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QUANTITATIVE DETERMINATION OF TRAMADOL IN HUMAN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A gas chromatographic—mass spectrometric method for the quantitative determination of tramadol in human serum, plasma or whole blood samples is described. The method involves the use of [$^2H_2$,^{15}N]tramadol hydrochloride as an internal standard and chemical ionization with isobutane, employing single-ion monitoring for quantification. It is specific, sensitive and precise, and has high accuracy. The within-run coefficient of variation is about 1% between 25 and 200 ng/ml and 1.8—2.9% at the lowest concentrations tested (6.25 and 12.5 ng/ml). The between-run coefficient of variation increases from 1.6% to 5.2% with decreasing concentration from 200 to 12.5 ng/ml. The calibration graphs were linear in the tested concentration range, and the accuracy of the assay was not dependent on the sample volume used. The detection limit was about 4 ng/ml for serum samples of 1 ml. The method proved suitable for pharmacokinetic studies. Its high sensitivity allows measurements of serum concentrations for at least 30 h after the single administration of therapeutic doses of tramadol hydrochloride.

INTRODUCTION

Tramadol hydrochloride [*rac*-1(e)-(*m*-methoxyphenyl)-2(e)-(dimethylaminomethyl)cyclohexan-1(a)-ol hydrochloride, CG 315, Tramal; Grünenthal, Stolberg, F.R.G.] is a centrally acting analgesic [1—8] with an activity comparable to that of dextropropoxyphene, tilidine and codeine [2, 3, 9, 10], and is well tolerated [4—7, 9, 11, 12]. After single oral doses, tramadol is well and rapidly absorbed in man and animals, as was demonstrated by administration of [^{14}C]tramadol hydrochloride [13, 14]. The main metabolic pathways in man and all animal species tested are N- and O-demethylation followed by glucuronidation and sulphation of O-demethylated compounds [14, 15].

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Analytical methods for quantification of the unlabelled drug in body fluids have not previously been published.

This paper describes a gas chromatographic—mass spectrometric (GC—MS) method for the determination of tramadol in human serum, plasma and other biological fluids, which is suitable for pharmacokinetic studies following single administration of 50—100 mg tramadol hydrochloride. The method uses [$^2H_2$, ^{15}N]tramadol hydrochloride as an internal standard and the single-ion monitoring (SIM) technique after chemical ionization (CI) with isobutane.

EXPERIMENTAL

Standards

Drug standard. Tramadol hydrochloride of 99% purity (Grünenthal; Lot No. 1009/2) was used as a drug standard.

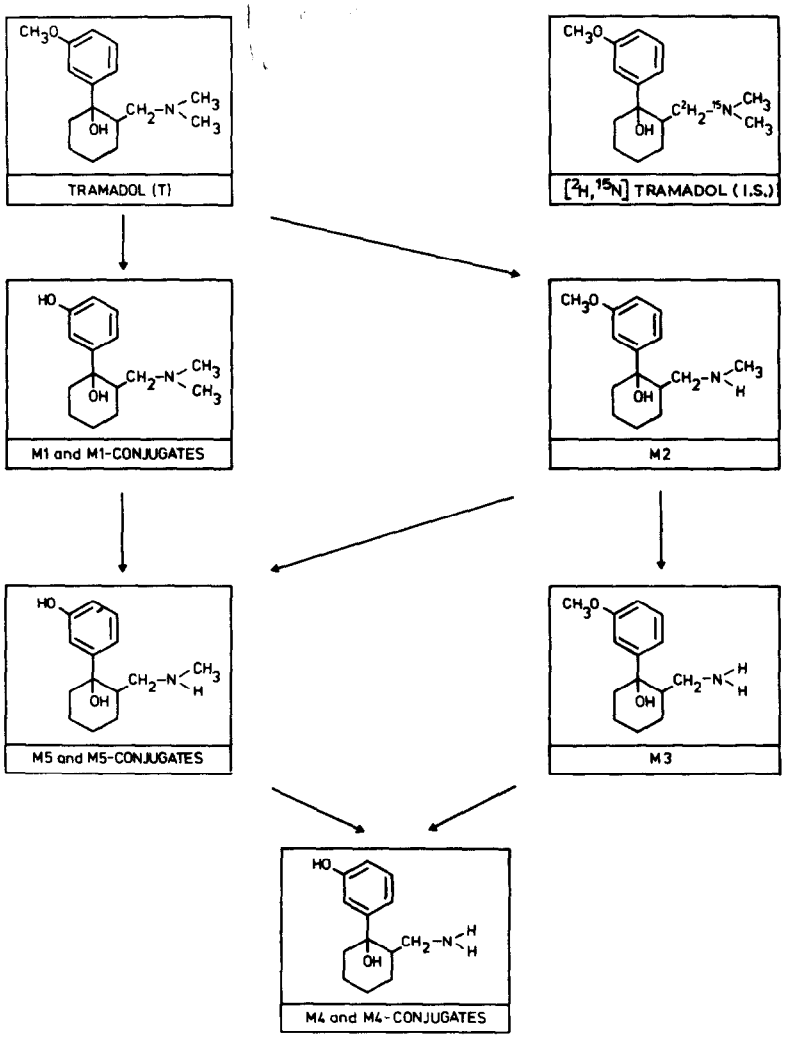

Fig. 1. Metabolic pathways of tramadol and structural formula of the internal standard.

Internal standard. [$^2H_2$,^{15}N]Tramadol hydrochloride (Fig. 1) as the internal standard was synthesized by the method originally described for unlabelled tramadol [1, 16]. The labelled intermediate compound was prepared via Mannich condensation from cyclohexanone, [$^2H_2$]paraformaldehyde (98 atom-% deuterium; Merck Sharp & Dohme, Montreal, Canada) and [^{15}N]dimethylamine hydrochloride (99 atom-% ^{15}N; Stohler Isotope Chemicals, Innerberg, Switzerland). Purification was performed by recrystallization with a

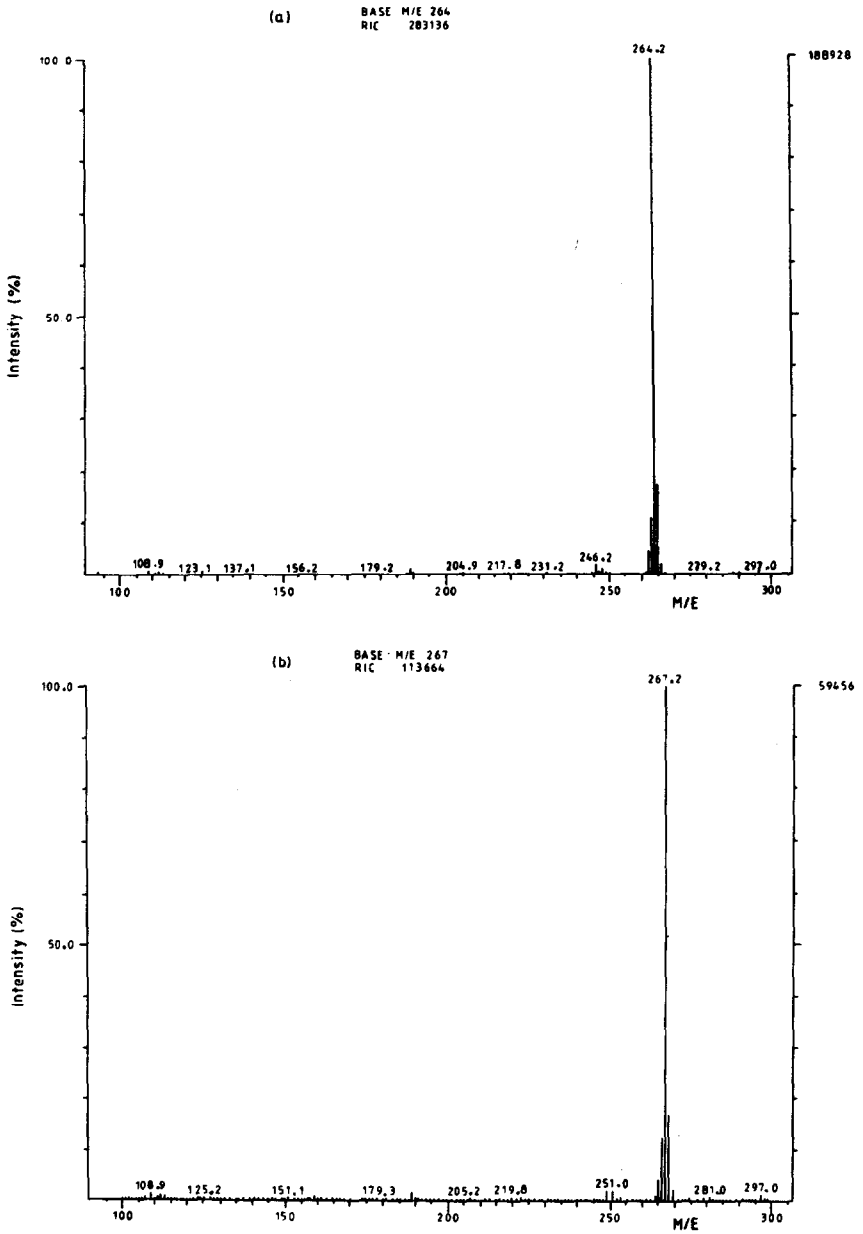

Fig. 2. Isobutane CI mass spectra of (a) tramadol, base peak *m/e* 264, and (b) [$^2H_2$,^{15}N]-tramadol, base peak *m/e* 267.

dioxane—water mixture (98:2). The chemical purity determined by thin-layer chromatography with chloroform—methanol—25% ammonia (90:8:2) as solvent system (hR_F = 74) exceeded 99%; the amount of the concomitant *cis*-isomer [1], as determined by high-performance liquid chromatography (HPLC), was 1—2%. The HPLC separation was achieved with a Nucleosil 10-C_{18} column (30 cm × 3 mm I.D.; 10 μm particle size; Macherey, Nagel & Co., Düren, F.R.G.) at an elution rate of 2.0 ml/min and a nominal pressure of 20.7 MPa. The elution system consisted of methanol—water (60:40) with 0.01% sodium bicarbonate, the temperature of the column was maintained at 40°C and the detector wavelength was 270 nm. The isotopic purity of [$^2H_2$,^{15}N]tramadol hydrochloride was about 99%, as shown by MS of pure standard samples (Fig. 2 and Table I).

TABLE I

RELATIVE ISOTOPE ABUNDANCE (RA) AND RECONSTRUCTED ION CURRENT (RIC) OF TRAMADOL AND [$^2H_2$,^{15}N]TRAMADOL (MASS RANGE: 264—269)

Mass	Tramadol (300 ng)		[$^2H_2$,^{15}N]Tramadol (300 ng)	
	RA (%)	RIC (%)	RA (%)	RIC (%)
264	100.00	66.73	0.97	0.50
265	17.19	11.47	4.41	2.31
266	2.00	1.34	12.59	6.59
267	0.20	0.14	100.00	52.31
268	0.04	0.03	17.17	8.98
269	0.06	0.04	2.32	1.21

Reference substances (drug metabolites). To determine the selectivity of the method, serum samples from several healthy volunteers, and all metabolites known so far, were used. The metabolites M1 to M5 were synthesized as described previously [14, 16]. The structures of these compounds are shown in Fig. 1.

Solvents

All solvents used in sample preparation were of analytical-reagent grade. Ammonia (25%), acetic acid (100%), *n*-hexane and chloroform were purchased from E. Merck (Darmstadt, F.R.G.). Doubly glass-distilled water was used to prepare the ammonia and acetic acid solutions.

Glassware

All glassware (tubes, stoppers and reaction vials) was rinsed with acetone and tap water, soaked overnight in 0.1 mol/l hydrochloric acid, thoroughly rinsed with tap water, then rinsed with doubly distilled water.

Sample preparation

Preparation of standard samples. Standard solutions of 1.00 mg/ml tramadol hydrochloride in water were prepared in 10-ml volumetric flasks and diluted to concentrations of 0.125, 0.25, 0.50, 1.00, 2.00 and 4.00 μg/ml. The diluted standard solutions of tramadol hydrochloride were then dissolved in pooled

drug-free serum to give final concentrations of 6.25, 12.5, 25, 50, 100 and 200 ng/ml tramadol hydrochloride. The final solutions were divided into 1.5-ml portions and stored at −20°C until used.

Preparation of quality control samples. Quality control samples with final concentrations of 12.5, 50 and 200 ng/ml tramadol hydrochloride were prepared and stored, as described for standard samples, by using independent standard solutions of tramadol hydrochloride in water and another serum pool.

Preparation of the internal standard solution. A 10.0-mg amount of [$^2H_2$,^{15}N]tramadol hydrochloride (internal standard, Fig. 1) was dissolved in 10.0 ml of water and diluted to a final concentration of 6.00 μg/ml. The stock solution was stored at −20°C, whereas the working solutions were kept at 4°C for two months at the most.

Extraction procedure

Samples of either 0.1 or 0.2 ml of serum were diluted to 1 ml with drug-free pool serum in 10-ml glass-tubes, whereas samples of 1.0 ml were used without dilution. About thirty unknown samples, six serum standards and three quality control samples were included in each extraction series. The internal standard solution (50 μl), containing [$^2H_2$,^{15}N]tramadol hydrochloride in water (6 μg/ml), was added to give a final concentration of 300 ng/ml. After mixing, the samples were alkalinized with 50 μl of ammonia (25%) and 3 ml of *n*-hexane were added. The samples were shaken for 20—30 min on a rotatory shaker and centrifuged for 5 min at 1400 *g*. As much as possible of the solvent phase was transferred into clean tubes containing 3 ml of 0.1 mol/l acetic acid.

The solvent phase was extracted with the acid by shaking for 20—30 min, followed by centrifugation as before. Thereafter the solvent phase was removed. The remaining acid phase was alkalinized with 200 μl of ammonia (25%) and extracted by gentle rotation (20—30 min) with 3 ml of *n*-hexane. After centrifugation the solvent phase was transferred into clean 10-ml glass tubes and evaporated to dryness under nitrogen at 37°C.

A 500-μl volume of chloroform was added and the samples were vortexed for 30 sec. The solutions were transferred into clean 0.3-ml V-shaped glass tubes (Micro Product V-vials, Wheaton Scientific, Melville, NJ, U.S.A.), evaporated under nitrogen at 37°C and the samples stored at 4°C. Prior to analysis, the samples were reconstituted in 20 μl of chloroform. Aliquots of 1—2 μl were injected into the gas chromatograph—mass spectrometer.

Gas chromatography—mass spectrometry

The measurements were carried out in a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a multiple-ion monitoring device (Finnigan 6100 computer data system, Finnigan, Munich, F.R.G.). The injector of the gas chromatograph was a Grob-type split—splitless system for capillary columns, which operated in the splitless mode. The injector was equipped with valves that were programmed to vent the injector 48 sec after injection. The glass capillary column used was a 25-m ARNC-SE-30 glass capillary column (Macherey, Nagel & Co.) and a pressure of 345 kPa (50 p.s.i.) of helium was applied to the column, which was directly faced with the ion source.

For quantification of tramadol in body fluids, the following GC—MS

parameters and conditions were established: injection port temperature, 250°C; oven temperature, programmed from 80°C to 240°C at 25.5°C/min; transfer line temperature, 240°C; carrier gas (helium) flow-rate, approx. 2 ml/min; ionization gas, isobutane; ionization pressure, 47 Pa (0.35 Torr); ionizer temperature, 180°C; emission current, 0.25 mA; electron energy, 100 eV; electron multiplier voltage, 1800—2200 V; time interval between two injections, 7—8 min; scanning time, 0.419 sec per mass unit.

Quantification

Peak areas were integrated by using the "cross-hairs" method of the Finnigan 6100 computer data unit. The ratio of the peak area of *m/e* 264 (tramadol) to that of *m/e* 267 ([$^2H_2$,^{15}N]tramadol) at t_R = 5.2 min was calculated for each sample. Calibration graphs were constructed by linear regression analysis of the calculated ratios versus concentration of drug added. They were calculated using a $1/y^z$ weighting scheme for each set of standard samples, where y denotes the response (ratio of the peak areas) and z ($0<z<2$) represents the concentration-dependent within-run variations in the responses of the corresponding standard samples. Control and unknown samples were calculated by using the regression equations of each experimental series.

Selectivity, sensitivity, accuracy and precision of the method

Selectivity. In order to determine the selectivity criteria of the method, it was first applied to tramadol-free serum from ten healthy volunteers. Further, the GC separation of tramadol from its metabolites M1—M5 was tested.

Sensitivity. Sensitivity tests were performed by using blank samples and standard samples of known tramadol concentration, which were diluted to values near the detection limit with drug-free pooled serum.

Accuracy and precision (within-run, between-run). All studies to determine within-run and between-run variations in accuracy and precision were performed with identical serum samples prepared in the same manner as the standard samples. In addition, the influence of the metabolite M2 on the accuracy and precision of the tramadol determinations was investigated after the addition of this metabolite in quantities double those of tramadol.

Application of the method

To characterize the practical value of the method, the time course of the tramadol concentration in serum, after intravenous application of 32.8 and 100 mg of tramadol hydrochloride, was determined in two human volunteers. After the bolus injection of the dose within 1 min, blood samples were taken via an indwelling cannula over the first 4 h, and by venipuncture over the following 20 h. After clotting at room temperature the samples were centrifuged. The supernatant serum was then separated and stored frozen at −20°C until analysed.

RESULTS AND DISCUSSION

Gas chromatography

Amines and amino alcohols tend to adsorb on glass capillary columns. We

nevertheless considered it appropriate to develop a GC—MS method for tramadol itself because (a) derivatization involves more time and expense in the preparation of the samples, and (b) pilot tests showed that almost symmetrical GC peaks also result with non-derivatized tramadol after deactivation of acid centres on the surface of glass capillary columns.

Glass capillary columns with a liquid phase are just as suitable for quantitative determination of tramadol as glass capillary columns with chemically bound phases (e.g., SE-30 or other polydimethylsiloxane phases) if the active centres of the glass surface are sufficiently deactivated. All the columns tested were deactivated by pre-treatment of the glass surface with hydrochloric acid and then silylation of the OH groups. The results presented in this paper were obtained with an SE-30 column that was not optimally deactivated. Although better deactivation of the columns gives completely symmetrical tramadol peaks, the results as regards selectivity, sensitivity, precision and accuracy are not significantly better, as the internal standard used compensates for all the problems caused by chromatography, if its serum concentration is at least 50 ng/ml.

Internal standardization

[$^2H_2$,^{15}N]Tramadol (Fig. 1) is a highly satisfactory internal standard for the GC—MS determination of tramadol after CI with isobutane for the following reasons:

(1) The bonding of both deuterium atoms is sufficiently stable. Exchange reactions during sample preparation and GC were not observed.

(2) Owing to the relatively small difference in the molecular weight of tramadol and [$^2H_2$,^{15}N]tramadol, isotope effects that could considerably affect reaction rates, distribution coefficients and chromatographic results are certainly irrelevant in practice. [$^2H_2$,^{15}N]Tramadol additionally offers carrier

TABLE II

WITHIN-RUN COEFFICIENTS OF VARIATION (C.V., n = 10) AND ACCURACY FOR STANDARD SAMPLES OF TRAMADOL (ADDED AMOUNT: 25 ng) IN HUMAN POOL SERUM WITH AND WITHOUT THE TRAMADOL METABOLITE M2 (METHOD A), AND FOR THREE METHODS OF SAMPLE PREPARATION (METHODS B, C AND D)

Method B: without dilution; method C: after dilution to 1.0 ml with water; method D: after dilution to 1.0 ml with pooled human serum.

Method	Sample volume (ml)	Amount of M2 added (ng)	Amount of tramadol found (mean ± S.D.) (ng)	C.V. (%)	Accuracy (mean) (%)
A	0.2	50.0	25.0 ± 0.200	0.80	100.0
A*	0.2	—	25.1 ± 0.279	1.11	100.4
A	0.2	—	24.8 ± 0.327	1.32	99.2
B	0.1	—	25.4 ± 0.340	1.34	101.6
C	0.1	—	25.1 ± 0.434	1.73	100.4
D	0.1	—	25.1 ± 0.323	1.29	100.4

*Repeated injection of same sample.

functions that are advantageous to overcome adsorption losses in the lower detection range during sample preparation and GC.

(3) The *m/e* shift by the isotope labelling of tramadol is three mass units; for this reason, it is sufficiently large to overcome the natural isotope abundance of tramadol on the quasi-molecular ion $[M + H]^+ = 264$ (*m/e* 267 content of tramadol = 0.20%; Table I).

(4) The isotope purity of [$^2H_2$,^{15}N]tramadol was about 99%, i.e., the tramadol content of [$^2H_2$,^{15}N]tramadol is lower than 1%, as was shown by MS of pure standard samples (Table I).

(5) CI of tramadol with isobutane guarantees high sensitivity because the quasi-molecular ions *m/e* 264 and 267 are the base peaks [reconstructed ion current (RIC), 50—70%; Fig. 2 and Table I].

(6) The mass fragmentography of tramadol and [$^2H_2$,^{15}N]tramadol by isobutane CI can be reproduced with high precision. As shown by the results in Table II, the coefficients of variation for repeated injections of identical tramadol—[$^2H_2$,^{15}N]tramadol mixtures into the GC system are lower than 2%.

Calibration graphs

Calibration graphs obtained for serum samples spiked with increasing amounts of tramadol hydrochloride and a constant amount of internal standard (300 ng) were linear for concentrations ranging from 6.25 to 200 ng/ml. All calibration graphs were obtained by a weighted least-squares method of fitting (see *Quantification*). Results for the parameters a and b of ten weighted linear regression lines $y = a + bx$ are listed in Table III, where y is the peak-area ratio, x is the concentration of tramadol (ng/ml) and a and b are the y-intercept and the slope of the regression line, respectively. The mean values and standard deviations of a and b and also the correlation coefficients ($r > 0.999$) indicate reasonable linearity between the detector response and the amount added to serum.

TABLE III

COEFFICIENTS OF VARIATION (C.V.) OF TEN WEIGHTED LINEAR REGRESSION LINES, $y = a + bx$, FOR SIX STANDARD SAMPLES

y = Peak-area ratio; x = amount of tramadol added (ng/ml); tramadol concentrations of each calibration line, 6.25, 12.5, 25.0, 50.0, 100 and 200 ng/ml; weighting factor, $1/y^{1.0915}$.

Parameter	Mean ± S.D.	C.V. (%)
y-Intercept (a)	0.00935 ± 0.00440	47.1
Slope (b)	0.00365 ± 0.00011	2.92
Correlation coefficient (r)	0.99971 ± 0.00030	0.030

Selectivity

Theoretically a very high selectivity might be expected, because isotope-labelled tramadol was used as an internal standard and the quantification was performed by SIM. In fact, under these conditions tramadol-free human serum does not cause any signal in addition to the tramadol content of the internal standard, as was shown by testing blank serum samples from ten healthy volun-

teers against water samples (Fig. 3 and Table IV). For the same reason, interference by other drugs is extremely improbable, the method affording three selectivity stages with ascending separation efficacy: even the first stage, sample preparation by extraction, guarantees extensive separation of acidic and neutral compounds from the alkaline drug tramadol. GC using glass capillary columns in the second stage should be able to separate all alkaline drugs that differ in polarity from tramadol. Finally, the last stage should overcome the residual separation problems, especially as it is characterized by very high selectivity. Hence only substances with great structural similarity could interfere with tramadol.

To confirm these considerations, we checked the influence of all metabolites of tramadol so far known on the separation and quantification of tramadol. As expected in view of the partition coefficients [14], the metabolites M1, M4 and M5 (Fig. 1) were almost completely separated by the extraction procedure, owing to their amphoteric character. Furthermore, traces of M1, M4 or M5

TABLE IV

COEFFICIENTS OF VARIATION (C.V.) OF THE *m/e* 264 BACKGROUND (ng/ml TRAMADOL EQUIVALENTS) FOR AQUEOUS BLANK SAMPLES AND BLANK SERUM SAMPLES FROM TEN HEALTHY VOLUNTEERS

Samples	Mean ± S.D.	C.V. (%)
Blank water samples	1.75 ± 0.225	12.9
Blank serum samples	2.04 ± 0.740	36.2

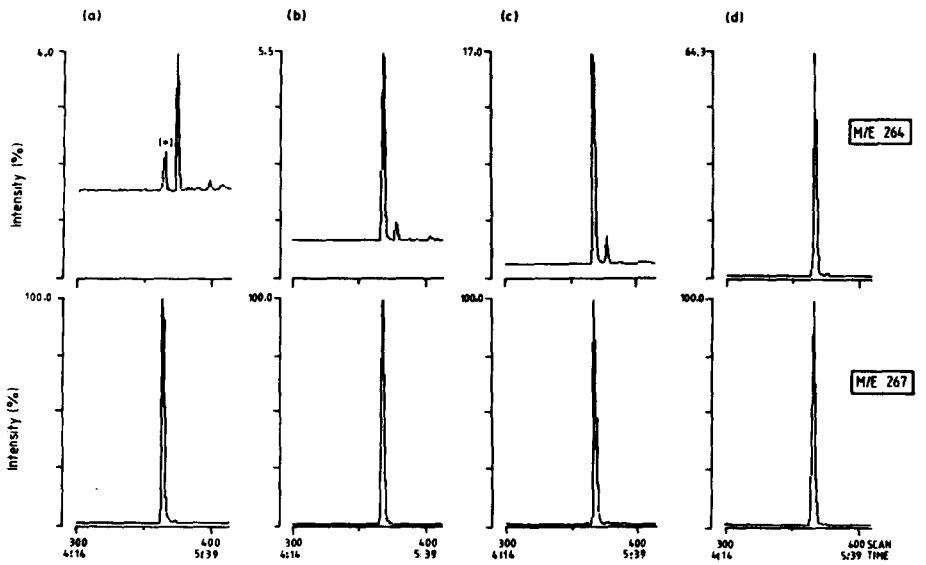

Fig. 3. Typical mass fragmentograms of human serum extracts spiked with different amounts of tramadol hydrochloride (*m/e* 264) (T), and 300 ng of internal standard (*m/e* 267): (a) blank serum; (b) 12.5 ng/ml T; (c) 50 ng/ml T; (d) 200 ng/ml T. The peak marked with an asterisk in mass fragmentogram (a) corresponds to the T content of the internal standard (cf., Table IV). Note the differences in the intensities at *m/e* 264 compared with those at *m/e* 267.

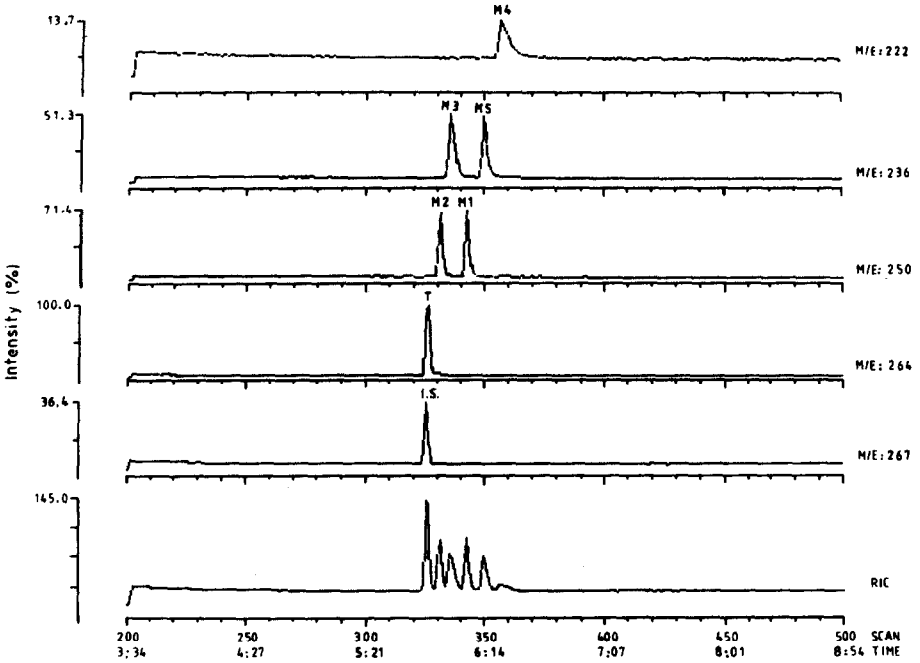

Fig. 4. Selected-ion monitoring (SIM) and reconstructed ion current (RIC) records after injection of 1 μl of a pure solution of tramadol, its metabolites M1—M5 and the internal standard [^{2}H,^{15}N]tramadol in chloroform. Concentrations, about 0.2 mg/ml; *m/e* values: 264 (T = tramadol); 267 (I.S. = [^{2}H,^{15}N]tramadol); 250 (M2 and M1); 236 (M3 and M5); 222 (M4).

extracted together with tramadol did not interfere with tramadol, the retention times of these metabolites under the GC conditions being greater than those of tramadol (Fig. 4). Appreciable amounts of the basic metabolites M2 and M3, however, are extracted together with tramadol; but there is no danger of interference in quantification, because the GC resolution between tramadol and these metabolites is satisfactory (Fig. 4). To confirm these considerations by experiment, we compared the results for tramadol-spiked serum samples with or without addition of a two-fold excess of M2. As shown in Table II, there was no significant difference between the samples containing M2 and those without M2. Hence the results confirm the expected high selectivity of the method, especially as M2 was added in amounts that are appreciably greater than those found in human serum following intravenous or oral administration of tramadol hydrochloride [14, 17].

Finally, glucuronides and sulphates of the metabolites M1, M4 and M5, which are known metabolites of tramadol in human serum [14], did not disturb the drug determination because they are not transferred to the solvent phase prior to enzymatic cleavage, and because they yield M1, M4 and M5, respectively, after cleavage.

Sensitivity

Maximum sensitivity for quantification was achieved by SIM using CI with isobutane and focusing on the base peaks of tramadol and [$^2H_2$,^{15}N]tramadol

at *m/e* 264 and 267, respectively (Fig. 2). The detection limit of the optimized MS detector with a freshly cleaned ion source is about 50 pg of tramadol, as was shown by injection of pure solutions of tramadol into the GC—MS system. This value corresponds to a theoretical detection limit of 0.5 ng/ml after extraction of 1 ml of serum and injection of one tenth of the final extract into the GC—MS system on condition that the overall recovery of tramadol is about 100%. As the recovery is reduced by the extraction procedure, and also by adsorption losses on the glassware and the GC column (see below), the actual detection limit would be about 1.0 ng/ml for 1-ml serum samples and injection of one tenth of the final extract, if the *m/e* 264 content of the internal standard were much lower than the actual detection limit. However, as [$^2H_2$,^{15}N]tramadol has an isotopic purity of about 99% and its tramadol content is about 1% (Table I), the amount of tramadol is about 3 ng when 300 ng of the internal standard are used, corresponding to 1.5 ng/ml at overall recoveries of about 50%.

Indeed, when pure water samples spiked with 300 ng of [$^2H_2$,^{15}N]tramadol hydrochloride were analysed, the background peak at *m/e* 264 corresponded to 1.75 ± 0.23 ng/ml of tramadol hydrochloride in water (Table IV). Similar results were obtained by using tramadol-free serum samples from ten volunteers instead of pure water samples (Table IV). This indicates that the background peak originates exclusively from the internal standard added as carrier and for exact quantification. Further, in the chromatograms of the blank samples no additional background peak was observed to interfere with tramadol. From the statistical results ($\bar{x}$ ± S.D.) for the background of the internal standard at *m/e* 264, and for the *y*-intercept (*a*) of the linear calibration graphs (see above), the 99% detection limit of the method ($\bar{x}$ ± 3 S.D.) is 4.3 and 3.6 ng/ml, respectively, if 300 ng of internal standard and 1 ml of serum are used, but can be lowered to about 1 ng/ml if only 50 ng of the internal standard are applied.

Accuracy and precision

Both the within-run and between-run accuracy and precision of the assay were investigated over a six-month period and the results are presented in Tables V and VI. Acceptable accuracy and precision were achieved even at the lowest concentration. The within-run coefficient of variation was 0.5—1.1% at concentrations between 25 and 200 ng/ml and 1.8—2.9% at the lowest concentrations

TABLE V

WITHIN-RUN COEFFICIENTS OF VARIATION (C.V., n = 10) AND ACCURACY OF TRAMADOL DETERMINATION IN HUMAN POOL SERUM

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (mean) (%)
6.25	6.28 ± 0.181	2.88	100.5
12.5	12.1 ± 0.213	1.76	98.6
25.0	25.4 ± 0.280	1.10	101.6
50.0	50.9 ± 0.440	0.864	101.8
100	100.4 ± 0.436	0.434	100.4
200	198.6 ± 1.45	0.728	99.3

TABLE VI

BETWEEN-RUN COEFFICIENTS OF VARIATION (C.V., n = 10) AND ACCURACY OF TRAMADOL DETERMINATION IN HUMAN POOL SERUM

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (mean) (%)
12.5	12.3 ± 0.645	5.27	98.4
25.0	24.6 ± 0.931	3.78	98.4
50.0	50.4 ± 1.21	2.41	100.8
100	100.7 ± 1.68	1.67	100.7
200	199.5 ± 3.21	1.61	99.8

tested (6.25 and 12.5 ng/ml) (Table V). For this reason, the within-run accuracy also depends slightly on the concentration and varies from 98.6% to 101.8% in the concentration range from 6.25 to 200 ng/ml. As expected, the results for the between-run coefficients of variation were slightly inferior to the within-run results, increasing from 1.6% to 5.3% with decreasing concentrations in the range from 200 to 12.5 ng/ml, whereas the accuracies were as good as within the same run (98.4—100.8%) (Table VI). Hence the within-run and between-run experiments confirm that the GC—MS method is characterized by good precision and high accuracy.

Further characterization of the method

Stability of tramadol in serum. Since tramadol is very stable in aqueous solutions at room temperature, good stability in serum, especially at −20°C, can be expected. In fact, no decrease in tramadol concentration was detectable after storage of samples at −20°C over a period of one year (see Table VII).

Extraction recovery. The extraction recovery was determined on samples of serum spiked with [^{14}C]tramadol hydrochloride (50 ng/ml). As expected in view of the partition coefficients [14], the extraction recovery was almost 100% if alkaline aqueous phases (pH > 9) were extracted with n-hexane. Nevertheless, the overall recovery of tramadol during sample preparation was about half that (50—55%). This relatively low overall recovery is due to incomplete phase separations during sample preparation, and also by inevitable adsorption losses to the glass surfaces during the extraction and concentration procedures. Hence the extraction procedure reduces the sensitivity of the assay, but this effect is compensated for, the three-step extraction providing clean extracts for analysis.

Protein binding. Binding of tramadol to human serum proteins is slight (about 14%) [18]. Further, it does not influence the extraction recovery, as was shown by comparison of water and serum samples. Using [^{14}C]tramadol hydrochloride we obtained identical and nearly complete recoveries of radioactivity in the solvent phases of both sample types after extractions without re-extraction. This result indicates that the protein binding of tramadol is broken by the extraction procedure. Consequently, the results of quantitative determination are independent of the sample size, as was shown by analysing samples containing equal amounts of tramadol hydrochloride in 0.1 and 1.0 ml of human serum (Table II).

TABLE VII

SERUM CONCENTRATIONS OF TRAMADOL HYDROCHLORIDE (ng/ml) AFTER INTRAVENOUS INJECTION OF 32.8 (A) AND 100 mg (B) TO TWO MALE VOLUNTEERS (A AND B), ASSAYED ON TWO DIFFERENT DAYS

Time delay between the first (c_1) and the second (c_2) tramadol determination; three days (A) and about one year (B); c_1/c_2 = ratio of first to second tramadol determination.

Time (h)	Serum concentration (ng/ml)					
	Volunteer A			Volunteer B		
	c_1	c_2	c_1/c_2	c_1	c_2	c_1/c_2
0.017	N.D.*	N.D.	N.D.	1149	1145	1.003
0.045	2075	2062	1.006	1011	962	1.051
0.080	484	475	1.019	941	981	0.959
0.112	340	328	1.037	N.D.	N.D.	N.D.
0.16	236	240	0.983	635	610	1.041
0.250	173	173	1.000	582	602	0.967
0.333	160	154	1.039	N.D.	N.D.	N.D.
0.500	138	140	0.986	579	578	1.002
0.750	110	113	0.973	N.D.	N.D.	N.D.
1.04	101	103	0.981	490	N.D.	N.D.
1.3	99.0	98.0	1.010	N.D.	N.D.	N.D.
1.5	89.9	91.4	0.984	447	410	1.090
2.0	84.2	80.5	1.046	394	407	0.968
2.5	78.7	80.5	0.978	N.D.	N.D.	N.D.
3.0	74.4	74.2	1.003	334	337	0.991
4.0	61.3	60.8	1.008	284	292	0.973
5.0	57.3	56.5	1.014	N.D.	N.D.	N.D.
6.0	52.1	54.4	0.958	232	237	0.979
7.0	49.2	48.1	1.023	N.D.	N.D.	N.D.
8.0	45.1	47.3	0.953	185	189	0.978
10.0	41.6	N.D.	N.D.	144	143	1.007
12.0	34.1	36.9	0.924	122	114	1.070
14.0	N.D.	N.D.	N.D.	104	106	0.981
24.0	N.D.	N.D.	N.D.	41	42	0.976
Mean			0.996			1.002
S.D.			0.031			0.040

*N.D. = Not determined.

Other biological fluids. Tests with blank samples of human urine, human bile, human milk and tissue samples from rats showed that the method described is also applicable to other biological fluids and tissue homogenates, without essential modifications to the sample preparation. In addition, identical blank chromatograms were obtained by using whole blood or plasma samples instead of serum samples. This means that it is possible to apply the method to blood or plasma in addition to serum.

Application of the assay

The utility of the method was demonstrated by applying it to the determination of tramadol in the serum of two healthy volunteers after intravenous

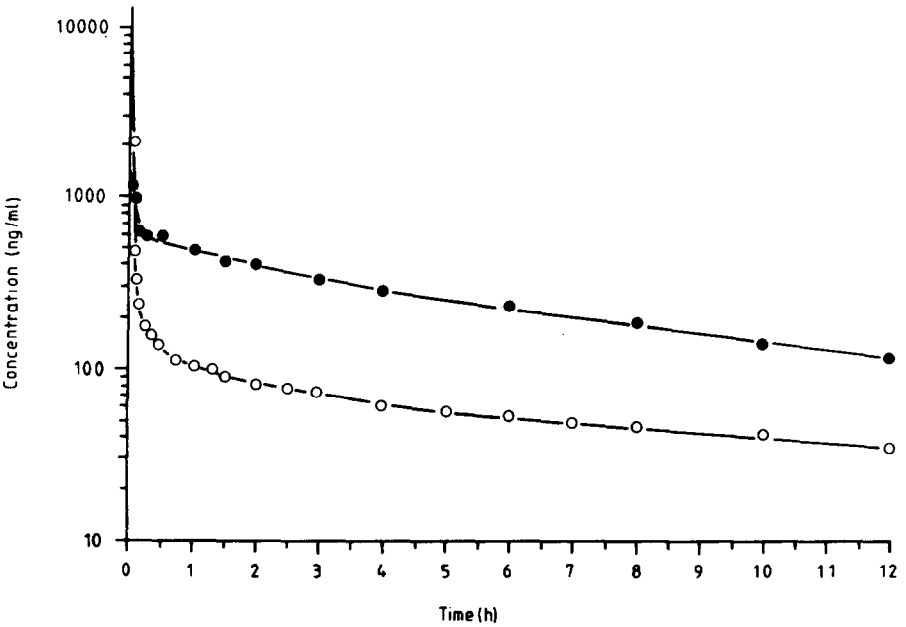

Fig. 5. Time course of serum concentrations of tramadol hydrochloride (mean values of two assays) following a single intravenous dose of 32.8 mg (volunteer A, ○) and 100 mg (volunteer B, ●) of tramadol hydrochloride administered to two healthy male volunteers. For exact values, see Table VII.

injection of tramadol hydrochloride. The results are given in Fig. 5 and Table VII. The serum concentration curves are fairly consistent, indicating a relatively short distribution phase with an apparent half-life of about 0.5 h and a terminal half-life of approximately 6 h. The time courses show that the technique described is sufficiently precise for the determination of serum concentrations of tramadol hydrochloride in humans, and that it is sensitive enough to allow the determination of tramadol over five to six half-lives, i.e., 30—36 h after the administration of therapeutic doses (50—100 mg).

CONCLUSION

With the GC—MS method described it is possible to determine tramadol quantitatively in serum, plasma, whole blood and other biological samples. The method offers the advantage of combining precision, accuracy, sensitivity and selectivity, and eliminates any interference from endogenic compounds in the biological fluids. Therefore, it appears satisfactory for all pharmacokinetic and drug monitoring studies after therapeutic dosage, allowing accurate determination of tramadol concentrations up to at least five biological half-lives.

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