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DETERMINATION OF TRAMADOL IN HUMAN SERUM BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

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SUMMARY

A specific, sensitive and precise method for the determination of tramadol in human serum is described. It comprises a three-step extraction procedure and a specific determination by capillary gas chromatography with nitrogen-selective detection, using a homologue of tramadol as an internal standard. The specificity of the method was checked by gas chromatography—mass spectrometry. Precision parameters were 1.7—5.5% (within-run) and 3.2—5.7% (between-run) in the concentration range 12.5—200 ng/ml. The detection limit was about 3 ng/ml.

INTRODUCTION

Tramadol [rac-1(e)-(*m*-methoxyphenyl)-2(e)-(dimethylaminomethyl)cyclohexan-1(a)-ol hydrochloride, Tramal®] is a centrally acting analgesic with an activity comparable to that of dextropropoxyphene, tilidine, codeine and pentazocine [1—5].

Investigations involving ^{14}C-labelled tramadol have shown that the drug is rapidly and well absorbed [6]. Tramadol is metabolized mainly by N- and O-demethylation followed by conjugation of the O-demethylated metabolites [7, 8]. In order to carry out pharmacokinetic and bioavailability studies, very sensitive and specific assays are required. The first tramadol assay to be developed involved gas chromatography—mass spectrometry (GC—MS) with stable isotope-labelled tramadol serving as an internal standard [9]. Due to its sensitivity, specificity and precision, this method has proved valuable and has been successfully employed in several pharmacokinetic investigations in our laboratory.

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The availability of the GC—MS method, however, is restricted by the costly equipment required. To overcome this limitation, the present study describes the development of a gas chromatographic procedure with nitrogen-selective flame ionization detection (GC—N-FID) for the sensitive and selective determination of tramadol. Assay parameters and correlation of the GC—N-FID method with the GC—MS method are presented.

EXPERIMENTAL

Reagents and chemicals

Tramadol hydrochloride (Fig. 1, I), the internal standard (Fig. 1, II) and the metabolites M1 (mono-O-demethyltramadol hydrochloride), M2 (mono-N-demethyltramadol hydrochloride), M3 (di-N,N-demethyltramadol hydrochloride), M4 (tri-N,N,O-demethyltramadol hydrochloride) and M5 (di-N,O-demethyltramadol hydrochloride) were synthesized by methods described previously [7, 10].

Ammonia (25%), acetic acid, *n*-hexane and methanol, all of analytical grade, were obtained from Merck (Darmstadt, F.R.G.).

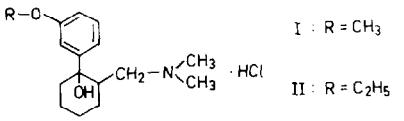

Fig. 1. Structural formulae of tramadol (I) and the internal standard (II).

Preparation of standard and control 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 and 2.00 μg/ml. The diluted standard solutions of tramadol hydrochloride were then dissolved in pooled drug-free serum to give final concentrations of 12.5, 25, 50, 100 and 200 ng/ml tramadol hydrochloride. The single pools were divided into 1.5-ml portions and stored at −20°C until used.

From the stock solution of the internal standard (1.00 mg/ml in water), working solutions of 3 μg/ml were prepared. The stock solution was stored at −20°C, whereas the working solutions were kept at 4°C for a maximum of one month.

Extraction procedure

All glassware used in sample preparation was thoroughly rinsed with tap water, acetone and double-distilled water. An extraction series included about 25 unknown serum samples, 5 serum standards (12.5—200 ng/ml) and 3 control samples. The unknown samples were used either undiluted or diluted with 0.9% sodium chloride solution to give tramadol hydrochloride concentrations not exceeding 200 ng/ml.

Samples (1 ml) were placed in 10-ml glass tubes. Then 0.1 ml of the internal standard solution (300 ng), 0.05 ml of ammonia solution and 3 ml of *n*-hexane were added. The samples were shaken for 20—30 min on a rotary shaker and centrifuged for 5 min at 1400 *g*. The organic phase was transferred to 10-ml

tubes containing 3 ml of 0.1 *M* acetic acid. After extraction (20–30 min) and phase separation, the organic layer was discarded. The remaining aqueous phase was alkalinized with 0.2 ml of ammonia solution and extracted by gentle rotation (20–30 min) with 3 ml of *n*-hexane. After centrifugation, the organic phase was transferred to 10-ml glass tubes and evaporated under nitrogen at 37°C.

A 0.5-ml volume of methanol was added and the samples were vortexed for 30 s. The solutions were transferred to 0.5-ml V-shaped glass tubes (Microproduct V-vials, Wheaton Scientific, Melville, NJ, U.S.A.) and evaporated under nitrogen at 37°C.

The samples were reconstituted in 25 μl of methanol and stored at 4°C until analysed. Aliquots of 1–2 μl were injected into the gas chromatograph.

Gas chromatography

The analyses were performed on a Hewlett-Packard 5730/A gas chromatograph equipped with a Grob-type split–splitless injector and a nitrogen–phosphorus-selective detector (Model 188481). An integrator (Model 3390 A) printed peak heights and peak half-widths after each run. Fused-silica columns of dimension 25 m × 0.32 mm I.D. (FS-SE30-CB-0,25), coated with chemically bonded SE 30, were obtained from Macherey-Nagel (Düren, F.R.G.).

Operating conditions were: injector temperature 250°C, detector temperature 300°C. After injection in the splitless mode, the split was opened 35 s later and the temperature programme was started. The column temperature was programmed from 100 to 240°C at 32°C/min. Gas flow-rates were: helium (carrier), 60 cm/s; hydrogen, 4 ml/min; air, 80 ml/min; helium (auxiliary gas), 50 ml/min.

For the injections, 10-μl microsyringes from Glenco Scientific (Houston, TX, U.S.A.) were used. Between injections, they were thoroughly cleaned with methanol which was sucked through by vacuum.

Quantification

The ratios of peak height of tramadol to that of the internal standard (y) were calculated and the tramadol concentrations were determined from standard curves derived from analysis of the five standard serum samples.

Standard curves were constructed by linear regression analysis of the weighted ratios versus the amount of drug added (12.5–200 ng/ml). A weighting scheme for the y ratios was calculated using a computer program developed and supplied by Varoqui [11]. First, the exponent p in eqn. 1 representing relative standard deviation (S.D./y) as a function of y was determined by least-square fitting of the measured S.D./y and y values at five standard serum concentrations (12.5–200 ng/ml).

$$\frac{\text{S.D.}}{y} = k\, y^p \qquad (1)$$

As shown by others [12], the weighting factor w is related to the variance according to eqn. 2.

$$w = \frac{K}{(\text{S.D.})^2} \quad (2)$$

Eqn. 1 rearranged to eqn. 3

$$\text{S.D.} = k\, y^{p+1} \quad (3)$$

and substitution of eqn. 3 into eqn. 2 yields eqn. 4.

$$w = \frac{K}{k^2\, y^{2(p+1)}} \quad (4)$$

K can be freely selected. By setting $K = k^2$ and substituting $2(p + 1)$ by q, eqn. 4 may be simplified to eqn. 5.

$$w = \frac{1}{y^q} \quad (5)$$

Gas chromatography—mass spectrometry

The method involves the use of [$^2H_2$,^{15}N]tramadol hydrochloride as an internal standard and chemical ionization with isobutane. The base peaks of tramadol (*m/e* 264, $[M + H]^+$) and internal standard (*m/e* 267, $[M + H]^+$) were monitored. For more details, see ref. 9.

RESULTS AND DISCUSSION

Extraction procedure

The three-step pH-dependent procedure allows the extraction of lipophilic basic compounds such as tramadol, the internal standard and metabolites M2 and M3. The amphoteric metabolites M1, M4 and M5, however, were not extracted by *n*-hexane [7]. In order to extract the latter metabolites from serum, more polar solvents such as methylene chloride or ethyl acetate were required. The low background of serum blanks shows that the extraction of other lipophilic basic compounds from serum is of no importance for the tramadol assay (Fig. 2).

The extraction yield of tramadol and the internal standard (II) from serum was determined by GC analysis. In order to allow quantification of the serum extracts, II or tramadol was added as standard to the serum extracts after the last extraction step. The extraction yield of tramadol was 87.0 ± 2.2% ($n = 5$, $c = 100$ ng/ml), whereas the extraction yield of II was slightly superior (95.1 ± 5.7%; $n = 5$, $c = 100$ ng/ml). Thus the extraction recovery of both compounds was nearly complete. The overall recovery, i.e. the amounts found in the methanolic solution to be injected into the GC apparatus, is decreased due to absorption losses on the glass surface of the reaction vials. Storage of evaporated extracts in reaction vials decreases tramadol recovery to 62 ± 10% in two days. Recovery was also incomplete (77%) when silanized reaction vials were employed. On the other hand, the possible minor absorption losses apparently had no effect on the assay's precision and accuracy, as tramadol and II are chemically very similar.

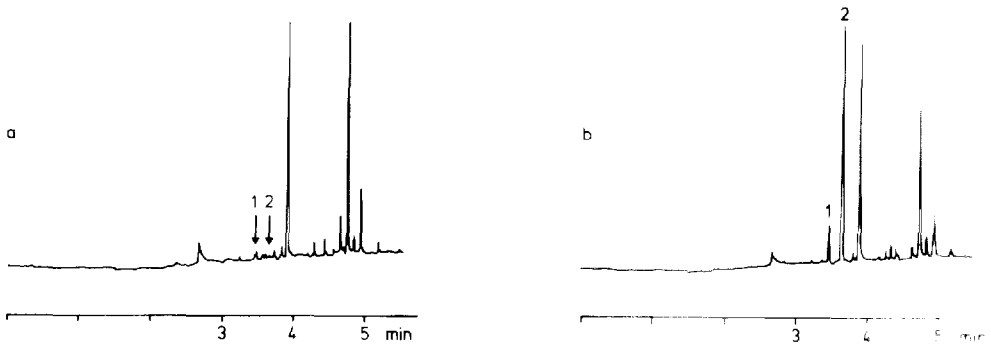

Fig. 2. Chromatograms of human serum samples. (a) Blank serum; (b) serum containing 12.5 ng/ml tramadol and 300 ng/ml internal standard. Peaks: 1 = tramadol; 2 = internal standard

Gas chromatography

Bioanalysis in the low nanogram range requires a careful choice of solvents, injection techniques, deactivated columns, sensitive detection and, last but not least, an appropriate internal standard. The solvent must possess sufficient strength to dissolve the sample and minimize sample losses due to absorption on the glass surface of the sample vials. The solvent must also be compatible with the column and the detector. For example, chloroform, the solvent employed in the GC–MS method, has to be ruled out because of its damaging effect on the nitrogen-selective flame ionization detector. Polar solvents such as methanol only became compatible with the advent of non-extractable column coatings.

The fused-silica columns used in this study have proved stable towards methanol and were well deactivated. Inertness of the columns allowed the detection of about 15 pg of pure substance. Efficiency of the column permitted the separation of tramadol, the internal standard and the five metabolites M1–M5 (Fig. 3).

Actually, only the separation of tramadol and M2 was required, since of the

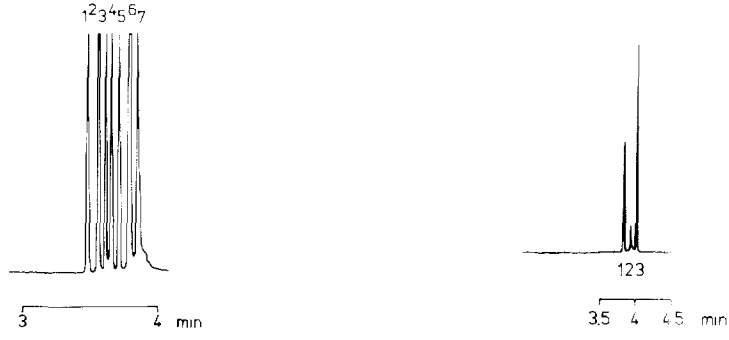

Fig. 3. Separation of tramadol, internal standard and five metabolites. Peaks: 1 = tramadol, 2 = M2; 3 = M3; 4 = internal standard; 5 = M1; 6 = M5; 7 = M4.

Fig. 4. Chromatogram of a human serum sample (8 h after oral administration of 100 mg of tramadol) showing low amount of metabolite M2. Peaks: 1 = tramadol; 2 = M2; 3 = internal standard.

metabolites extracted by the presented method (M2 and M3), only M2 occurred in appreciable amounts (Fig. 4).

Special attention was given to sample introduction. Splitless injection was used since none of the sample is wasted. When methanol was used as a solvent, injection at column temperatures below the boiling point of methanol resulted in peak splitting, which became more pronounced with increasing volumes of solvent. Unsplit peaks were generated only at column temperatures above 80°C. The narrow peaks of approx. 1 s half-widths, produced with an initial column temperature of 100°C, demonstrate that sample reconcentration by cold trapping was quite effective and no solvent effect was required. Injections at 100°C column temperature have also proved to be advantageous with regard to the sample volumes. Injections of 1—7 μl of methanolic solutions did not distort tramadol or internal standard peaks and peak half-width did not increase even with 7-μl volumes. But, since the high sensitivity of the chromatographic system proved fully adequate with small injection volumes, volumes greater than 2 μl were not injected routinely.

Standard curves

The calibration curve obtained from serum samples spiked with increasing amounts of tramadol hydrochloride and a constant amount of internal standard (300 ng) was linear in the range 12.5—200 ng/ml tramadol. It can be described by the equation $y = a + bx$, with y denoting the peak-height ratio and x the concentration of tramadol hydrochloride. The mean values and standard deviations of parameters a and b as derived from eight standard curves were: $a = 0.00003 \pm 0.00228$ and $b = 0.00348 \pm 0.00009$. The mean coefficient of correlation was 0.9986.

These results were obtained from weighted calibration curves with a weighting factor $w = 1/y^{1.058}$. A weighting factor of $w = 1/y$ can equally be employed without considerably changing the calibration curve.

Linearity of standard curves may exceed 200 ng/ml provided the amount of tramadol injected into the gas chromatograph does not surpass 50 ng, since the detector response has been shown to be linear up to at least 50 ng of tramadol. Nevertheless, serum samples of high tramadol concentrations should be diluted to fit into the concentration range of the standard curve in order to ensure good precision and accuracy of the determination.

Assay parameters

Selectivity of the method with respect to metabolites is determined by the extraction procedure that eliminates the metabolites M1, M4 and M5, and by gas chromatography, which efficiently separates tramadol, the internal standard and all the metabolites known so far (Fig. 3). Selectivity with respect to the blanks is shown in Fig. 2; the biological background only corresponded to about 1–2 ng/ml.

We further analysed serum samples after intravenous administration of 100 mg of tramadol hydrochloride by both the GC—N-FID and the GC—MS method. The unweighted regression line was calculated by the sum of least squares (Fig. 5). The coefficient of correlation was 0.996 and the slope was 0.98. This result confirms that selectivity is not influenced by unknown metabolites.

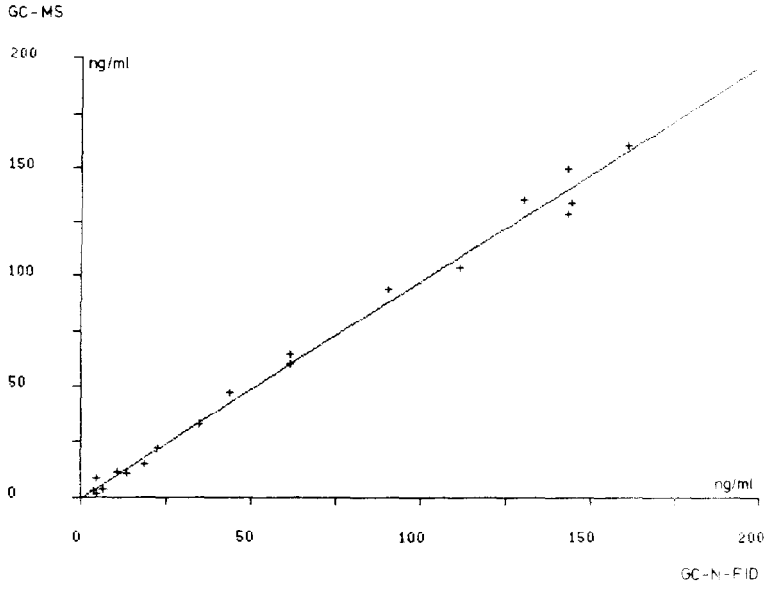

Fig. 5. Regression line of the tramadol serum levels from a pharmacokinetic study determined both by GC—N-FID and GC—MS.

Sensitivity of the assay is limited by blank serum values and the standard deviation of the y intercept of the calibration curve, not by the low detection limit of the nitrogen-selective detector of about 15 pg of tramadol. Determinations of blank serum samples from eight volunteers gave values of 1.22 ± 0.62 ng/ml. From determinations of the blanks, a detection limit (mean + 3S.D.) of approx. 3.1 ng/ml was calculated. The detection limit as calculated from the standard deviation of the y intercepts of eight calibration curves was about 2 ng/ml.

Precision and accuracy of the assay have been investigated within run as well as between runs on eight days over a four-week period (Tables I and II). Acceptable precision and accuracy were obtained even at the lowest concentration measured. As expected, between-run precision was slightly inferior to within-run precision.

The presented assay parameters show some differences in comparison with

TABLE I

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) (%)
12.5	13.4 ± 0.74	5.52	107.2
25	25.1 ± 1.38	5.50	100.4
50	48.4 ± 1.87	3.86	96.8
100	95.3 ± 2.49	2.61	95.3
200	201.1 ± 3.44	1.71	100.6

TABLE II

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	13.1 ± 0.74	5.65	104.8
25	24.3 ± 0.78	3.20	97.2
50	47.7 ± 2.18	4.58	95.4
100	97.2 ± 3.21	3.30	97.2
200	207.4 ± 6.97	3.36	103.7

the GC—MS method: precision and accuracy of the GC—MS method is generally superior. Coefficients of variation were between 0.73 and 1.73% and the accuracy was 98.6—101.8% (within-run). This is not surprising, since the internal standard used was isotope-labelled tramadol, which is extracted and chromatographed exactly like tramadol itself. Despite better selectivity with respect to serum blanks, the sensitivity of the MS detection could not fully be utilized and was not superior to the sensitivity of the GC assay, because the internal standard used with the GC—MS method contained about 1% tramadol [9].

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