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Journal of Chromatography B, 804 (2004) 369–374
JOURNAL OF CHROMATOGRAPHY B
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Synthesis of d_1-*N*-ethyltramadol as an internal standard for the quantitative determination of tramadol in human plasma by gas chromatography–mass spectrometry

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Received 2 September 2003; received in revised form 13 January 2004; accepted 23 January 2004

Abstract

A gas chromatography–mass spectrometry (GC–MS) assay for the determination of tramadol in human plasma is presented. The synthesis of an *N*-ethyl analogue of the drug is described and its use as an internal standard for the quantitative measurement of tramadol in human plasma is described. The method involves extraction at plasma pH and analysis of the underivatized drug by gas chromatography–electron ionization mass spectrometry using *m/z* 58 and 73 for detection of tramadol and internal standard, respectively. The calibration curve was linear in the range of 5–640 ng/ml plasma ($r = 0.9999$). The method was validated in the abovementioned calibration range. Data on solution stability, long- and short-term stability of tramadol in plasma samples, freeze–thaw-stability, as well as inter- and intra-day precision and accuracy have been evaluated and are presented. The application of the method to the pharmacokinetic profiling of the drug is demonstrated.

Keywords: Tramadol; d_1-*N*-Ethyltramadol

1. Introduction

Tramadol, (±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol (**I**, Fig. 1) is a widely used centrally-acting analgesic [1]. Its analgesic potency ranges between weak opioids and morphine [2,3]. Previously reported methods for the determination of tramadol in biological samples were gas chromatography with nitrogen-selective detection [4], gas chromatography (GC)–mass spectrometry (MS) [5–7] or liquid chromatography (LC) methods with UV [8,9], fluorometric [10–12] or electrochemical detection [13]. Methods involving capillary electrophoresis [14,15] or LC–MS [16,17] and LC–MS/MS [18] were also reported.

The choice of the internal standard is of crucial importance since it affects precision and accuracy of the method. In most of the described procedures, internal standards were used that differ significantly in their chemical structure from tramadol. Thus, physicochemical properties may vary to some degree and cause high variability during sample pretreatment and differences in detector response. Ideally, isotope-labeled analogues should provide the best results, but also structurally closely related compounds may be of similar usability. It was thus the aim of this study to prepare a suitable internal standard for the measurement of tramadol by GC–MS. Additionally, a rapid and simple sample work-up procedure was elaborated that allows high throughput analysis. The method was applied to the determination of tramadol in the course of a pharmacokinetic study.

2. Materials and methods

Tramadol·HCl (<99%) was from Lannacher Heilmittel, Lannach, Austria. Platinum(IV) oxide hydrate, acetaldehyde, and sodium borodeuteride were from Sigma, Vienna, Austria. All other solvents and reagents of analytical grade were from Merck, Darmstadt, FRG.

2.1. Preparation of d_1-*N*-ethyltramadol

d_1-*N*-Ethyltramadol was prepared from tramadol by *N*-demethylation and subsequent *N*-d_1-ethylation. *N*-Demethyla-

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doi:10.1016/j.jchromb.2004.01.045

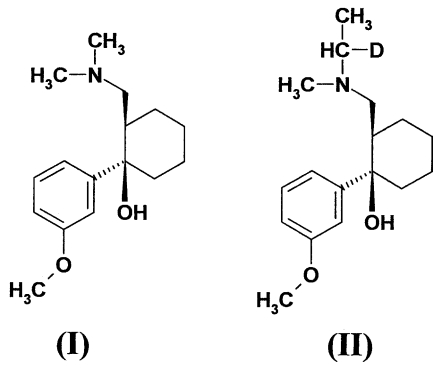

Fig. 1. Chemical structures of tramadol (**I**) and d_1-*N*-ethyltramadol (**II**).

tion was accomplished by the method of Birkenmeyer and Dolak [19] and Davis [20]. For this purpose, 20 mg of tramadol hydrochloride were dissolved in 1 ml of water in a conical glass vial. In a separate vial 218 mg of platinum oxide hydrate were dissolved in 1 ml of methanol and reduced with hydrogen gas for 5 min; the resulting mixture was centrifuged, and the precipitated platinum black catalyst washed twice with 0.5 ml water. The tramadol hydrochloride solution was added immediately to the catalyst, the vial equipped with a small magnetic stirrer, and the mixture stirred for 90 h. The reaction mixture was centrifuged, the liquid layer removed and the residue washed with 1 ml of water. The combined aqueous phases were treated with 0.5 ml of sodium hydroxide solution (3 M) and extracted twice with 2 ml of *n*-hexane. The hexane phase was directly used for the second step in the synthesis after evaporation of the solvent under nitrogen.

N-Ethylation was accomplished by reductive amination of *N*-demethyltramadol. The free base was dissolved in 0.5 ml methanol and 100 μl of acetaldehyde were added. After 20 min 4 mg of sodium borodeuteride were added, followed by a second portion after another 10 min. The mixture was then left at room temperature for 15 min. Then the solvent was removed under nitrogen, 500 μl of water were added along with sodium hydroxide (1 ml, 3 M), and extracted with *n*-hexane. The product was converted to the hydrochloride salt by treatment with HCl. The product was free of tramadol. No other products could be detected by GC–MS.

2.2. Gas chromatography–mass spectrometry

A TRACE 8000 gas chromatograph coupled to a Finnigan TRACE quadrupole mass spectrometer (ThermoQuest, Vienna) was used. The GC was equipped with a SGE BPX5 fused silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness; 5% phenyl polysilphenylene-siloxane, ultra low bleed) from ThermoQuest. The injector was operated in the splitless mode at 260 °C. Helium was used as a carrier gas at a constant flow rate of 1.5 ml/min. Initial column temperature was 100 °C for 1 min, followed by an increase of 40 °C/min to 300 °C and an isothermal hold of 2 min. Split valves were opened 1 min after injection. The mass spectrometer transfer line was kept at 310 °C. Electron ionization (EI) was performed at an electron energy of 70 eV and an emission current of 0.250 A. During single ion monitoring, *m/z* 58.03 and 73.09 were recorded for target and internal standard, respectively, with a dwell time of 30 ms.

2.3. Plasma sample preparation

Fifty microliter of the methanolic solution of the internal standard, containing 28 ng *N*-ethyl-d_1-tramadol were pipetted into a 4 ml disposable glass tube and 0.5 ml of plasma was added. After short, vigorous shaking 2.5 ml *n*-hexane were added. The tubes were stoppered and shaken at a reciprocal shaker for 10 min. After centrifugation at 1950 × *g* for 5 min, the (upper) organic layer was transferred to fresh disposable glass tubes (4 ml) and the solvent evaporated under a stream of nitrogen at 40 °C. The residue was dissolved in 100 μl acetonitrile, vortexed and transferred to autosampler vials (0.8 ml, amber). The vials were closed with crimp-top caps and stored at −20 °C until analysis.

2.4. Analytical method validation

Calibration graphs were established in the range of 5–640 ng/ml plasma. For this purpose, 0.5 ml blank plasma was spiked with the appropriate amounts of tramadol by adding 50 μl of the corresponding methanolic solution. Standard solutions of tramadol were prepared by serial dilution in methanol to yield concentrations of 640, 320, 160, 80, 40, 20, 10 and 5 ng/50 μl. Standard solutions were stored at −20 °C. Blank plasma was checked for possible tramadol content before use. Internal standard was added to calibration samples and work-up accomplished as described above. Peak area was used for quantification.

The *specificity* of the method was examined by the analysis of blank plasma samples derived from six different human volunteers. The samples were worked up without addition of the internal standard.

Inter-assay precision was determined at 5 ng/ml plasma (LOQ), 20.48 ng/ml plasma, 102.4 ng/ml plasma and 512 ng/ml plasma by carrying identical samples throughout the analytical sequence at 1 single day. Spiked samples were prepared from blank plasma. Five-fold determinations were carried out.

Intra-assay precision was determined at 5 ng/ml plasma (LOQ), 20.48 ng/ml plasma, 102.4 ng/ml plasma and 512 ng/ml plasma by multiple GC–MS analysis of one prepared sample. Spiked samples were prepared from blank plasma. Five-fold determinations were carried out.

Accuracy of the methods was also tested at the abovementioned concentrations after five-fold determinations. Thus, the data from inter-assay precision measurements were used to calculate the deviation of the values measured from the actual spiked values.

To measure *freeze–thaw stability*, three plasma samples at each of 20.48 and 512 ng/ml plasma were analyzed immediately after spiking with the indicated amount of internal standard, and again after three freeze–thaw cycles.

For assessment of *short-term stability* (i.e. stability of spiked plasma samples at room temperature), five samples at each of 20.48 and 512 ng/ml plasma were thawed and left to stand at room temperature for 3 h. After that, another five identical samples of the same concentrations were thawed. All samples were spiked with the internal standard and analyzed. Concentrations were calculated and the corresponding means were compared.

For determination of *long-term stability*, five samples at each of 20.48 and 512 ng/ml plasma were analyzed immediately. Another five identical samples of the same concentrations were analyzed after 8 weeks storage time at −20 °C. Concentrations were calculated and the corresponding means were compared.

To measure *methanolic solution stability* of tramadol, five samples at each of 1080 ng/ml were analyzed together with equivalent amounts of the internal standard after 12 weeks storage and compared to five identical samples of a freshly prepared solution. Area ratios were calculated and the corresponding means were compared.

Injection repeatability (i.e. the precision of the GC–MS measurements under reproducibility conditions) is measured by analyzing five spiked samples at 20.48 and 512 ng/ml concentration levels immediately after work-up and repeating the analysis 24 h later. Concentrations are calculated and the means are compared.

3. Results and discussion

3.1. Preparation of d_1-*N*-ethyltramadol

By using an internal standard with extraction and fragmentation properties very similar to those of tramadol, the drawbacks of a chemically significantly different internal standard can be avoided. We have previously applied this method of deamination–amination to the preparation of an analogue of clindamycin [21]. Since the procedure is suitable for tramadol as well due to the mild conditions employed, it can be regarded as a general strategy for the preparation of highly homologous compounds from substituted amines, as are frequently encountered in pharmaceutical analysis. Due to the addition of deuterium to the ethyl side-chain, the mass of the corresponding fragment is increased by 15 U instead of 14 U (Fig. 2B), which guarantees a minimum of interference. Although an increase of 14 U by the use of an ethyl- homologue would be satisfactory for use as an internal standard, additional deuteration allows to rule out possible interference from homologue contamination of the samples and/or the reference standards used at the time of method development. Since we never could observe such impurities, the non-deuterated homologue seems to be satisfactory as well. Nevertheless, deuteration allowed us to verify the proposed reaction mechanism of the amination procedure. The procedure was simple and rapid and found to be sufficiently pure to yield a standard without any interferences during analysis.

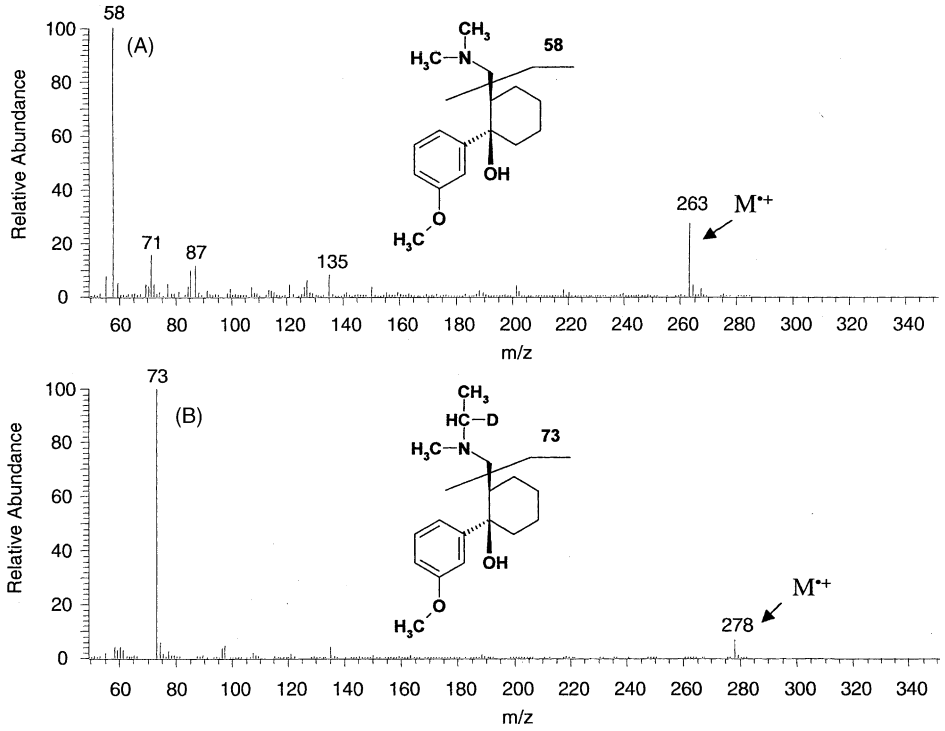

Fig. 2. EI mass spectrum of: (A) tramadol and (B) d_1-*N*-ethyltramadol.

3.2. Sample processing

The extraction procedure described here offers a rapid way to isolate tramadol from the plasma matrix. Usually, the methods referenced herein use solid phase extraction or liquid–liquid extraction with subsequent purification by back-extraction into HCl. Surprisingly, tramadol is equally well extracted from plasma at plasma pH as compared to extraction after addition of carbonate buffer at pH 9.3. Extraction with *n*-hexane produced the cleanest extract in terms of background interference in the chromatograms (Fig. 3). Samples were stored at −20 °C to permit sample re-analysis over a whole time range of batch sample processing.

3.3. Gas chromatography–mass spectrometry

The EI mass spectra of tramadol and d_1-*N*-ethyltramadol are shown in Fig. 2A and B. Both compounds show a highly abundant fragment ion arising from α-cleavage to the amino nitrogen, yielding *m*/*z* 58 and 73 for tramadol and the internal standard, respectively. It should be noted that the quality of the capillary column is critical, since detection at this low masses is prone to be influenced by column bleeding and excessive chromatographic noise. We have observed, that split injection does not alter this noise level on a "bad" column, thus tracing its origin to the column. Due to the excellent sensitivity, the only limitation is in fact this particular noise level. Typical single-ion monitoring (SIM) mass chromatograms obtained after analysis of tramadol in human plasma are given in Fig. 3.

3.4. Analytical method validation

The calibration graphs established were linear within the tested range of 5 and 640 ng/ml plasma, using 56 ng/ml of internal standard. The calibration curves typically show

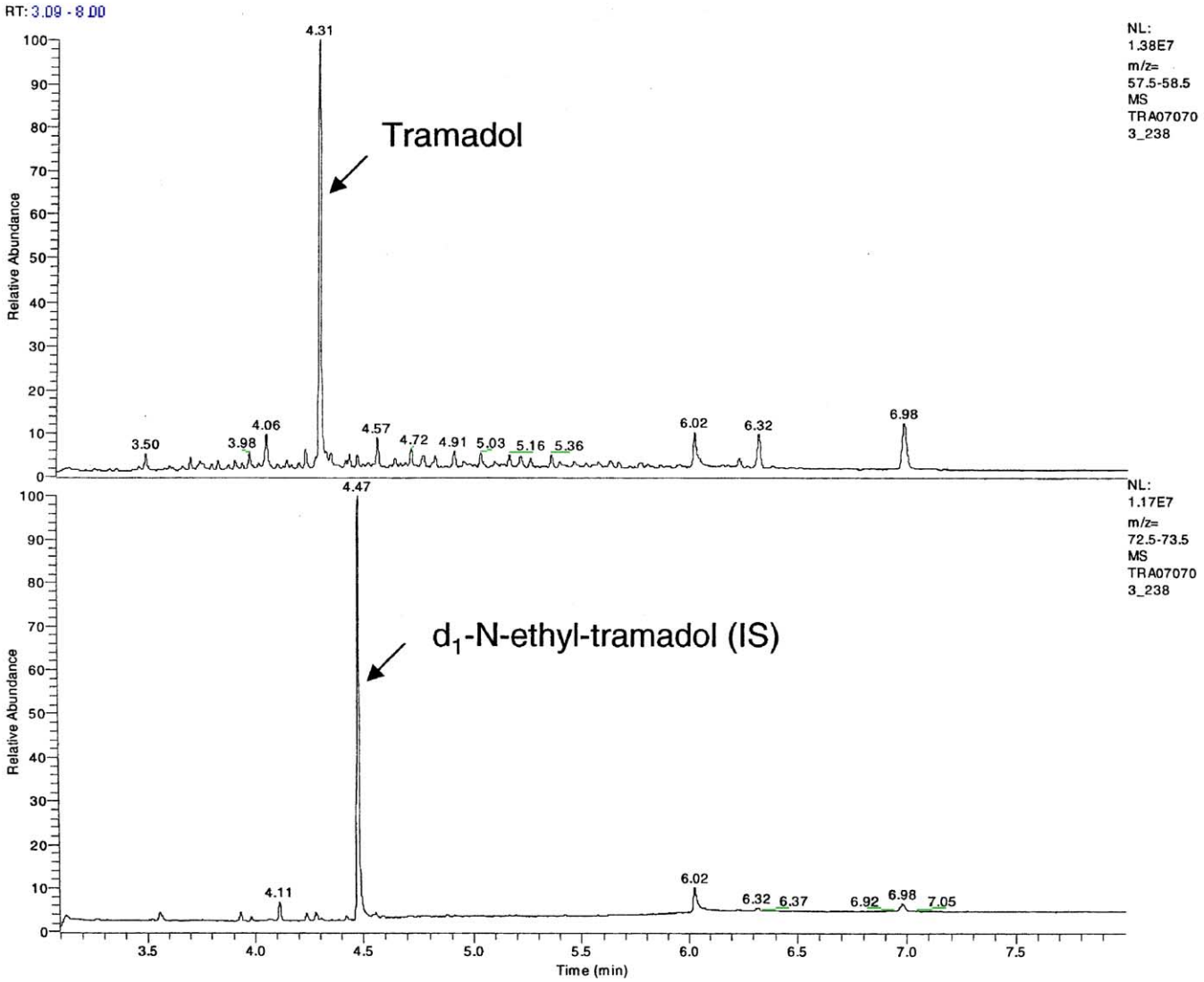

Fig. 3. Selected ion monitoring (SIM) mass chromatogram obtained after analysis of a plasma sample from a human volunteer (A) receiving 100 mg tramadol orally. The concentration measured in this sample was 58 ng/ml plasma.

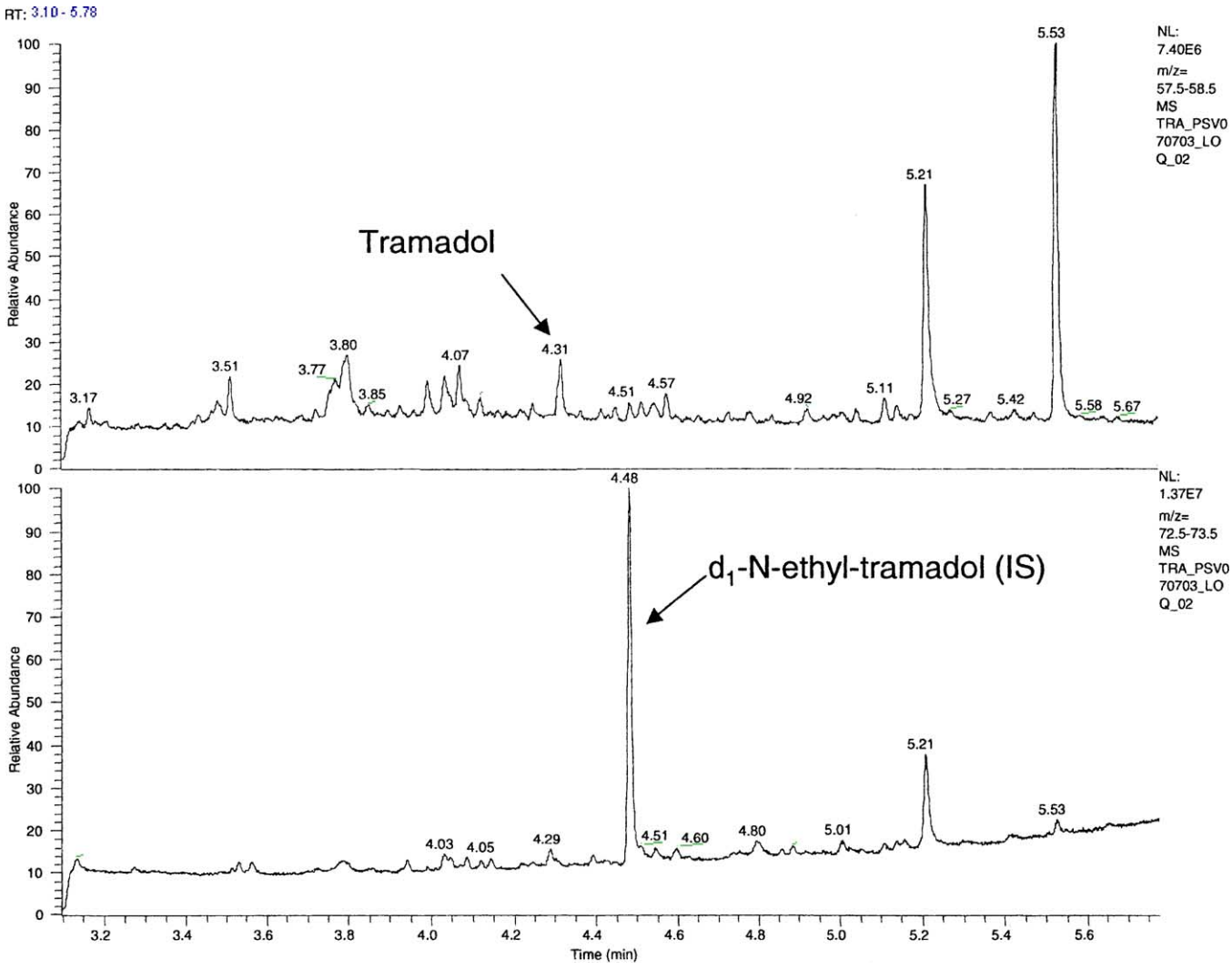

Fig. 4. Selected ion monitoring (SIM) mass chromatogram obtained after analysis of a spiked plasma sample containing 5 ng/ml plasma tramadol (LOQ).

r-values of 0.9993–1.0000 ($n = 6$). The lower limit of detection for the complete procedure was set to 5 ng/ml plasma (based on a signal-to-noise ratio of 3:1), estimated by analyzing spiked plasma samples (Fig. 4).

The coefficients of inter- and intra-day variation and accuracy of the spiked samples are presented in Tables 1 and 2. It can be seen from these data, that the method provides a highly precise and accurate assay for tramadol in human plasma. This can be attributed to the use of d_1-*N*-ethyltramadol as an internal standard. No decomposition of standard and stock solutions was measurable after 3 months of storage at −25 °C. Investigations on freeze–thaw stability of plasma samples at three different concentrations did not indicate any sample degradation after three freeze–thaw cycles. The results are shown in Table 3. Plasma samples containing tramadol were stable for at least 12 weeks when stored below −20 °C, as shown in Table 4. Under analysis conditions, derivatized samples were stable for at least 48 h. Six different blank matrices were checked for interferences. In none of the samples was there background

Table 1
Intra-day precision and accuracy of tramadol determination in human plasma

Spiked amount (ng/ml)	5	20.48	102.4	512
Mean	5.08	20.66	99.39	512.81
S.D.	0.08	0.41	1.31	2.60
C.V. [%]	1.66	1.98	1.31	0.51
Accuracy [%]	1.58	0.88	−2.94	0.16
n	5	5	5	5

Table 2
Inter-day precision and accuracy of tramadol determination in human plasma

Spiked amount (ng/ml)	5	20.48	102.4	512
Mean	5.11	20.54	99.32	514.89
S.D.	0.18	0.49	1.263	10.39
n	5	5	5	5
C.V. [%]	3.45	2.38	1.27	2.02
Accuracy [%]	2.14	0.31	−3.01	0.56

Table 3
Freeze–thaw stability of plasma samples containing tramadol

Spiked amount (ng/ml)	Amount found before three F–T cycles (ng/ml)	Amount found after three F–T cycles (ng/ml)	Percentage found after three F–T cycles
20.48	21.38	20.31	95.00
512	509.61	509.98	100.07

Plasma samples were analyzed immediately after spiking with the indicated amounts of tramadol and after three freeze-thaw (F–T) cycles.

Table 4
Long-term stability of plasma samples containing tramadol

	Nominal concentration (ng/ml plasma)					
	20.48	512	20.48	512	20.48	512
Replicate #	Immediate		After 5 weeks		After 12 weeks	
	Concentration found (ng/ml plasma)					
1	20.57	516.55	20.47	531.01	22.18	516.57
2	21.22	514.10	20.05	515.39	21.98	483.00
3	20.26	510.58	21.06	521.71	21.34	483.43
4	20.32	510.28	20.16	519.13	21.14	523.12
5	20.94	512.52	20.66	477.00	20.93	516.16
Mean	20.66	512.81	20.48	512.85	21.51	504.46
S.D.	0.41	2.60	0.41	20.85	0.54	19.59
Deviation [%]			−0.89	2.00	4.13	−1.63

Plasma samples being stored below −20 °C in a freezer for 5 and 12 weeks were compared to immediately analyzed samples.

contribution above 25% LOQ. Short-term stability measurements of tramadol revealed no difference between freshly prepared samples and samples worked up after 3 h at room temperature, as shown in Table 5. We have applied this method to the analysis of tramadol in human plasma in the course of a randomized crossover pharmacokinetic study to compare the relative bioavailability of two different formulations of tramadol. Volunteers received 100 mg of tramadol and blood samples were drawn at different time points. Values for all samples were within the calibrated range. A typical mass chromatogram obtained after analysis of a plasma sample after oral administration of 100 mg of tramadol is given in Fig. 3. The assay proved to be useful in the batch analysis of more than 900 plasma samples. Under the conditions described, tramadol and internal standard elute after 4.31 and 4.48 min from the GC, respectively. The total analysis cycle including cooling of the GC and equilibration time is 12 min per sample.

Table 5
Short-term stability of plasma samples containing tramadol

	Nominal concentration (ng/ml plasma)			
	20.48	512	20.48	512
Replicate #	Immediate analysis		Analysis after 3 h	
	Concentration found (ng/ml plasma)			
1	21.63	526.78	20.99	515.71
2	20.66	513.90	21.75	516.69
3	20.83	514.30	20.43	515.68
4	21.10	517.60	20.90	518.21
5	21.55	521.70	20.42	518.45
Mean	21.15	518.85	20.90	516.95
S.D.	0.43	5.42	0.54	1.33
Deviation [%]			−1.21	−0.37

Plasma samples subjected to the extraction immediately after thawing and those kept at room temperature for 3 h were analyzed.

The analysis of tramadol from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly specific assays with high sample throughput capacity. The facile preparation of a suitable ethyl- analogue described herein also allows access to compounds highly useful as internal standards, that show validation data comparable to isotope labeled analogues.

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