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Short communication

Improved sample preparation for the quantitative analysis of paroxetine in human plasma by stable isotope dilution negative ion chemical ionisation gas chromatography–mass spectrometry

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Abstract

An improved sample work-up and derivatisation procedure for the quantitative determination of paroxetine in human plasma by gas chromatography–negative ion chemical ionisation mass spectrometry is presented. Solvent extraction from plasma samples at alkaline pH was combined with derivatisation to the pentafluorobenzyl carbamate derivative in one step and subsequently analysed without any further purification. Thus, lengthy and time-consuming solvent evaporation steps are avoided to assure high-throughput analysis. Complete validation data are presented. The method is rugged, rapid and robust and has been applied to the batch analysis of paroxetine during pharmacokinetic profiling of the drug.
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1. Introduction

The inhibitor of 5-hydroxytryptamine uptake paroxetine (3*S*-*trans*)-3-[(1,3-benzodioxol-5-yl-oxy)-methyl]-4-(4-fluorophenyl)piperidine (Fig. 1), is used widely as an antidepressant drug [1]. It is mainly metabolised by cytochrome P450 2D6 isoform (CYP2D6). Genetic polymorphism of this CYP2D6 causes wide inter-individual variations of steady-state plasma paroxetine levels after the same dosage [2–4]. Thus, reliable methods for pharmacokinetics and drug level monitoring of parox-

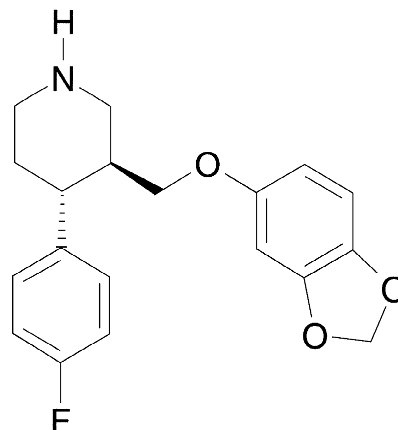

Fig. 1. Chemical structure of paroxetine.

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etine are highly desirable. High-performance liquid chromatography (HPLC) using UV, coulometric and, mainly, fluorescence detection [5–13] has been used for the determination of paroxetine in plasma, whereas GC assays have been used to a lesser extend [10,14]. We have recently developed a sensitive and specific method for the quantitative measurement of paroxetine from human plasma using gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICI-MS) of the pentafluorobenzyl carbamate derivative, using stable isotope-labelled paroxetine as an internal standard [15]. The method involves solvent extraction from plasma with hexane–ethyl acetate (1:1) at alkaline pH, evaporation of solvent, derivatization to the pentafluorobenzyl carbamate derivative, evaporation of reagents followed by GC–NICI-MS analysis.

For pharmacokinetic applications, short analysis time is a major concern, since they involve the processing of a large number of samples. It was therefore the aim of this study to elaborate a method for the determination of paroxetine in human plasma that meets the requirements of sensitivity, specificity, thereby enhancing sample throughput for pharmacokinetic applications. Based on this application, the elaborated method should also be applicable to a wider range of analytical targets.

2. Experimental

2.1. Chemical and reagents

Paroxetine tartrate hemihydrate was from Gedeon Richter (Budapest, Hungary). Deuterium oxide was from Fluka (Vienna, Austria), palladium on calcium carbonate (5%) from Merck (Darmstadt, Germany). Sodium borodeuteride was from Aldrich (Vienna, Austria). All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

2.2. Gas chromatography–mass spectrometry

A Finnigan TRACE 2000 GC coupled to a Finnigan TRACE quadrupole MS (ThermoQuest, Vienna, Austria) was used. The GC was fitted with a DB5-MS fused-silica capillary column (15 m×0.25 mm I.D., 0.25 μm film thickness, ThermoQuest). The injector was operated in the splitless mode at 290 °C. Helium was used as a carrier gas at a constant flow-rate of 1.5 ml/min. Initial column temperature was 160 °C for 1 min, followed by an increase of 40 °C/min to 310 °C and an isothermal hold of 3 min. The mass spectrometer transfer line was kept at 315 °C. Negative ion chemical ionization (NICI) was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 250 μA. During single ion recording, m/z 372 and m/z 378 were recorded for target and internal standard, respectively, with a dwell time of 50 ms.

2.3. Preparation of [$^2H_6$]paroxetine

Deuteration of paroxetine was accomplished as previously described [15]. Briefly, paroxetine tartrate hemihydrate was dissolved in ^2H_2O and palladium on calcium carbonate (5%) were added. After addition of sodium borodeuteride the glass vial was closed and heated to 160 °C for 5 h, followed by cooling and extraction with ethyl acetate. The solvent was evaporated under nitrogen and HCl (1 *M*) was added, shaken, and dried under nitrogen. The residue was dissolved in 100 ml of methanol. To check purity and isotope distribution an aliquot of this solution was derivatised with and without addition of native paroxetine solution and analysed by GC–NICI-MS.

2.4. Preparation of pentafluorobenzyl chloroformate

Pentafluorobenzyl chloroformate was prepared by reaction of phosgene with pentafluorobenzyl alcohol in the presence of dimethyl aniline as described elsewhere [16]. The product was distilled twice and stored anhydrous at −20 °C.

2.5. Plasma sample preparation

Samples were thawed at room temperature in the dark and processed immediately after thawing. Fifty μl of the methanolic solution of the internal standard, containing 11.2 ng [$^2H_6$]paroxetine were pipetted into a 5-ml polypropylene tube and 0.5 ml of plasma was added. After short, vigorous shaking at

the Reax 2000, 0.9 ml NaOH (0.3 *M*) was added, followed by 200 μl toluene (containing 5% pyridine, v/v) and 200 μl of reagent solution (pentafluorobenzyl chloroformate (PFBCLF, 0.5% in toluene, v/v). After shaking for 10 min at the reciprocal shaker (Reax 2), the samples are stored at −20 °C until further processing, or centrifuged at 4000 rpm for 10 min. The clear supernatant solution was directly transferred into autosampler vials with a 200-μl pipette. The vials were closed with crimp-top caps and stored at −20 °C until analysis.

2.6. Recovery of paroxetine after extractive derivatisation

Recovery of paroxetine after extractive derivatization was examined as follows. Blank plasma was spiked with 20 ng/ml paroxetine and samples were processed as described above. Previously derivatised [$^{2}H_{6}$]paroxetine internal standard was added and the mixture analyzed by GC–NICI-MS. To estimate the 100% recovery value, the same amount of paroxetine was directly derivatised and analyzed together with the same amount of pre-derivatized internal standard. Five-fold determinations were carried out.

2.7. Analytical method validation

Calibration graphs were established within a range of 0.469–120 ng/ml plasma. For this purpose, blank plasma was spiked with the appropriate amounts of paroxetine by adding 50 μl of the corresponding methanolic solution. Standard solutions of paroxetine were prepared by serial dilution in methanol to yield concentrations of 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, and 0.469 ng/50 μl. Standard solutions were stored at −20 °C. Blank plasma was checked for possible paroxetine content before use.

Intra-day precision was determined at 0.469, 4, 20, and 80 ng/ml by carrying identical samples throughout the analytical sequence. Spiked samples were prepared from blank plasma. Five-fold determinations were carried out.

Inter-day precision was determined at 0.469 (LOQ), 4, 20, and 80 ng/ml by analyzing one replicate at each concentration on five consecutive days.

Inter- and intra-day accuracy of the method was also tested at the above-mentioned concentrations after 5-fold determinations. Thus, the data from inter- and intra-day precision measurements were used to calculate the deviation of the values measured from the actual spiked values.

Specificity was tested by analyzing 10 different blank plasma samples.

3. Results and discussion

3.1. Sample preparation

The combined extractive derivatisation procedure described here offers a rapid way to isolate paroxetine from the plasma matrix and conversion to the pentafluorobenzyl carbamate derivative in one single step. Thus, we have achieved extraction and derivatisation of batches with 240 samples routinely within 3 h, with two technicians working on it.

Derivatisation occurred quantitatively within 10 min using the reagent–solvent system described. The derivative was stable for at least 5 weeks at −20 °C and 12 days at room temperature. Nevertheless, samples were stored at −20 °C to assure possibility of sample re-analysis over a whole time range of batch sample processing. Compared to the original procedure [15] reagent and solvent consumption are minimal. The tremendous sensitivity of the derivative to NICI detection allows injection of only 0.2% of the total extraction mixture onto the GC. Derivatisation occurs smoothly at a reagent concentration of only 0.25%. These conditions assure also constant system performance due to decreased sample and reagent load.

The procedure yields clean extracts, as shown in typical chromatograms in Fig. 2A,B.

Chloroformates can also react with tertiary amines and in some cases with hydroxyl groups, although the conditions for these reactions are rather harsh. Under the conditions of extractive derivatisation employed, no reactions of the reagent with tertiary amines and hydroxyl containing functionalities was observed, thus enhancing specificity of the assay.

Extractive derivatisation yielded quantitative recovery of the analyte (98.4%) and is thus compar-

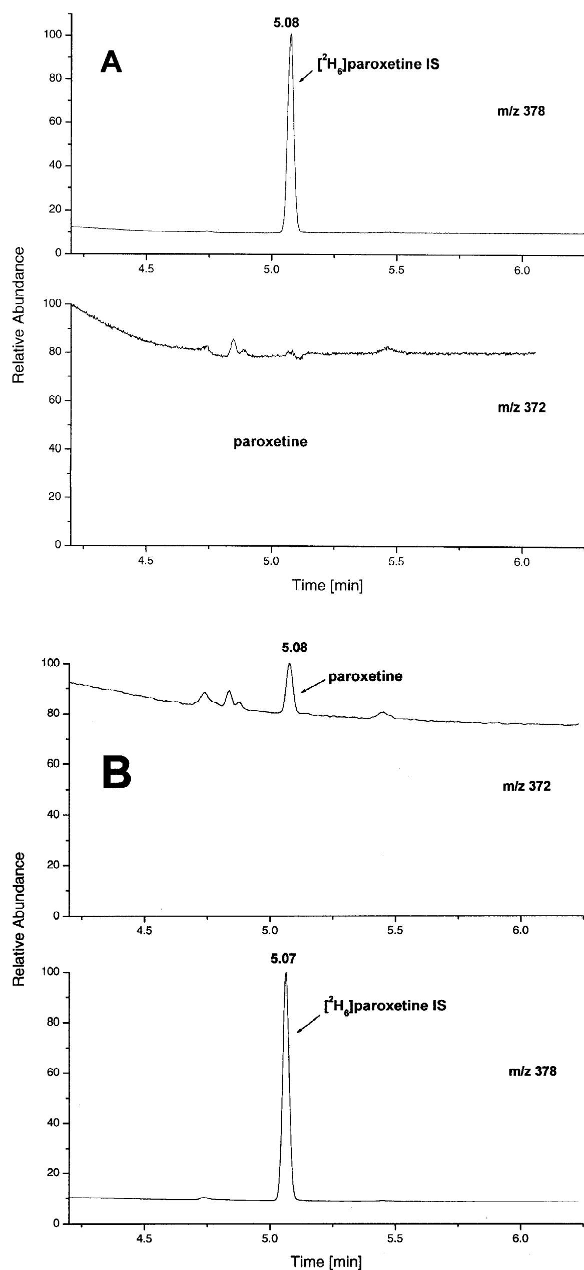

Fig. 2. (A) SIR mass chromatogram obtained after analysis of a blank plasma sample. (B) SIR mass chromatogram obtained after analysis of a plasma sample spiked with 0.469 ng/ml paroxetine (LOQ).

able to our previously published method for the determination of paroxetine after solvent extraction and separate derivatisation reaction [15].

3.2. Analytical method validation

The calibration graphs established were linear within the tested range of 0.469–120 ng/ml using 11.2 ng [$^2H_6$]paroxetine internal standard. Weighted linear regression analysis (weighting$=1/s^2$, s=SD of duplicates) results in correlation coefficients of r^2= 0.99948±0.000502 (five calibration curves). The slope and intercept values were 0.03929±0.005120 and 0.00708±0.00267, respectively (determined from five calibration curves). The lower limit of detection for the complete procedure was 0.2 ng/ml plasma at a signal-to-noise ratio of 4:1, estimated by analyzing spiked plasma samples. For pharmacokinetic measurements, the lower limit of quantitation (LOQ) was set to 0.469 ng/ml plasma.

The coefficients of inter- and intra-assay variation and the accuracy of the spiked samples are presented in Table 1. It can be seen from these data, that the method provides a highly precise assay for paroxetine in human plasma. This can be attributed to the inherent sensitivity of NICI detection in combination with a stable isotope-labeled internal standard. Mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and assays based on this technique can be regarded as reference procedures to validate other analytical methods. Ten different blank matrices were checked for interferences. In none of the samples was there background contribution above 10% LOQ.

Data on freeze–thaw stability of plasma samples, short-term stability and long-term stability have been previously presented [15]. Autosampler stability was checked over a period of 24 h, where no deviation from the first analysis batch was observed.

We have applied this method to the analysis of paroxetine in human plasma in the course of a pharmacokinetic study. Typical mass chromatograms obtained after analysis of a blank plasma sample and of a spiked plasma sample at LOQ (0.469 ng/ml plasma) are given in Fig. 2A,B, respectively. The assay proved to be useful in the batch analysis of more than 1000 plasma samples. The simple workup protocol allows rapid sample preparation and assures a high throughput in analysis. Under the conditions described, the paroxetine derivative elutes after 5.08 min from the GC. The total analysis cycle including

Table 1
Intra- and inter-day precision and recovery of paroxetine determination in human plasma

	Intra-day (ng/ml plasma)				Inter-day (ng/ml plasma)			
Spiked amount	0.469	4	20	80	0.469	4	20	80
Mean	0.463	3.993	19.423	77.578	0.486	4.162	20.389	82.744
SD	0.017	0.036	0.400	1.762	0.011	0.136	1.151	3.861
n	5	5	5	5	5	5	5	5
C.V. (%)	3.6	0.9	2.1	2.3	2.2	3.3	5.7	4.7
Accuracy (%)	98.7	99.8	97.1	97.0	96.4	104.1	102.0	103.4

cooling of the GC and equilibration time is 13.4 min per sample.

The analysis of paroxetine from human plasma is of major interest in pharmaceutical research and clinical monitoring of the drug. For monitoring single dose applications, the sensitivity of our previously elaborated method is sufficient [15]. As for paroxetine, pharmacokinetic profiling is often done under steady-state conditions. In this case, the method presented herein can be applied with the benefits of rapid sample preparation. If a lower limit of detection is necessary, sample volume can be increased and/or the amount of extraction solvent–reagent mixture further minimized.

In summary, the method presented allows the highly specific detection of the drug in human plasma, taking advantage of the stable isotope-labeled internal standard. The speed, robustness and ruggedness of the method allows batch processing of large sample numbers making it useful in routine and pharmacokinetic applications.

References

[1] G.C. Dunbar, J.B. Cohn, L.F. Fabre, J.P. Feighner, R.R. Fieve, J. Mendels, R.K. Shrivastave, J. Psychiatry 150 (1991) 394.

[2] S.H. Sindrup, K. Brosen, L.F. Gram, Clin. Pharmacol. Ther. 51 (1992) 288.

[3] S.H. Sindrup, K. Brosen, L.F. Gram, J. Hallas, E. Skielbo, A. Allen, G.D. Allen, S.M. Cooper, G. Mellows, T.C.G. Tasker, B.D. Zussman, Clin. Pharmacol Ther. 51 (1992) 278.

[4] J.C. Bloomer, F.R. Woods, R.E. Haddock, M.S. Lennard, G.T. Tucker, Br. J. Clin. Pharmacol. 33 (1992) 521.

[5] J.G. Shin, K.A. Kim, Y.R. Yoon, I.J. Cha, Y.H. Kim, S.G. Shin, J. Chromatogr. B 713 (1998) 452.

[6] R.N. Gupta, J. Chromatogr. B 661 (1994) 362.

[7] C. Lopez-Calull, N. Dominguez, J. Chromatogr. B 724 (1999) 393.

[8] L. Kristoffersen, A. Bugge, E. Lundanes, L. Slordal, J. Chromatogr. B 734 (1999) 229.

[9] M.A. Breit, H.D. Dierdorf, B.D. Zussman, P.E. Coates, J. Chromatogr. 419 (1997) 438.

[10] E. Lacassie, J.M. Gaulier, P. Marquet, J.F. Rabatel, G. Lachatre, J. Chromatogr. B 742 (2000) 229.

[11] D.S. Schatz, A. Saria, Pharmacology 60 (2000) 51.

[12] J. Knoeller, R. Vogt-Schenkel, M.A. Brett, J. Pharm. Biomed. Anal. 13 (1995) 635.

[13] S. Hartter, B. Hermes, A. Szegedi, C. Hiemke, Ther. Drug Monit. 16 (1994) 400.

[14] E.N. Petersen, E. Bechgaard, R.J. Sortwell, L. Wetterberg, J. Pharmacol. 52 (1978) 115.

[15] H.J. Leis, W. Windischhofer, G. Raspotnig, G. Fauler, J. Mass Spectrom. 36 (2001) 923.

[16] J.T. Simpson, D.S. Torok, S.P. Markey, J. Am. Soc. Mass Spectrom. 6 (1995) 525.