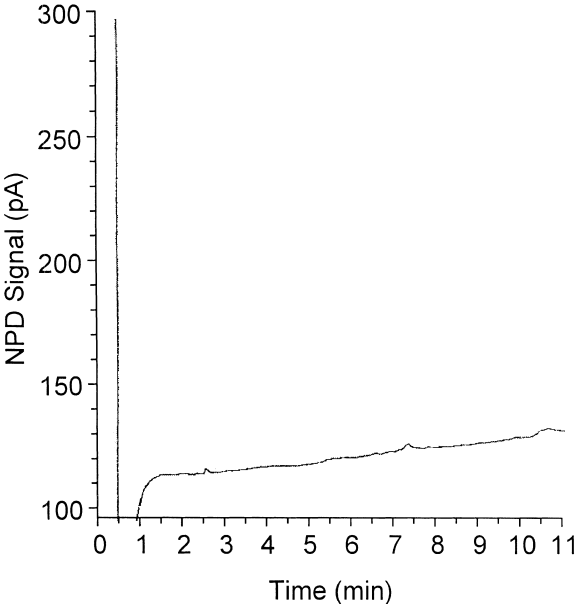

Fig. 2. A typical chromatogram of an extract of a blank plasma sample.

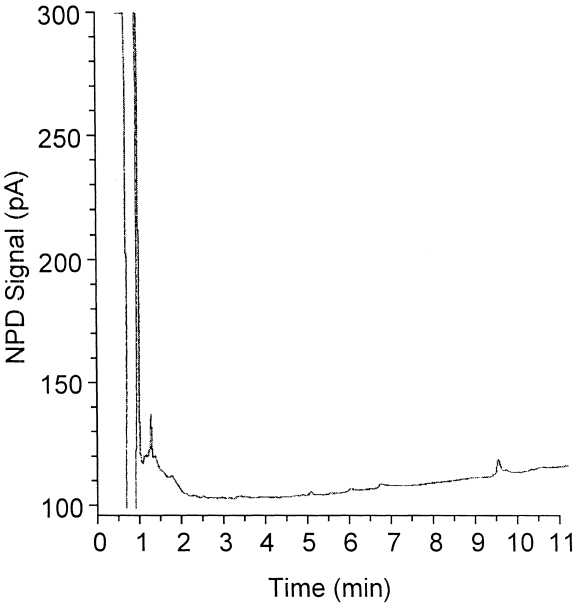

Fig. 3. A typical chromatogram of an extract of a blank brain tissue sample.

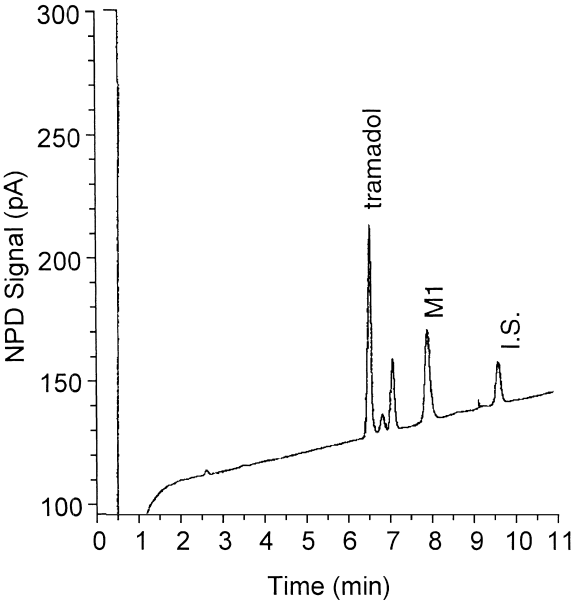

Fig. 4. A typical chromatogram of an extract of a plasma sample collected from a rat dosed with 40 mg/kg of tramadol 30 min prior to sample collection.

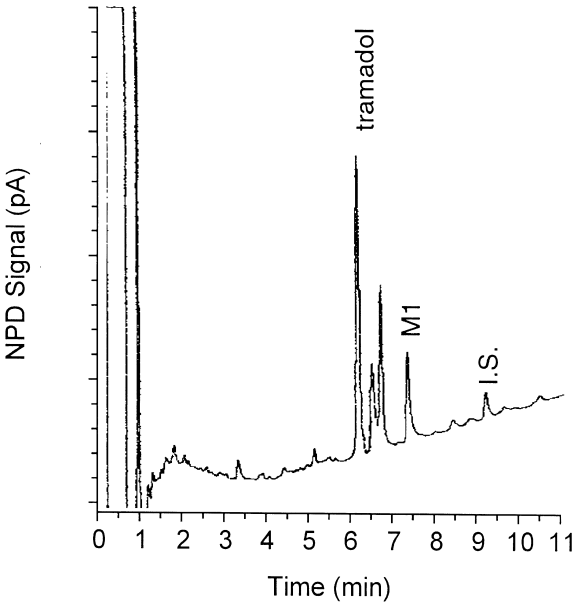

Fig. 5. A typical chromatogram of an extract of a brain sample collected from a rat dosed with 40 mg/kg of tramadol 20 min prior to sample collection.

tions of plasma extracts or 100 injections of brain tissue extracts.

Tramadol and M1 concentrations of 2–10 000 ng/ml for plasma and 2–10 000 ng/g for brain tissue were used to generate calibration curves. The curves were found to be linear over the range 10–10 000 ng/ml or ng/g, with a mean correlation coefficient of >0.99, for tramadol and M1 in both plasma and brain tissue. The results of linear regression analysis of five calibration curves generated during the course of the study are shown in Table 1. The RSD values of the peak ratios at each calibration point from all five calibration curves were $\leq 5.50\%$ for tramadol/I.S. and $\leq 7.96\%$ for M1/I.S. in plasma, and $\leq 7.16\%$ for tramadol/I.S. and $\leq 6.45\%$ for M1/I.S. in brain tissue. To evaluate the predicted concentrations, the observed responses (peak area ratios) for the individual standards were substituted back into the equations in order to calculate the predicted values based on the calibration curves. The results indicated an excellent reproducibility of the calibration curves as well as an acceptable accuracy of the assay. The limit of quantification for either tramadol or M1 was 10 ng/ml in plasma and 10

Table 1
Linear regression analysis of calibration curves of tramadol and M1

		Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Mean	SD	RSD
Tramadol in plasma	Slope	0.0100	0.0102	0.0103	0.0104	0.0104	0.0103	0.0002	1.54
	Intercept	0.0041	−0.0272	0.0031	0.0044	0.0047	−0.0022	0.0140	–
M1 in plasma	Slope	0.0057	0.0056	0.0056	0.0053	0.0055	0.0055	0.0001	2.27
	Intercept	−0.0081	0.0076	−0.0155	0.0043	0.0012	−0.0021	0.0095	–
Tramadol in brain tissue	Slope	0.0081	0.0082	0.0082	0.0084	0.0086	0.0083	0.0002	2.65
	Intercept	−0.0102	−0.0110	−0.0284	−0.0064	−0.0027	−0.0117	0.0099	–
M1 in brain tissue	Slope	0.0062	0.0062	0.0062	0.0063	0.0065	0.0063	0.0001	2.21
	Intercept	−0.0044	−0.0046	−0.0097	−0.0015	0.0006	−0.0039	0.0039	–

The number of calibration points was 10 in all cases.

ng/g in brain tissue, with both precision (RSD) and accuracy (% bias from theoretical value) values of $\leq \pm 15\%$.

The intra- and inter-day precision and accuracy during the validation study were within acceptable limits. The RSD and % bias values for precision and accuracy, respectively, of all three levels of quality control samples during the routine sample assay were: $\leq 9.21\%$ and $\leq \pm 5.97\%$ for tramadol in plasma; $\leq 10.6\%$ and $\leq \pm 4.76\%$ for M1 in plasma; $\leq 8.74\%$ and $\leq \pm 4.81\%$ for tramadol in brain tissue; and $\leq 8.20\%$ and $\leq \pm 7.38\%$ for M1 in brain tissue.

Tramadol and M1 were found to be stable in rat plasma or brain tissue samples for at least 8 weeks when stored at −70°C and subjected to three freeze–thaw cycles. The assay % bias from the initial values over the test period for tramadol and M1 were $\leq \pm 5.16\%$ and $\leq 9.81\%$, respectively, for the 10 plasma samples tested and $\leq \pm 10.88\%$ and $\leq \pm 11.93\%$, respectively, for six brain tissue samples tested. Tramadol and M1 in plasma and brain tissue extracts were also stable for at least 1 week under the conditions evaluated (overall % bias from the initial tested values $\leq \pm 8.76\%$).

Table 2
Peak plasma and brain levels (ng/ml and ng/g, respectively) (±S.E.M.) of tramadol and M1 metabolite in mice and rats administered 20 mg/kg tramadol by oral gavage

	Tramadol	M1
Mice		
Plasma	431.44 (82.22)	585.24 (55.72)
Brain	1043.1 (219.02)	397.77 (77.84)
Rats		
Plasma	204.60 (36.87)	748.65 (153.03)
Brain	274.66 (21.72)	173.40 (27.63)

Mice and rats were administered 20 mg/kg tramadol by oral gavage and the plasma and brain levels of tramadol and M1 metabolite were measured. The results are shown in Table 2.

4. Discussion

We report the development of an efficient and accurate method for the extraction and quantification of tramadol and its major metabolite (*O*-desmethyl tramadol; M1) from plasma and brain tissue of mice and rats. The method involves the use of a GC–NPD technique and to our knowledge is the first report of a method for the determination of tramadol or M1 in brain tissue. With regard to plasma samples, the extraction method is simpler than others reported previously, without sacrifice of peak resolution, sensitivity or robustness. Recovery was high and frozen samples could be stored for extended periods before testing. Tramadol and M1 could be quantified from 10 to 10 000 ng/ml, which is sufficient to measure plasma levels in mice or rats administered antinociceptive (analgesic) doses of tramadol (manuscript submitted for publication). With regard to brain tissue samples, the washing step using MTBE appears to be effective in eliminating substances which might otherwise interfere with the determination of either tramadol or M1. The presence of blood in brain tissue does not appear to affect accuracy of the analysis nor the estimate of concentration (possibly because blood in rodent brain accounts for less than

10% of brain mass [20]). No measurable degradation and/or metabolism of samples was noted during the analysis period.

Some notable features of the present methodology include (i) the short retention times for tramadol, M1 and internal standard (<10 min), allowing for multiple determinations per hour, and (ii) the small amount of sample required (0.25 ml of plasma or 0.25 g of brain tissue). The latter makes it possible to determine tramadol or M1 in plasma and brain tissue from an individual mouse or rat and to perform repeat determinations from one sample. These features should allow the successful characterization of the kinetic profile of tramadol and M1 in the plasma and brain tissue samples of mice and rats following oral administration of tramadol (manuscript submitted for publication).

In summary, a GC–NPD method was successfully developed and validated that measures levels of tramadol and its *O*-desmethyl metabolite (M1) in plasma and brain tissue samples of mice and rats. This is the first such reported method for determination of tramadol in brain tissue and it has important potential use in the further exploration and explanation of the clinical attributes of tramadol.

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