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High-performance liquid chromatographic assay for the simultaneous determination of tramadol and its metabolites in microsomal fractions of human liver

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Abstract

A high-performance liquid chromatographic assay for the quantitative determination of the opioid analgesic tramadol and its metabolites is described. A homologue of tramadol [1-(*m*-hydroxyphenyl)-2-(N-ethyl-N-methylaminomethyl)cycloheptane-1-ol hydrochloride] is used as internal standard. The assay allows the determination of tramadol O- and N-demethylation activity in vitro in microsomal fractions of human liver. Tramadol and its in vitro generated Phase I metabolites are extracted by a one-step extraction procedure from microsomal incubation mixtures using methylene chloride. Extraction efficiencies of tramadol, O-demethyltramadol and mono-N-demethyltramadol were 70, 91 and 94% respectively. The isocratic high-performance liquid chromatographic system employs a C_{18} reversed-phase column. The mobile phase is a mixture of methanol, ammonium hydrogencarbonate solution and ammonium hydroxide solution. Sensitivity of the assay was 0.5, 0.2 and 0.2 μg/ml for tramadol, O-demethyltramadol and mono-N-demethyltramadol, respectively. Within-run precision of the overall assay was 13, 3.1 and 7.6% for tramadol, O-demethyltramadol and mono-N-demethyltramadol, respectively. Accuracy of the assay was determined as mean differences of concentrations added and found in microsomal fractions. It was −2.4% for tramadol, −0.85% for O-demethyltramadol and 0.32% for mono-N-demethyltramadol.

Keywords: Tramadol; O-Demethyltramadol; Mono-N-demethyltramadol

1. Introduction

Tramadol (T) is a synthetic analgesic of the aminocyclohexanol group (Fig. 1) acting at opioid receptors [1,2]. In addition to the opioid mechanism (predominantly μ receptors) of antinociception tramadol modifies transmission of pain impulses by an inhibition of monoamine re-uptake [3]. The compound has two chiral centers. The marketed drug is a racemate of the *trans* isomers. Tramadol undergoes extensive hepatic metabolism. Approximately 85% of an oral dose are metabolized in healthy humans. Phase I hepatic biotransformation results in the formation of five metabolites (Fig. 1) [4]. A further six metabolites arise from conjugation (Phase II reactions) [4]. O-Demethyltramadol (M1 in Fig. 1) shows an analgesic activity in animals, which is superior to the parent compound [2,5].

Tramadol has some structural similarities with codeine. The analgesic activity of the weak opioid codeine is thought to be mediated by its O-demethylated metabolite morphine [6]. It has been

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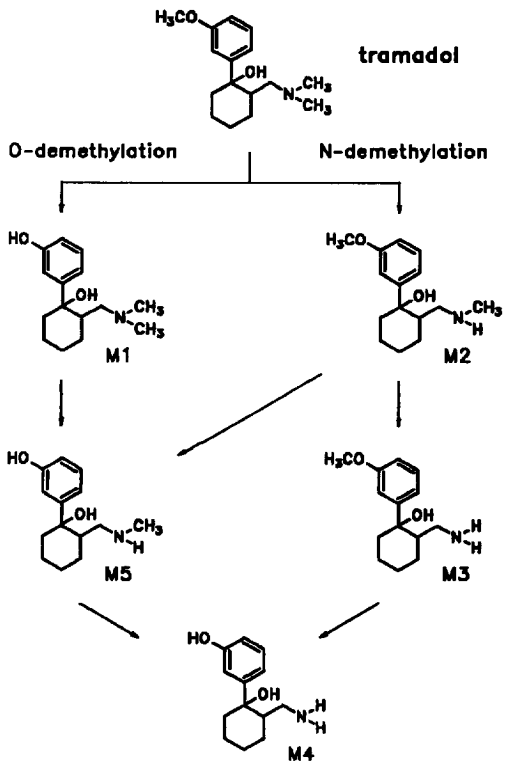

Fig. 1. Phase I metabolism of tramadol.

shown that the hepatic O-demethylation of codeine is carried out by the isozyme cytochrome P-450IID6 (CYPIID6) [7]. The gene encoding for CYPIID6 is known to show polymorphism [8,9]. The existence of different alleles of the CYPIID6 gene results in functionally different enzymes. This is the basis of large interindividual differences of the metabolism of those drugs requiring CYPIID6 to be eliminated from the body. CYPIID6 polymorphism is the most extensively studied oxidation polymorphism, which was discovered by the identification of so called "poor metabolizers" of the antihypertensive drug debrisoquine and the antiarrhythmic and oxytocic drug sparteine [10,11]. More than 25 drugs are substrates of CYPIID6, including drugs with a narrow therapeutic range (e.g. antiarrhythmic agents) [12,13]. In the case of codeine, poor metabolizers of sparteine/debrisoquine (6–10% of the white population) are virtually unable to demethylate codeine to the potent analgesic morphine [7,14,15]. Therefore poor metabolizers exhibit an either absent or at least weaker analgesic efficacy after intake of the weak analgesic codeine [16]. Possible co-segregation of the tramadol O-demethylation with the debrisoquine/sparteine oxidation polymorphism might have consequences for poor metabolizers because of the lack of formation of an active metabolite and/or alterations in tramadol disposition.

In order to study the possible impact of CYPIID6 in tramadol metabolism in vitro, a HPLC assay was developed to determine tramadol, O-demethylated-tramadol and mono-N-demethylated-tramadol in microsomal preparations of human liver.

2. Experimental

2.1. Materials

Tramadol hydrochloride, the internal standard (Fig. 2) (E 353) [1-(*m*-hydroxyphenyl)-2-(N-ethyl-N-methylaminomethyl)cycloheptan-1-ol hydrochloride] and the metabolites M1 (Fig. 1; mono-O-demethyltramadol hydrochloride), M2 (Fig. 1; mono-N-demethyltramadol hydrochloride), M3 (Fig. 1; di-N,N-demethyltramadol hydrochloride), M4 (Fig. 1; tri-N,N,O-demethyltramadol hydrochloride) and M5 (Fig. 1; di-N,O-demethyltramadol) were a gift of Grünenthal (Aachen, Germany). All reagents were of analytical grade.

2.2. Preparation of microsomes

Human liver tissue was obtained from tumor-free areas of liver tissue taken on the occasion of partial liver resection because of liver metastases. The liver samples were immediately frozen in liquid nitrogen and kept at −80°C until assayed. Duration of storage prior to preparation was about 3 months. Microsomes were prepared according to a method de-

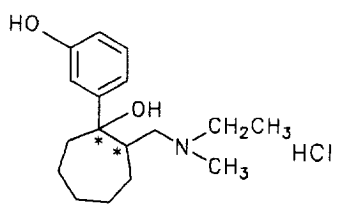

Fig. 2. Structural formula of the internal standard.

scribed by Dayer et al. [17]. The protein content of the microsomal preparations was estimated using the Lowry method [18], and the cytochrome P-450 content was determined by spectrophotometry [19]. Portions of the microsomal preparation could be stored up to 3 months at −80°C after preparation before to use them for incubation.

2.3. Incubation conditions

The incubation mixture (final volume 250 μl) contained: microsomal protein (150 μg, 25–60 pmol cytochrome P-450, 30 μl), Tris buffer (0.05 *M*, pH 7.5, 145 μl), $MgCl_2$ (0.06 *M*, 25 μl), tramadol (concentration range 50–2000 μ*M*, 25 μl). The reaction was started by the addition of NADPH (0.004 *M*, 25 μl), which was prepared fresh for each assay. Incubation was carried out at 37°C for 40 min. The reaction was terminated by the addition of 25% ammonia solution (10 μl).

2.4. Sample preparation

A 200-μl volume of the microsomal incubation mixture were pipetted into a clean tube and 0.5 μg of the internal standard (200 μl aqueous solution; concentration=2.5 μg/ml) and 400 μl of ethanol were added. The sample was mixed with the aid of a whirlmix and centrifuged at 2250 *g* for 15 min. The supernatant was transferred into a clean tube. After alkalization using 100 μl 25% NH_4OH, 5 ml methylene chloride was added. The sample was vortexed for 1 min and then centrifuged at 2500 *g* for 15 min. The aqueous phase was aspirated and discarded. The methylene chloride was blown down with nitrogen at 37°C. After addition of 200 μl of an ethanol–water mixture (3:1) the sample was redissolved by agitating for 0.5 min on a whirlmix. Prior to analysis the sample was stored at room temperature for up to 72 h without loss of stability.

2.5. Chromatographic system

The HPLC equipment consisted of an LC-6A pump (Shimadzu) fitted with a 502 autosampler (Beckmann, Düsseldorf, Germany) with a 100-μl sample loop. The analytical column (30 cm×4 mm I.D.) was filled with Nucleosil RP 18 (100-10) (Machery and Nagel, Düren, Germany). The analytical column was protected by a precolumn (125× 3 mm I.D.) containing the same material. Fluorescence detection was carried out at λ_{em} 310 nm and λ_{ex} 280 nm with an RF 530 fluorescence spectromonitor (Shimadzu). Recording of the peaks detected was done with a strip-chart recorder. The mobile phase was a mixture of methanol–0.1 *M* ammonium hydrogencarbonate solution–25% ammonium hydroxide solution (300 ml; 150:147:3, v/v) containing 30 μl triethylamine.

3. Results

3.1. Extraction procedure

The extraction efficiencies were determined at four different concentrations in duplicate (n=8). They were 70% for tramadol (S.D.=11.5%), 91% for M1 (S.D.=8.5%) and 94% for M2 (S.D.=7.4%) under the conditions reported in Section 2.4.

3.2. High-performance liquid chromatography

Efficiency of the chromatographic system allowed base line separation of T, internal standard and the metabolites M1–M5. The following retention times were determined: M4 9 min, M5 12.5 min, M1 18 min, M3 24 min, internal standard 35 min, M2 42 min, T 64 min. Fig. 3 shows an HPLC chromatogram of the extract from a denaturated microsomal fraction after addition of 0.5 μg T, 0.5 μg internal standard and 0.5 μg M1, M2, M3, M4 and M5.

3.3. Calibration

The calibration curve obtained from human liver microsomal fractions spiked with increasing amounts of T, M1 and M2 and a constant amount of the internal standard (0.5 μg) was linear in the range 0.5–4.0 μg/ml (Fig. 4).

3.4. Sensitivity

The limit of detection for tramadol was 0.5 μg/ml, the corresponding value for the metabolites M1 and M2 was 0.2 μg/ml (signal-to-noise ratio=5:1).

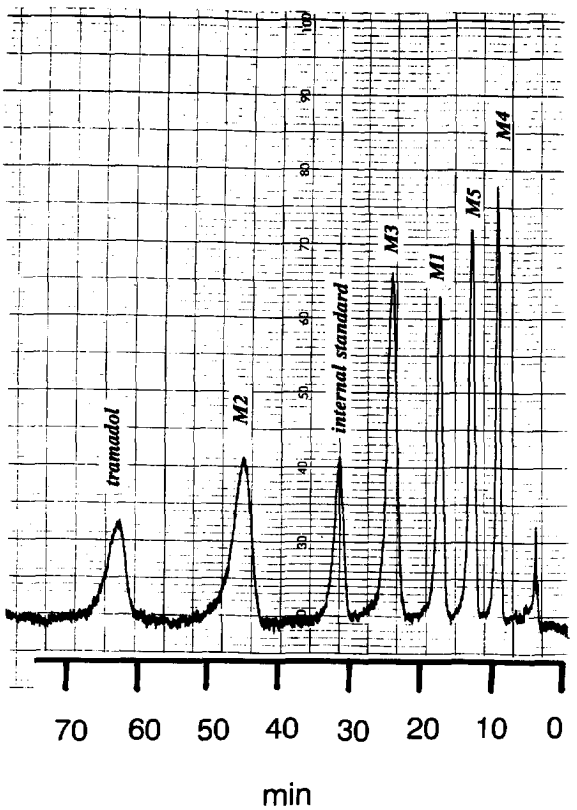

Fig. 3. HPLC chromatogram of the extract from a denaturated microsomal fraction after addition of 0.5 μg tramadol, 0.5 μg internal standard and 0.5 μg M1–M5.

3.5. Precision

Within-run precision of the HPLC assay was determined by analysing ten spiked denaturated human microsomal fractions. Concentration of tramadol and M1–M5 amounted to 0.5 μg/ml. Results are shown in Table 1.

3.6. Accuracy

Ten spiked microsomal fractions were analysed by HPLC. Table 2 shows the concentrations added and found. Mean deviations between concentrations added and found were −2.4% (range −14.7 to +12%), −0.8% (range −7.11 to +6.7%) and 0.32%

Table 1
Precision of the HPLC assay

Substance added (0.5 μg/ml)	Concentration found (mean±S.D.) (μg/ml)	R.S.D. (%)
Tramadol	0.47±0.061	13
M1	0.51±0.016	3.1
M2	0.49±0.037	7.6
M3	0.51±0.024	4.7
M4	0.53±0.036	6.8
M5	0.51±0.016	3.1

Within-run relative standard deviation (R.S.D., $n=10$) of the determination of tramadol and M1–M5 in microsomal fractions.

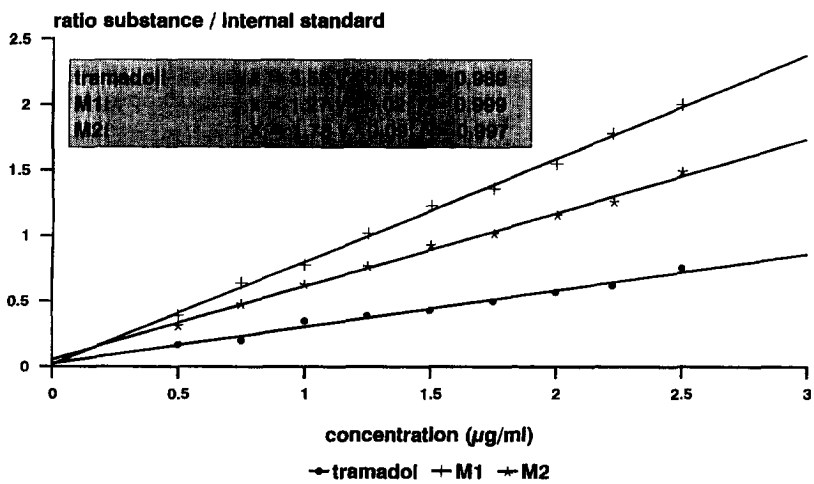

Fig. 4. Calibration curve for the determination of tramadol, O-demethyltramadol and mono-N-demethyltramadol in microsomal fractions of human livers.

Table 2
Accuracy of the HPLC-assay

Tramadol			M1			M2		
Added (μg/ml)	Found (μg/ml)	Difference (%)	Added (μg/ml)	Found (μg/ml)	Difference (%)	Added (μg/ml)	Found (μg/ml)	Difference (%)
0.05	0.054	8	0.05	0.049	−2	0.05	0.051	2
0.075	0.064	−14.7	0.075	0.08	6.7	0.075	0.079	5.3
0.1	0.112	12	0.1	0.098	−2	0.1	0.103	3
0.125	0.125	0	0.125	0.128	2.4	0.125	0.126	0.8
0.15	0.138	−8	0.15	0.154	2.7	0.15	0.153	2
0.175	0.16	−8.6	0.175	0.17	−2.9	0.175	0.167	−4.6
0.2	0.182	−9	0.2	0.194	−3	0.2	0.19	−5
0.225	0.208	−7.6	0.225	0.209	−7.11	0.225	0.21	−6.7
0.25	0.243	−2.8	0.25	0.25	0	0.25	0.244	2.4
0.3	0.32	6.7	0.3	0.29	3.33	0.3	0.312	4
Mean		−2.4			−0.85			0.32

Comparison of concentrations added and found in microsomal fractions ($n=10$).

(range −6.7 to +5.3%) for tramadol, M1 and M2 respectively.

3.7. Application of the high-performance liquid chromatography for the determination of tramadol and its metabolites in microsomal fractions

The HPLC assay proved to be suitable for the quantification of in vitro derived Phase I tramadol metabolites. Fig. 5 shows an HPLC chromatogram of the extract of a microsomal fraction after incubation with 500 μ*M* (131.5 ng) tramadol in the presence of NADPH (150 μg microsomal protein, 40 min).

The reproducibility of the incubation procedure and the HPLC-chromatography was determined by incubating ten samples containing 500 μ*M* tramadol under the conditions reported. Rate of tramadol-O-demethylation (M1 formation) had a coefficient of variation of 10.2%, corresponding value for the tramadol-N-demethylation (M2 formation) was 9.2%.

The linearity of the M1 and M2 formation was investigated by incubating 500 μ*M* tramadol for 5, 10, 20, 30, 40, 50 and 60 min. Linear formation of both metabolites was observed up to 60 min.

Protein dependency of the in vitro assay was determined in incubation mixtures containing the following microsomal protein concentrations: 50, 100, 200 and 300 μg. In this experiment 500 μ*M* tramadol were incubated for 40 min. Metabolite

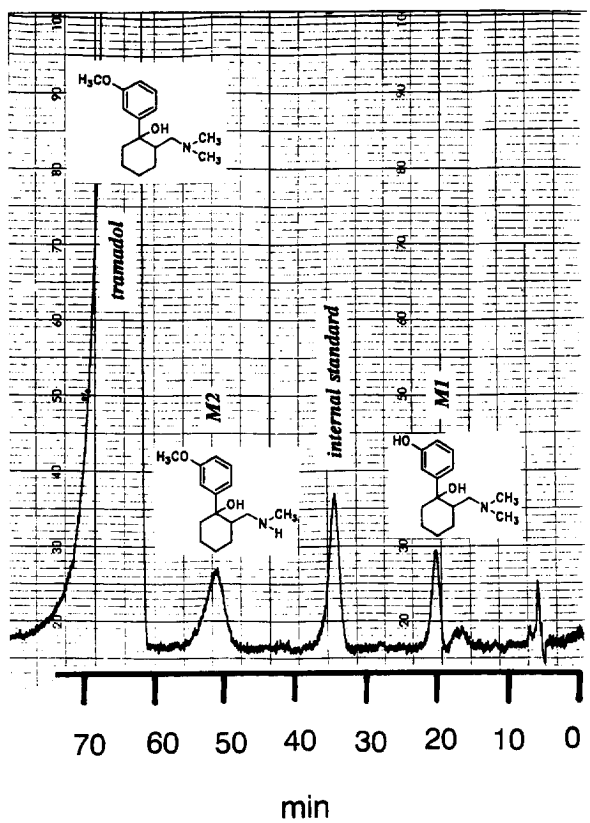

Fig. 5. HPLC chromatogram of the extract of a microsomal fraction after incubation with 500 μ*M* tramadol (131.5 ng tramadol, 150 μg microsomal protein, 40 min).

formation (M1 and M2) was found to be linear in the range up to 300 μg of microsomal protein/sample.

4. Discussion

The HPLC assay described allows the sensitive and precise determination of Phase I tramadol metabolites in supernatants of human liver microsomal fractions.

A gas-chromatographic assay described earlier by Becker and Lintz [20] has not been found suitable for the determination of the in vitro formed metabolites. In this assay tramadol has a shorter retention time as compared to its Phase I metabolites. Comparable to other drugs [21] high substrate concentrations of tramadol are required in the microsomal assay in order to gain sufficiently high metabolite concentrations. The resulting broad tramadol peak interferes in the gas chromatographic assay with the peak of M2. In order to avoid chromatographic interference with other metabolite peaks of interest, a longer retention time for tramadol peak was achieved by proper selection of the stationary and mobile phases, and optimization of chromatographic conditions (Fig. 5).

Sensitivity of the HPLC assay was found to be inferior to gas chromatography or gas chromatography–mass spectrometry [20,22]. Precision and accuracy were satisfactory for the in vitro investigation of tramadol Phase I metabolism.

The in vitro formed Phase I metabolites of tramadol were determined with the HPLC assay in microsomal fractions of human liver in the presence or absence of specific inhibitors of cytochrome P-450 isoenzymes. By this approach CYP-450IID6 was identified to carry out tramadol-O-demethylation [23]. Thus the sparteine/debrisoquine polymorphism is involved in the metabolism of tramadol. In addition, the HPLC analysis of in vitro formed M1 and M2 after separate incubations of tramadol enantiomers gave evidence for marked stereoselectivity of tramadol metabolism [24,25].

Both the finding of the involvement of a polymorphic cytochrome-P-450 isozyme, and the identification of stereoselectivity in tramadol metabolism might be of significance for the therapeutic use of tramadol.

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