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Rapid and simple method for the analysis of nateglinide in human plasma using HPLC analysis with UV detection

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Abstract

Nateglinide (NA) is a novel oral mealtime glucose regulator, recently approved for the treatment of type II diabetes mellitus. To facilitate clinical studies investigating the dependence of NA elimination on the genotype of cytochrome P450 isoenzymes, we developed a rapid HPLC method for determination of NA in human plasma samples. The validated limit of quantitation (LOQ) of 0.1 μg/ml is low enough to allow determination of pharmacokinetic parameters of the substance. The intra-assay coefficients of variation (CV) ranged from 1.6 to 12.9% at NA concentrations of 0.5–7.5 μg/ml. The inter-assay variation for the same plasma concentrations ranged from 3.8 to 8.4%. The calibration was linear in the range of 0.1–20 μg/ml. For the quantitation of NA, only 50 μl of plasma were needed. Following protein precipitation in human plasma, the samples were separated by isocratic reversed phase HLPC and analyzed using ultraviolet detection at 210 nm. Sample preparation time and analysis time are both short and allow rapid analysis of large sample sets.
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1. Introduction

Nateglinide (NA) [*N*-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine] is a novel non-sulfonylurea oral antidiabetic agent (Fig. 1) for the treatment of type II diabetes mellitus [1–3]. NA increases the insulin release from pancreatic ß-cells through inhibition of potassium-ATP channels [4]. After oral administration, NA is rapidly absorbed and peak plasma concentrations are reached after 0.5–1.0 h [5,6]. The elimination of the substance is fast, with a half-life of approximately 1.4 h [2]. NA undergoes extensive biotransformation resulting in hydroxylated and carboxylated derivatives. Certain metabolites are subsequently conjugated with glucuronic acid [7]. The participation of hepatic cytochrome P450 isoenzymes such as CYP2C9 and

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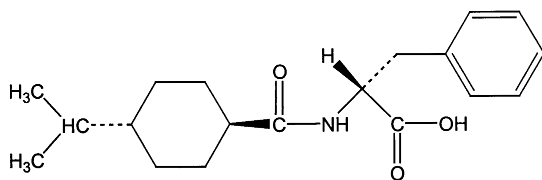

Fig. 1. Chemical structure of NA (mol. wt. 317.42).

CYP3A4 has been described [6]. For a pharmacokinetic study estimating the influence of different cytochrome P450 genotypes on the elimination of NA, we developed an HPLC method for the quantitation of the parent drug in human plasma. Ono et al. described the estimation of NA [8] and of NA together with its main metabolites [9] with column switching HPLC after solid phase sample preparation. These methods employ a specially manufactured column that is not commercially available and require expensive column switching equipment. Since the plasma concentrations of NA are comparably high with peak concentrations in the low micromolar range, it was possible to develop a more simple and very rapid method with sufficient sensitivity, which only requires small amounts of plasma.

2. Experimental

2.1. Chemicals and reagents

NA was kindly provided by Novartis Pharma AG (Basel, Switzerland), Carbamazepine was obtained from RBI (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Potassium hydroxide pellets, potassium hydrogen phosphate, methanol, and acetonitrile were of HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Chromatography

NA was quantified using a Shimadzu HPLC system (Duisburg, Germany) consisting of a pump LC 9A, an automatic sampler SIL 6A, a column heater CTO 6A and an ultraviolet detector SPD 6A. The CLASS LC10 software Version 1.6 (Shimadzu) was used for data analysis and processing. The compounds were separated at 50 °C on a ProntoSIL 120-5-C18 AQ (5 µm, 250 × 3 mm I.D.) (BISCHOFF Chromatography, Leonberg, Germany) with guard column and quantified by UV detection at 210 nm. For preparation of the mobile phase, a 0.1 M potassium hydrogene phosphate solution was adjusted to a pH of 4.0 with potassium hydroxide (30% in water). Seven hundred ml of this aqueous solution was mixed with 80 ml of methanol and 300 ml of acetonitrile to constitute the mobile phase. The mobile phase was prepared weekly and was delivered at a flow rate of 1.0 ml/min. The substance was quantified using its peak height ratio to an internal standard (carbamazepine).

2.3. Sample preparation

In a 1.5 ml plastic tube, 50 µl of plasma were mixed with 10 µl of internal standard working solution and 100 µl of acetonitrile for 10 min in an Eppendorf thermomixer 5437 (Hamburg, Germany). After centrifugation for 10 min at 3000 × *g*, the supernatants were transferred into an injection vial. Samples were evaporated to dryness at 50 °C under stream of nitrogen. The residues were reconstituted with 30 µl of the mobile phase and 20 µl were injected into the HPLC.

2.4. Preparation of stock solutions, calibration standards and quality control sample

2.4.1. Nateglinide

A stock solution was prepared by dissolving 2.00 mg NA in methanol in a 5 ml volumetric flask. The solution was stored at 4 °C. For preparation of calibration standards and quality control samples, aliquots of the diluted stock solution were evaporated to dryness and reconstituted in blank human plasma. The final concentrations of NA calibration standards were 0.1, 0.2, 0.4, 0.6, 1.0, 3.0, 5.0, 10.0, 15.0 and 20.0 µg/ml. Final NA quality control concentrations were 0.5, 2.0, and 7.5 µg/ml. Calibration standards, blank plasma samples and quality control samples were stored in aliquots of 50 µl at −20 °C until analysis.

Table 1
Data for the LOQ for NA

Concentration (μg/ml)	*n*	CV (%)	Accuracy (%)
0.1	13	11.9	99
0.2	13	7.7	97
0.4	13	10.5	95
0.6	12	6.1	94
1.0	13	5.4	94
3.0	12	1.8	104
5.0	13	3.3	100
10.0	13	2.2	102
15.0	13	1.8	99
20.0	12	0.7	100

2.4.2. Carbamazepine

A stock solution was prepared by dissolving 2.00 mg of carbamazepine in methanol in a 100 ml volumetric flask. The solution was stored at 4 °C. The working solution was prepared by dilution with methanol to the final concentration of 0.5 mg/100 ml.

3. Results

3.1. Selectivity and specificity

NA and the internal standard carbamazepine were well separated under the HPLC conditions applied. Retention times were 14.1 min for NA and 6.7 min for carbamazepine. After addition of methanol to the mobile phase, it was possible to separate small interfering peaks from the analytes, so that no interferences were observed in four different blank plasma samples around the retention times of NA and carbamazepine. Fig. 2 shows the chromatograms of a blank plasma sample (a), a quality control standard with a NA concentration of 7.5 μg/ml (b), and plasma samples from a volunteer 0.8 and 4.8 h after administration of 120 mg of NA, respectively (c, d).

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD determined as the amount of drug corresponding to a signal-to-noise ratio of 3:1 was 1 ng. The LOQ was determined as the lowest concentration of the analyte in plasma that could be quantified with an inter-assay coefficient of variation (CV) of < 20% and an accuracy between 80 and 120%. The LOQ for NA was 0.1 ng/ml (Table 1).

3.3. Recovery and linearity

The recovery of NA after protein precipitation and reconstitution in mobile phase was approxi-

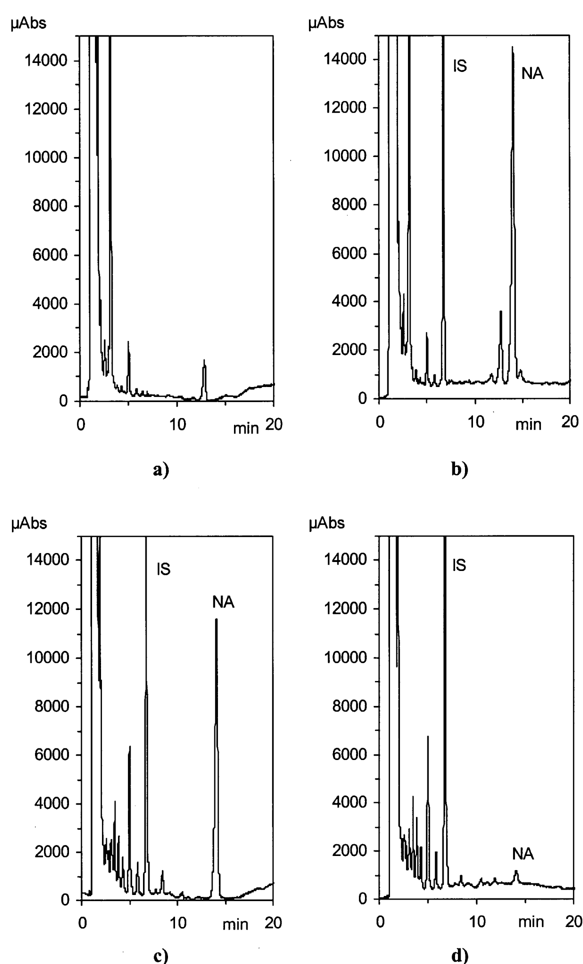

Fig. 2. HPLC trace of NA and the internal standard (IS) using ultraviolet detection at 210 nm. (a) Blank plasma sample; (b) quality control standard (7.5 μg/ml); (c) plasma sample 0.8 h post administration of 120 mg NA (6.5 μg/ml); (d) plasma sample 4.8 h post administration of 120 mg NA (0.3 μg/ml).

Table 2
Assay linearity for the quantitation of NA

	Mean	S.D	Range	*n*
Slope	0.1347	0.0132	0.1140–0.1560	13
r^2	0.9995	0.0006	0.9978–0.9999	13
y-Axis intercept	0.0023	0.0117	−0.0120–0.0301	13

Table 3
Intra-assay and inter-assay variability and accuracy of the quantitation of NA

ng/ml	Intra-assay-variability			Inter-assay-variability		
	n	CV (%)	Accuracy (%)	*n*	CV (%)	Accuracy (%)
0.5	8	12.9	107	13	8.4	96
2.0	9	1.9	104	13	3.8	103
7.5	9	1.6	98	13	4.3	98

mately 98%, tested at plasma concentrations of 0.6, 5.0 and 20.0 μg/ml. Assay linearity was evaluated up to NA concentrations of 20 μg/ml. The mean slopes, intercepts and r^2 values with standard deviation (S.D.) and ranges are reported in Table 2.

3.4. *Intra-assay and inter-assay variation and accuracy*

The intra-assay CV for the analyte ranged from 1.6 to 12.9% and the inter-assay CV from 3.8 to 8.4%. The values are reported in Table 3. The accuracy of the measurements was determined using the three quality control samples for each compound in every run and the results are reported in Table 3.

3.5. *Robustness*

The method has been used by three different analysts with between-person variability within the range of inter-assay variabilities observed for the same analyst. For lack of resources, the method could not be repeated in a different laboratory or using different equipment.

4. Discussion

We introduced a rapid method for the determination of NA in human plasma combining simple sample preparation with isocratic reversed phase HPLC analysis with UV detection. The LOQ of 0.1 μg/ml was sufficient to obtain plasma concen-

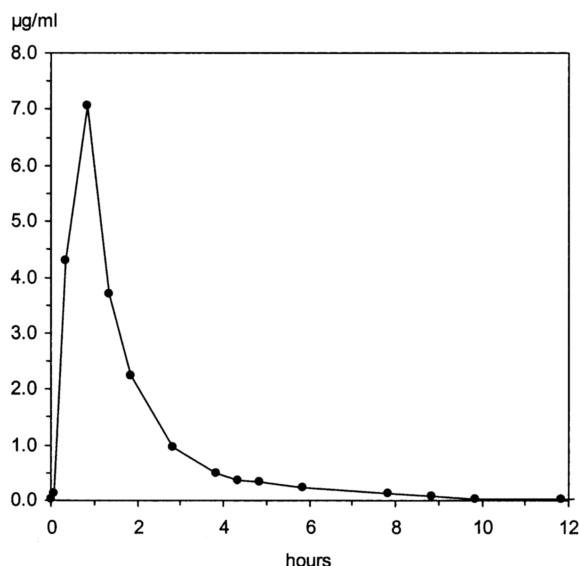

Fig. 3. Representative plasma concentration-time curve of NA 0–12 h after administration of 120 mg to a healthy volunteer.

tration curves over 12 h following administration of 120 mg NA to healthy volunteers (Fig. 3), which were used to determine pharmacokinetic parameters of NA (i.e. area under the curve, $t_{1/2}$, clearance).

The method described by Ono et al. [8,9] includes a solid phase extraction of 0.5 ml plasma. Solid phase extraction equipment and columns are expensive and not readily available in many laboratories. The comparably high plasma levels of NA allowed the simplification of sample preparation to a single precipitation step with acetonitrile, which also reduced the required sample volume to 50 μl.

After addition of methanol to the mobile phase that initially consisted of acetonitrile and potassium hydrogen phosphate buffer, it was possible to separate small interfering peaks from the analytes (Fig. 2) using a commercially available analytical column. These adjustments allowed the use of a simple isocratic system and eliminated the need for expensive column switching equipment.

The method has been applied to a pharmacokinetic study estimating the influence of different CYP2C9 genotypes on the elimination of NA in our department and was found to be reliable and reproducible.

References

[1] S. Fujitani, K. Okazaki, T. Yada, Br. J. Pharmacol. 120 (1997) 1191–1198.

[2] A.H. Karara, B.E. Dunning, J.F. McLeod, J. Clin. Pharmacol. 39 (1999) 172–179.

[3] L. Keilson, S. Mather, Y.H. Walter, S. Subramanian, J.F. McLeod, J. Clin. Endocrinol. Metab. 85 (2000) 1081–1086.

[4] S. Fujitani, T. Yada, Endocrinology 134 (1994) 1395–1400.

[5] S. Choudhury, Y. Hirschberg, R. Filipek, K. Lasseter, J.F. McLeod, J. Clin. Pharmacol. 40 (2000) 634–640.

[6] M.L. Weaver, B.A. Orwig, L.C. Rodriguez, E.D. Graham, J.A. Chin, M.J. Shapiro, J.F. McLeod, J.B. Mangold, Drug Metab. Dispos. 29 (2001) 415–421.

[7] H. Takesada, K. Matsuda, R. Ohtake, R. Mihara, I. Ono, K. Tanaka, M. Naito, M. Yatagai, E. Suzuki, Bioorg. Med. Chem. 4 (1996) 1771–1781.

[8] I. Ono, K. Matsuda, S. Kanno, J. Chromatogr. B Biomed. Sci. Appl. 678 (1996) 384–387.

[9] I. Ono, K. Matsuda, S. Kanno, J. Chromatogr. B Biomed. Sci. Appl. 692 (1997) 397–404.