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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 67–74
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Validated HPLC determination of 2-[(dimethylamino)methyl]cyclohexanone, an impurity in Tramadol, using a precolumn derivatisation reaction with 2,4-dinitrophenylhydrazine

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Received 18 February 2003; received in revised form 25 July 2003; accepted 26 July 2003

Abstract

A new method for the determination of 2-[(dimethylamino)methyl]cyclohexanone (DAMC) in Tramadol (as active substance or active ingredient in pharmaceutical formulations) is described. The method is based on the derivatisation of 2-[(dimethylamino)methyl]cyclohexanone with 2,4-dinitrophenylhydrazine (2,4-DNPH) in acidic conditions followed by a reversed-phase liquid chromatographic separation with UV detection. The method is simple, selective, quantitative and allows the determination of 2-[(dimethylamino)methyl]cyclohexanone at the low ppm level. The proposed method was validated with respect to selectivity, precision, linearity, accuracy and robustness.

Keywords: 2-[(Dimethylamino)methyl]cyclohexanone; Tramadol; Pre-column derivatisation; 2,4-Dinitrophenylhydrazine; RPLC; Validation

1. Introduction

Tramadol hydrochloride ((±*cis*-2-[dimethylamino] methyl-1-(3-methoxyphenyl) cyclohexanone hydrochloride, CAS 22204-88-2) is a centrally acting opioid type analgesic with additional non-opioid properties [1–4]. Its chemical structure is depicted in Fig. 1A.

Two main routes have been used for Tramadol hydrochloride synthesis. One of them is based on the catalytic hydrogenation of salicylic acid followed by oxidation to 2-carboxycyclohexanone and then Grignard addition with the organomagnesium compound of 3-bromoanisole. The resulting intermediate is condensed with dimethylamine and reduced to Tramadol. The second route consists of the Mannich addition [5] of cyclohexanone (enolic form) to the imminium ion formed during the reaction of formaldehyde and dimethylamine, resulting in the formation of 2-[(dimethylamino)methyl]cyclohexanone (referred to as DAMC or Impurity E—see Fig. 1B). The interaction of DAMC with the organomagnesium or

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doi:10.1016/j.japna.2003.07.003

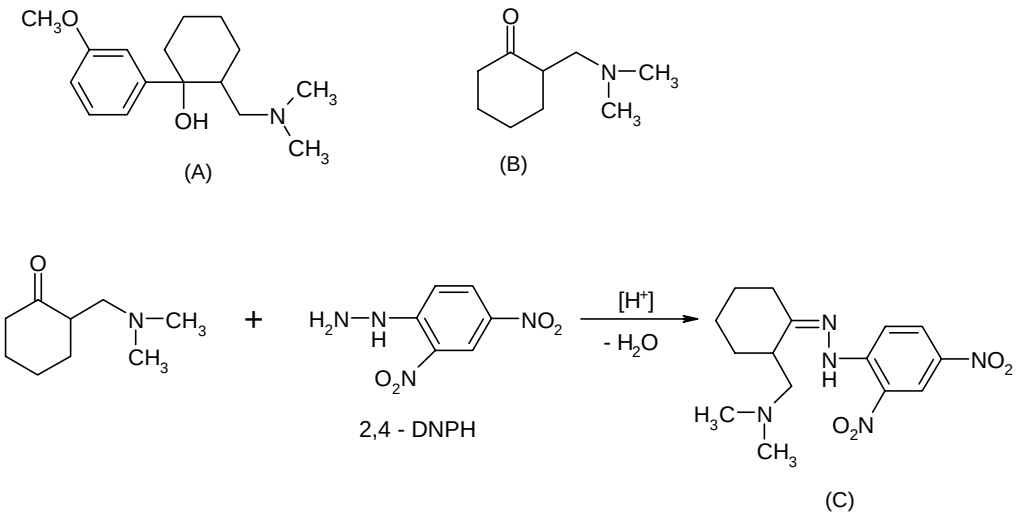

Fig. 1. Chemical structure of Tramadol (A), DAMC (B) and its 2,4-DNPH derivative product (C).

organolithium compounds of 3-bromoanisole leads to the formation of Tramadol [6,7].

This second synthetic route has been considered in the related Tramadol Hydrochloride monograph in the European Pharmacopoea, 4th ed., Addendum 2 [8], which requires the determination of DAMC (formally named Impurity E) as a related compound in the active substance, using a TLC method. The success of the TLC method depends strongly on the saturation of the chromatographic chamber with ammonia vapours. The visual comparison of the detected spots (generated after exposure to iodine vapours for 1 h and examination under UV 254 nm light) results in low precision and accuracy of the method.

DAMC could also represent a potential oxidative desalkylation degradation product of Tramadol. The quantitation of DAMC in the active substance as well as in its related pharmaceutical formulations is thus mandatory in order to control the quality of the finished pharmaceutical products.

The lack of a chromophore in the DAMC structure is the reason for its TLC determination using exposure to iodine vapours. Our method was focused on the derivatisation of DAMC with 2,4-dinitrophenylhydrazine (2,4-DNPH) as derivatisation reagent, knowing its recognised ability to react with the carbonyl function [9–12], prior to HPLC analysis.

In order to provide a viable alternative to the TLC method, the RPLC separation conditions were kept as close as possible to the conditions described in the HPLC method for related impurities in Tramadol hydrochloride from the monograph in the European Pharmacopoea, 4th ed., Addendum 2.

2. Experimental

All solvents were HPLC grade. Trifluoroacetic acid (TFA) ($d = 1.48$ g/ml), phosphoric acid (H_3PO_4) ($d = 1.71$ g/ml) and formic acid (FA) ($d = 1.22$ g/ml) were p.a. grade as well as 2,4-DNPH. Reagents were purchased from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). Water used for sample preparation and LC separation was MilliQ grade, with resistivity at least 18.2 MΩ and TOC maximum 30 ppb (Millipore GmbH, Eschborn, Germany).

Tramadol hydrochloride and Tramadol Impurity E (DAMC) are European Pharmacopoea certified reference standards (Council of Europe, Strasbourg, France–EPY 00 00 155, batch no.1 and EPY 00 00 157, batch no. 1, respectively).

Amorphus colloidal silica, sodium starch glycolate, lactose, magnesium stearate were pharmaceutical grade substances.

Volumetric glassware (volumetric flasks, pipettes and cylinders) are class A, gravimetrically in-house calibrated, purchased from Volac (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Experiments were carried out on an Agilent 1100 Liquid Chromatograph with autosampler and Diode array detector (Agilent Technologies, Waldbronn, Germany). The system is twice a year operationally qualified using its software built-in procedures.

Inertsil 5 ODS-2, 250 mm length, 4.6 mm internal diameter and 5 μm particle size was used as the analytical column (Varian Chrompack, Cat. no. CP 28408, batch 516404, Chrompack GmbH, Frankfurt am Main, Germany). Column validation made before starting the experiments revealed a reduced plate height ($\bar{h}$) of 2.3. Construction of the Van Deemter curve was made for fluoranthene (Test mixture no. 201 Varian Chrompack), having uracil as dead time indicator.

The mobile phase consisted of a mixture acetonitrile/aqueous 0.2% (v/v) trifluoroacetic acid (TFA) 60/40 (v/v) and elution was isocratic, at a flow rate of 1 ml/min. Injection volume was 20 μl.

The mobile phase and the column were maintained at 25 °C using the Agilent column thermostat Peltier based heater G1316A.

UV absorbtion at 270 ± 2 nm allowed simultaneous detection of Tramadol, the DAMC derivative and the 2,4-DNPH reagent and was used during initial derivatisation experiments. For routine work, detection at 358 ± 2 nm allowed the selective monitoring of the DAMC derivative and the 2,4-DNPH reageant only, with an almost double sensitivity. As a reference wavelength, 480 ± 10 nm was used.

A complete chromatographic run was achieved within 21 min.

3. Results and discussion

The condensation reaction between 2,4-DNPH and DAMC is given in Fig. 1C. The influence of time and temperature on the formation of the derivative product was first investigated. The reaction medium consisted of an acetonitrile/water mixture 7/3 (v/v) with the addition of 2.5% (v/v) H_3PO_4. Initial concentrations of the substrate and derivatisation reagent in the reaction medium were 50 and 1000 μg/ml, respectively. The formation of the derivative was expressed as percentage from the maximum peak area value integrated in the chromatograms obtained during the experiments. Results are given in Fig. 2.

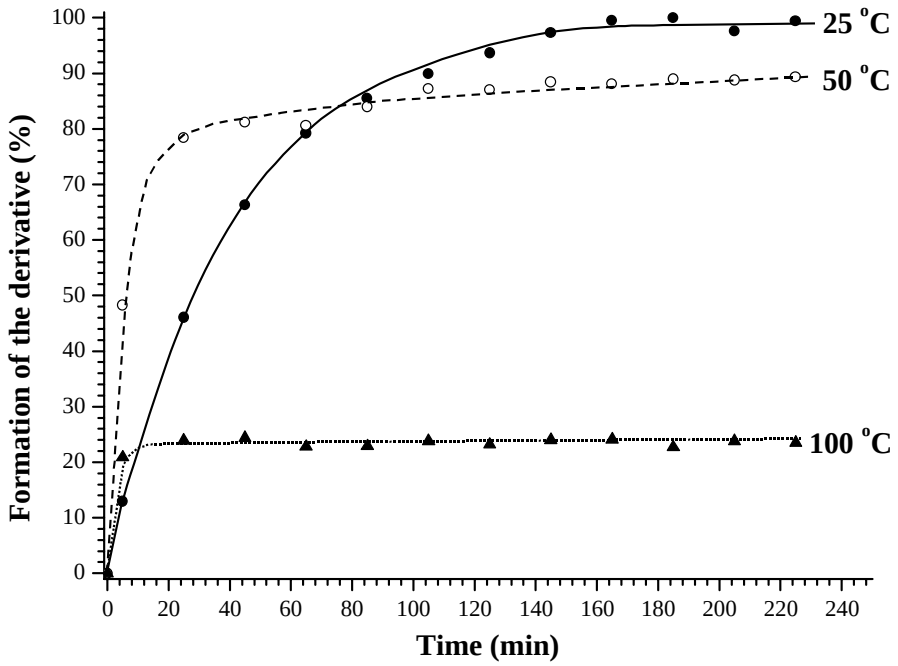

Fig. 2. Formation of the derivative as function of substrate/reagent contact time and temperature (experimental conditions are given in the text).

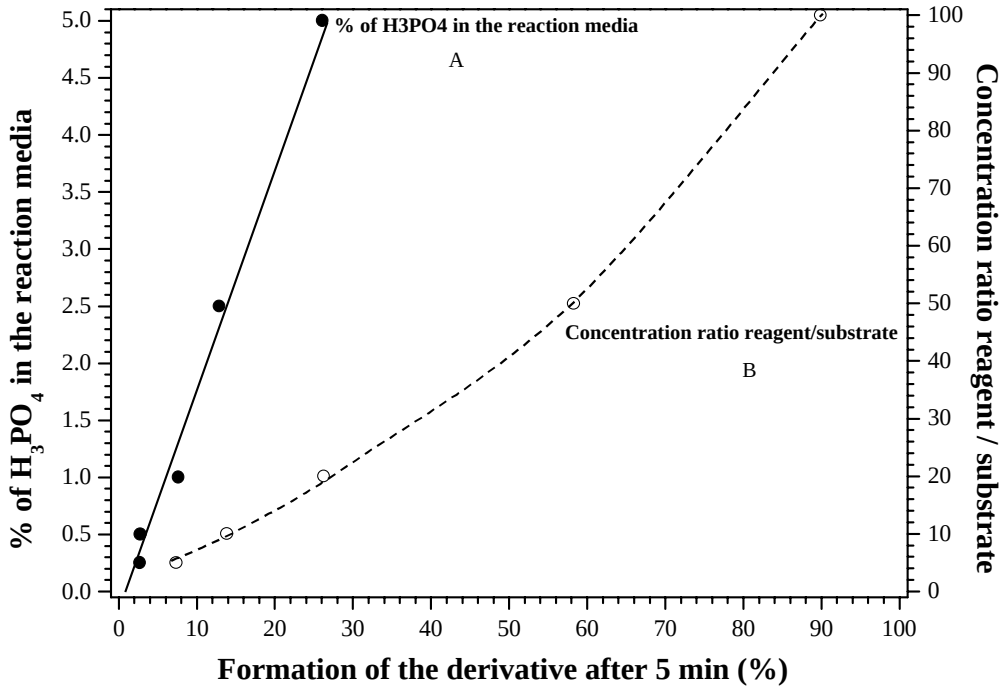

Fig. 3. Formation of the derivative as function of the amount of the acidic catalyst in the reaction medium (A) and concentration ratio reagent/substrate (B) (experimental conditions are given in the text).

From these data it seems obvious that, at higher temperatures, the derivative undergoes a concurrent decomposition, while at ambient temperature, equilibrium is reached after 145 min.

The derivatisation reaction is acid catalysed. Different amounts of phosphoric acid were added to the reaction media in order to evaluate this influence. The formation of the derivative was measured after 5 min of contact and was expressed according to the maximum peak area determined during the former experiment carried out at 25 °C. All the other conditions were kept constant. Results presented in Fig. 3A show that the formation of the derivative was linearly correlated to the concentration of the phosphoric acid in the reaction medium.

The excess of the reagent should also be emphasised, in order to control the derivative formation. Concentration ratios of 2,4-DNPH/DAMC ranging in the interval 5/1–100/1 were tested. The experiment was carried out at 25 °C, 5% (v/v) of H_3PO_4 in the reaction medium and with measurement of the derivatisation product 5 min after the start of the reaction. Results are presented in Fig. 3B. Although high excess of the derivating reagent induced rapid formation of the derivative, the overloading of the chromatographic column should be considered. It is also worthwhile to note that, when performing the derivatisation with 5% H_3PO_4 in the reaction medium and a 20/1 reagent excess, the reaction could be considered quantitative after 70 min and the formation yield after 165 min expressed according to the results of the first series of experiments was about 103%.

Due to the fact that Tramadol hydrochloride solutions can be made either in water or acetonitrile while the concentrated reagent solutions are mainly made in acetonitrile, it was obvious that the composition of the reaction medium could influence derivatisation.

The derivatisation reaction was thus carried out in different media (acetonitrile/water volumetric ratio varies from 1/4 to 19/1), at 25 °C, using 5% phosphoric acid and an excess of 2,4-DNPH of 20/1. Formation of the derivative was measured after 5 min. Results are given in Fig. 4.

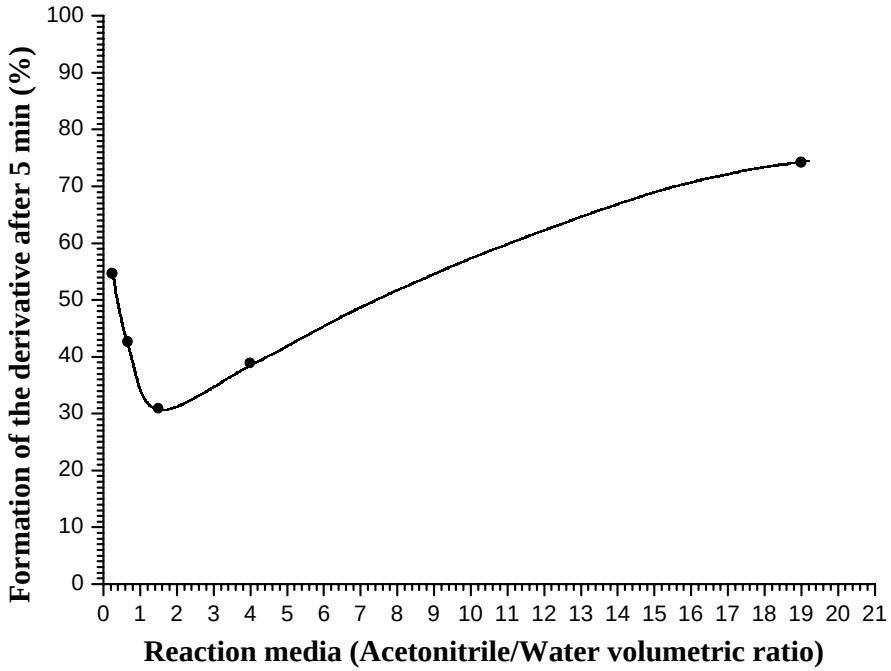

Fig. 4. Formation of the derivative as function of the composition of the reaction media (Experimental conditions are given in the text).

Interpretation of these experimental data is rather difficult. The results are probably simultaneously influenced by the real proton concentration in the reaction medium, the aqueous content and the intrinsic solubility of the derivatisation product. When derivatisation is achieved in almost pure acetonitrile, a peak shape degradation is also observed. Such distortion of the peak symmetry due to focusing phenomena on injection in the sample solvent, could strongly affect integration, resulting in some erratic recovery values of the derivative. It is therefore important to keep the composition of the reaction medium as constant as possible during experiments to ensure the reproducibility of the results.

One can conclude that the derivatisation procedure can be properly performed in the following manner: one volumes of water, one volume of aqueous sample solution (either Tramadol hydrochloride as tested active substance or reference standard of DAMC), 0.25 volumes of phosphoric acid and one volume of 2,4-DNPH solution in acetonitrile are mixed together and are brought to five volumes with acetonitrile. The resulting mixture was kept at ambient temperature for at least 70 min before injection to the LC system. For a concentration of 5 mg/ml of 2,4-DNPH in acetonitrile, accurate determination of DAMC up to 50 μg/ml in the sample solution can be observed. Tramadol hydrochloride does not interfere in the reaction, its concentration in the aqueous tested sample is limited only by its intrinsic solubility in water (a level of concentration of 5 mg/ml is allowed).

For generating more accurate results, the addition method is recommended for a routine procedure. Basically, the following solutions should be prepared: (a) the reference solution resulting after mixing two volumes of water, 1 volume of an aqueous stock solution of certified reference DAMC standard 7.5 μg/ml, 0.25 volumes of phosphoric acid, one volume of 5 mg/ml 2,4-DNPH reagent solution in acetonitrile and made up to five volumes with acetonitrile; (b) the sample solution resulting after mixing one volume of water, one volume of an aqueous stock solution of certified reference DAMC standard 7.5 μg/ml, one volume of the aqueous tested Tramadol hydrochloride solution 3.75 mg/ml, 0.25 volumes of phosphoric acid, one volume of 5 mg/ml 2,4-DNPH reagent solution in acetonitrile and made up to five volumes with acetonitrile.

For the previous given concentration values, the fulfillment of the acceptance criteria from the monograph Tramadol hydrochloride in European Pharmacopoeia, IVth ed., Addendum 2 should be considered as the following: the difference between peak areas corresponding to the derivative in the chromatograms of the sample and standard solutions, respectively, should be lower than or at the most equal to the peak area corresponding to the derivatisation product in the chromatogram of the standard solution (0.2%).

The sample preparation procedure briefly described earlier was applied together with the LC separation conditions given in the experimental section. To illustrate the selectivity of the method two blank samples were also obtained. The first one contained no substrate (only derivatisation reagent), the second one contained Tramadol hydrochloride free of DAMC. A few common excipients frequently used in pharmaceutical formulations were also tested. Their stock solutions were filtered before introduction in the derivatisation process, considering limitation on dissolving in aqueous medium. As expected, amorphous colloidal silica, magnesium stearate and sodium starch glycolate did not interfere. Starch and lactose, due to the acidic conditions in the derivatisation medium may undergo degradative hydrolysis, resulting in glycoside ring opening and the formation of a carbonyl moiety capable of reacting with 2,4-DNPH. This chemical interference is not a reagent consuming process, (low amounts of degradation products are formed) and the resulting by-products did not interfere in the chromatograms (their retention is lower than the target derivatisation product). Chromatograms illustrating the selectivity of the method are overlaid in Fig. 5.

The precision of the method was evaluated by repeating 10 times successively, at 70 min interval, both derivatisation procedure and LC separation, starting from the same stock solutions of 2,4-DNPH, DAMC and Tramadol hydrochloride. The concentrations of DAMC and Tramadol hydrochloride in the reaction medium were 5 and 750 μg/ml, respectively (this corresponds to 0.4% DAMC in the active substance). The variation of the retention time and the integrated peak area values corresponding to the derivatisation product were considered as indicating parameters for precision. As can be seen from Fig. 6, the relative standard deviations characterising the experimental series of data are 0.4% for retention time and 0.7% for the integrated peak area values.

It is also important to note that no trends in the variation of those parameters can be observed. The random distribution of the experimental data around the calculated mean values is obvious.

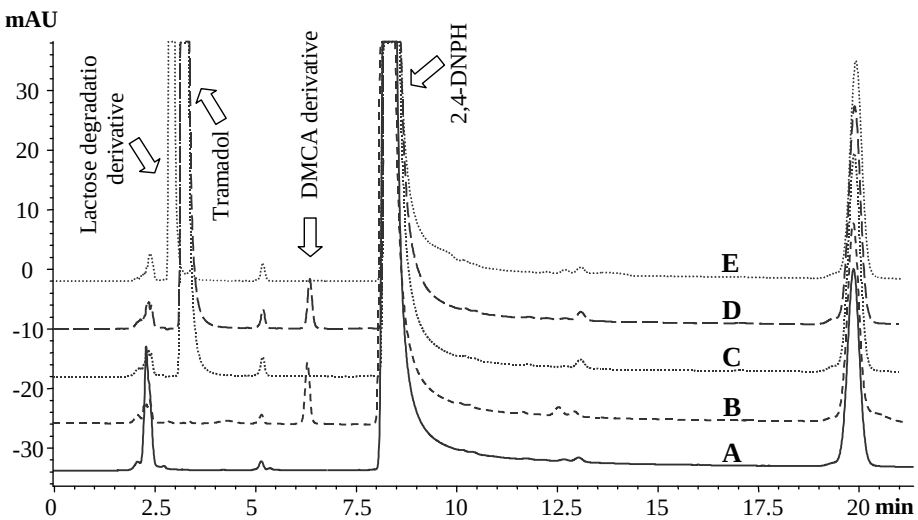

Fig. 5. Chromatograms of the derivating reagent (A), DAMC (B), Tramadol hydrochloride (C), both Tramadol and DAMC (D) and Lactose (E). All samples are treated according to the derivatisation procedure described in the text. Separation conditions are according to the experimental section.

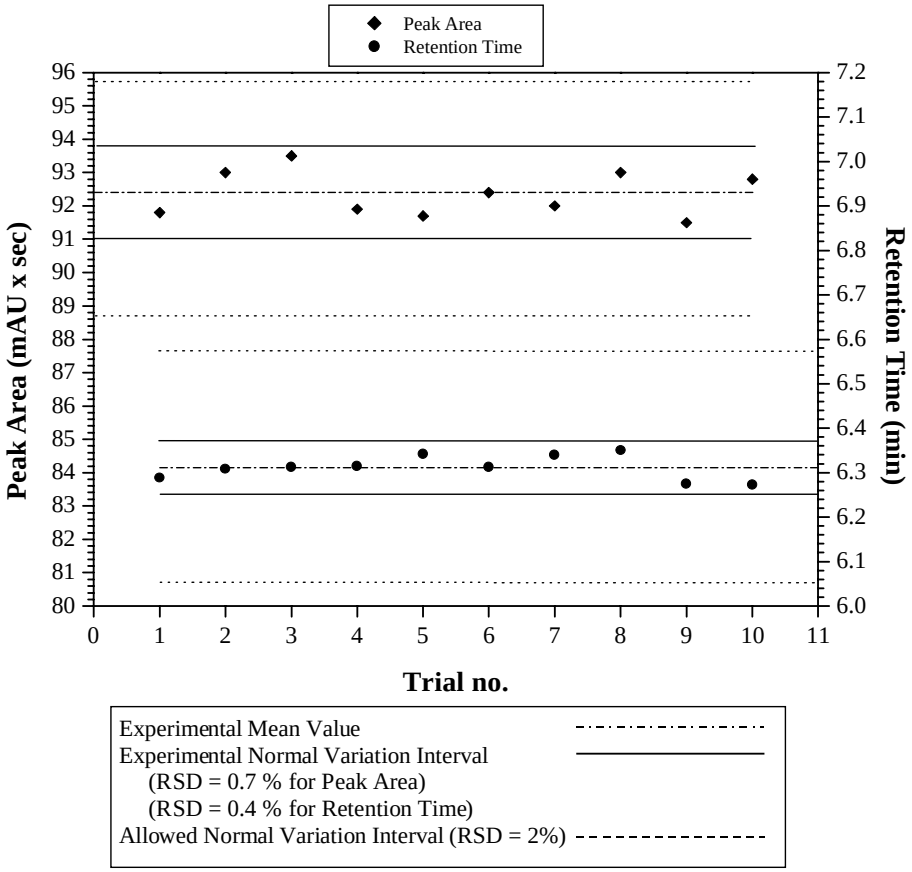

Fig. 6. Retention time and peak area values obtained after successive injections of ten samples prepared from stock solution containing Tramadol hydrochloride and DAMC (conditions are given in the text and in the experimental section).

Linearity was studied in the DAMC concentration range up to 10 μg/ml. Synthetic samples used during experiments contained Tramadol hydrochloride free of DAMC at a concentration of 0.75 mg/ml. Each sample was injected three times successively. The resulting linear functional dependence (peak area (mAU × s) = 27.91 × concentration (mg/ml) + 6.22) is characterised by a correlation coefficient of 0.9973. The limit of quantitation (LOQ) calculated for a confidence value of 95% was 1.17 μg/ml (corresponding to less than 0.1% DAMC in Tramadol hydrochloride active substance) with a limit of detection (LOD) of ca. 0.4 μg/ml (corresponding to 0.03% DAMC in the tested drug).

The accuracy of the proposed method was tested over the same DAMC concentration interval as for linearity. Three solid synthetic samples containing Tramadol hydrochloride, DAMC, colloidal anhydrous silica, magnesium stearate, sodium starch glycolate and lactose were obtained. These samples were prepared according to the above described derivatisation procedure (filtration of the aqueous sample stock solution is required) in order to obtain DAMC concentrations in the reacting medium of 1.5, 3 and 6.5 μg/ml, respectively. Each sample was injected three times consecutively to the LC system. For each of the three series of resulting peak areas, the mean value was calculated and introduced in the linear regression equation determined previously. The experimental concentration values were plotted against the theoretical values. The linear regression equation (experimental concentration (mg/ml) =

$0.9776 \times$ theoretical concentration (mg/ml) $+ 0.1192$) was characterised by a correlation coefficient of 0.9994.

The angle of the linear dependence calculated from the slope is 44.35°. The comparison of this result with the theoretical angle (45°) indicates the accuracy of the method.

The parameters considered for the evaluation of the LC separation robustness were: (i) the column temperature; (ii) the concentration of the TFA in the aqueous mobile phase constituent; (iii) the nature of the acid used as additive in the mobile phase; (iv) mobile phase composition and (v) the ODS stationary phase type material.

Making column temperature variations in the 20–30 °C range generated retention data for the target compound within the normal allowed variation interval, thus the column thermostatisation is not necessary.

Higher TFA amounts added in the aqueous constituent of the mobile phase did not affect retention (consequent deterioration of the stationary phase and column life time reduction should be however considered when using very acidic mobile phases). Diminution of the TFA percentage (0.15% instead of 0.2%) generated retention data for the target derivative outside the normally allowed variation interval (lower retention was observed). In such conditions, retention of the 2,4-DNPH reagent remained unaffected.

The use of H_3PO_4 or FA instead of TFA as additives in the mobile phase strongly reduced the retention of the derivative, making its separation from the Tramadol peak critical. Retention of the reagent remained unaffected.

Variation of the organic mobile phase constituent in the 55–65% interval significantly affects, as expected, the elution behaviour. Increasing the proportion of organic solvent in the mobile phase resulted in a decrease of retention for all analytes. In the studied interval, retention of the target derivative linearly varies with the mobile phase composition. Shifts of ±1% of the organic solvent in the mobile phase induce variation of retention within the normally allowed variation interval.

Retention of the analytes on a polymeric ODS Nucleosil 100-5 stationary phase instead of the Inertsil 5 ODS-2 column was strongly reduced. However, in such a situation, the elution profile could still be optimised.

The method appears to be robust and common HPLC instrumentation should be able to reproduce the operational parameters without affecting the resulting analytical data.

4. Conclusions

A validated, sensitive, precise, accurate and robust method for the determination of DAMC as a related impurity in Tramadol hydrochloride was developed. The method is based on the pre-column derivatisation of DAMC with 2,4-DNPH, in acidic media, followed by a RPLC separation method and UV detection.

The derivatisation reaction is quantitative within 70 min at room temperature. Tramadol hydrochloride and common pharmaceutical excipients do not interfere.

The proposed routine procedure allows the determination of DAMC in Tramadol hydrochloride down to 0.03%. The procedure represents an alternative to the TLC method, as recommended by the European Pharmacopoea 4th ed., Addendum. 2 for DAMC determination in Tramadol hydrochloride active substance.

References

[1] R.B. Raffa, E. Friderichs, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, J. Pharmacol. Exp. Ther. 260 (1992) 275–279.
[2] C.R. Lee, D. McTravis, E. Sorkin, Drugs 46 (1993) 313–315.
[3] A. Mattia, T. Vanderah, R.B. Raffa, J.L. Vaught, R.J. Talarida, F. Porreca, Drug Dev. Res. 28 (1993) 176–180.
[4] W. Reimann, H.H. Hennies, Biochem. Pharmacol. 47 (1994) 2289–2292.
[5] W.A. Benjamin, Modern Synthetic Reactions, second ed., Menlo Park, CA, 1972, p. 654.
[6] K. Flick, E. Frankus, E. Friderichs, Arzneim.-Forsch./Drug Res. 28 (1978) 107–113.
[7] K. Flick, S. Bochum, E. Frankus, U.S. Patent 3 652 589 (1972).
[8] European Pharmacopoea 4th ed., Addendum 2, Monograph 1681 Tramadol hydrochloride, 2002, p. 2805.
[9] G.A. Cordis, D. Bagchi, N. Maulik, D.K. Das, J. Chromatogr. A 661 (1994) 181–191.
[10] G. Chiavari, C. Bergamini, J. Chromatogr. 318 (1985) 427–432.
[11] M.X. Coutrim, L.A. Nakamura, C.H. Collins, Chromatographia 37 (1993) 185–190.
[12] G. Liebezeit, J. High Resolut. Chromatogr. Chromatogr. Commun. 5 (1982) 215–216.