Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 1, pp. 59–64, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

Determination of a catechol-*O*-methyltransferase inhibitor, nitecapone, in human plasma and urine by liquid chromatography

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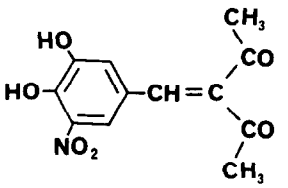
Abstract: Methods based on reversed-phase liquid chromatography with amperometric detection have been developed for determination of nitecapone, 3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione, a COMT inhibitor, in human plasma and urine. Nitecapone was extracted with ethyl acetate–hexane mixtures from plasma after acidification with hydrochloric acid and from urine as the tetrabutylammonium ion-pair of its diphenylborate derivative. The recoveries of both methods exceeded 70% and the relative standard deviations for within-day precision were less than 4% and 8% at 50 ng ml^{-1} and at the quantitation limits, respectively. The methods are selective, sensitive and precise enough for determination of 4–5 ng ml^{-1} of nitecapone in plasma and urine and are thus suitable for the kind of pharmacokinetic studies exemplified in this paper.

Keywords: *Catechol-O-methyltransferase inhibitor; nitecapone; reversed-phase liquid chromatography; electrochemical detection; plasma; urine.*

Introduction

Nitecapone, 3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione, is a new selective inhibitor of catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) [1].



COMT has an important role in the metabolic inactivation of catecholamine neurotransmitters and drugs with a catechol structure [2]. Inhibition of catechol methylation by nitecapone has been demonstrated in the rat as a reduction of 3-*O*-methyldopa formation after administration of levodopa [3]. Catecholamines have been suggested to be involved in the pathogenesis of duodenal ulcers [4, 5]. In rats, nitecapone has been shown to prevent cysteamine-induced duodenal ulcers [6] and to protect gastric mucosa against lesions induced by various necrotizing agents [7]. Presently, nitecapone is in clinical trials as a gastroprotective drug.

This paper describes methods for the determination of nitecapone in human plasma and urine by liquid chromatography with amperometric detection. The assay methods have been applied to study the pharmacokinetics of nitecapone in healthy volunteers [8].

Experimental

Chemicals and reagents

Nitecapone (OR-462) was synthesized by the Synthetic Department of Orion Pharmaceutica (Espoo, Finland). A stock solution of nitecapone was prepared every third day in 50 mM phosphate buffer pH 7.2 and stored at 4°C. For spiking of plasma and urine, dilutions of the stock solution were freshly prepared in the same buffer. Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (HPLC grade) was obtained from Orion Pharmaceutica. Tetrahydrofuran (HPLC grade) was obtained from Rathburn (Walkerburn, Scotland). Diphenylboric acid–ethanolamine complex (DPB, analytical grade) was from Aldrich (Steinheim, FRG). The DPB reagent solution (100 ml) was made by mixing 200 mg of diphenylboric acid–ethanolamine complex and

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500 mg of analytical grade disodium edetate dihydrate (Merck, Darmstadt, FRG) with a 500 mM disodium hydrogen phosphate solution. The diphenylboric acid dissolved as the pH was adjusted to 8.5 using 5 M NaOH solution. Tetrabutylammonium hydrogen sulphate (TBA) was from Fluka (Buchs, Switzerland). The TBA reagent solution (100 ml) was made by dissolving 1.6 g tetrabutyl ammonium hydrogen sulphate in 500 mM disodium hydrogen phosphate solution. All other reagents and solvents were of analytical grade and purchased from commercial sources.

Liquid chromatography

Chromatographic separation was performed using a 250 mm × 4 mm i.d. ≃ 10 μm Lichrosorb C-18 analytical column (Merck, Darmstadt, FRG) protected by a ≃10 μm μBondapak C-18 Guard-PAC guard column (Waters, Milford, MA, USA). The mobile phase was run isocratically and consisted of buffer (50 mM monosodium phosphate, 20 mM citric acid and 0.25 mM EDTA, pH adjusted to 2.0 using phosphoric acid)–methanol–tetrahydrofuran (36:20:1, v/v/v). The eluent was filtered through a 0.45 μm cellulose acetate membrane filter (Sartorius, Göttingen, FRG) and continuously degassed with a stream of helium. The eluent container and the column were immersed in a 30°C water bath. The flow rate of 1.5 ml min^{-1} was controlled with a Model 2150 pump (LKB, Bromma, Sweden). The injection volume of the Wisp 710B autosampler (Waters) was 20 μl. The working glassy carbon electrode of the LC 4B amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA) was configured in the dual-parallel mode. The detector potential was 600 mV relative to an Ag/AgCl reference electrode and the sensitivity was set at 10 nA. The integrator used was a Hewlett Packard Model 3392A.

Plasma sample preparation

The human plasma samples were stored at −20°C until the time of analysis, at which point they were thawed at room temperature. 1.0 ml of each plasma sample and 50 μl of 50 mM phosphate buffer pH 7.2 were pipetted into a 13 ml glass tube and vortex-mixed before the extraction step.

Nine calibration samples were made daily in 13 ml glass tubes by spiking 1.0 ml of human plasma with 4–2000 ng of nitecapone dissolved in 50 μl of 50 mM phosphate buffer pH 7.2, and mixing. The equilibration time before extraction was at least 5 min.

After addition of 100 μl of 2 M hydrochloric acid, the analyte was extracted from each plasma sample into 6.0 ml *n*-hexane–ethyl acetate (7:3, v/v) by mixing for 2 min using a multitube vortexer. After centrifugation (5 min, 3500 *g*), 5.0 ml of each organic phase was evaporated to dryness at 40°C using a gentle stream of nitrogen. The residues were dissolved in 50 mM phosphate buffer pH 2–methanol (6:4, v/v). When the expected concentration of the samples was less than 100 ng ml^{-1}, 200 μl of the solvent was used to dissolve each residue whereas 1000 μl was used when samples with a concentration expected to exceed 100 ng ml^{-1} were analysed.

Urine sample preparation

The human urine samples were stored at −20°C and were thawed in a water bath at approximately 30°C before analysis. Before extraction, 50 μl of 50 mM phosphate buffer pH 7.2 was added to 1.0 ml of urine in a 13 ml glass tube and briefly mixed.

Ten calibration samples were made daily in 13 ml glass tubes by spiking 1.0 ml of human urine with 5–500 ng of nitecapone dissolved in 50 μl of 50 mM phosphate buffer pH 7.2, and mixing.

A 200 μl aliquot of 500 mM disodium hydrogen phosphate solution and 50 μl of diphenylborate (DPB) reagent solution were added to each sample. 2.0 ml of water and 150 μl of tetrabutyl ammonium (TBA) reagent solution were added to each sample and vortex-mixed. Immediately, 1.0 ml of ethyl acetate was added and vortex-mixed for 5 s. 4.0 ml of *n*-hexane was added and the tubes were mixed for 2 min using a multitube vortexer. After centrifugation (5 min, 3500 *g*), 4.0 ml of each organic phase was separated into a conical tube and evaporated to dryness at 40°C with a gentle stream of nitrogen. The residues were dissolved in 200 μl of 50 mM phosphate buffer pH 2–methanol (6:4, v/v).

Determination of recoveries

The recovery of nitecapone was studied by analysing replicate spiked plasma and urine samples. The recoveries were calculated, after correcting for volume transfers, from the peak heights obtained for the extracts of the spiked

samples and from the peak heights of solutions of nitecapone in the solvent used to dissolve the evaporated extracts.

Quantitative determination

The range of calibration for plasma was 4–200 ng ml^{-1} or 20–2000 ng ml^{-1}, depending on the expected drug concentration of the plasma samples to be analysed. For urine, the range of calibration was 5–500 ng ml^{-1}.

For both methods the calibration curves were calculated by linear regression of the peak heights of the calibration samples vs their nitecapone concentrations. Separate calibration curves, each consisting of at least four points, were calculated for the high and low concentration ranges.

Results and Discussion

Extraction, recovery and specificity

The nitro-group of nitecapone lowers the pK_a of *o*-hydroxyl to 4.7. Extraction of the drug into an organic phase therefore requires acidification of the sample. Extraction of acidified plasma with ethyl acetate–hexane mixtures gave reasonable recovery and no interference with the chromatography and detection used. From acidified urine, however, many compounds giving rise to chromatographic peaks close to nitecapone were co-extracted. Therefore a more selective sample preparation method was necessary for urine.

An ion-pair extraction procedure, based on the method for catechols proposed by Smedes *et al.* [9], was developed for the isolation of nitecapone from urine. Diphenylborate forms covalent bonds with the catechol hydroxyls in an alkaline solution. This diphenylborate derivative was extracted as the tetrabutyl ammonium ion-pair, after dilution of the aqueous phase, in order to improve the selectivity of the extraction. At alkaline pH, the α-carbon of the side chain of nitecapone is susceptible to attack by nucleophiles contained in urine. Therefore, a rapid extraction was accomplished by using pure ethyl acetate in the first step of the extraction process while a good selectivity was obtained by subsequently adding *n*-hexane to the organic phase.

Typical chromatograms of plasma and urine extracts are shown in Figs 1 and 2, respectively. No interfering peaks were present in the chromatograms of unspiked plasma or urine. The recoveries of nitecapone from plasma and urine are given in Table 1.

The possibility that the metabolites of nitecapone interfered with the determination of the analyte was also studied. Nitecapone is metabolized mainly through *O*-glucuronidation and reduction of the unsaturated side chain groups [10]. A chromatogram of a mixture containing all of the major non-conjugated metabolites of nitecapone is shown in Fig. 3. Nitecapone is chromatographically well separated from the extractable metabolites. In practice, however, non-conjugated metabolites were not observed in plasma, and in urine their concentration was low compared to the concentration of nitecapone.

Limit of quantitation and linear range of calibration

Linear regression equations calculated from the peak height of spiked biological samples vs concentration data were used for calibration. The concentration range required for the analysis of plasma samples in pharmacokinetic studies was almost three orders of magnitude. To cover this range, different calibrations were used for low and high concentrations. Taking into consideration the background from different plasma samples, which corresponded to 0.6 ± 0.9 ng ml^{-1} (mean ±SD, $n = 10$) of nitecapone, and the precision of the method, the practical limit of quantitation was 3–5 ng ml^{-1}. The calibrated range used was from 4 ng ml^{-1} to 2000 ng ml^{-1}. To maintain linearity of detector response at concentrations exceeding 1000 ng ml^{-1}, the extraction residues were dissolved in 1000 μl instead of 200 μl of solvent. The correlation coefficients of all calibration curves were 0.999.

The quantitation limit and the lowest calibrated range were essentially the same for urine samples. The background from urine was lower due to the selective extraction, and the precision at the low concentration range was slightly better than in the plasma assay. The coefficient of correlation of the calibration curves were typically 0.998 for all concentration ranges up to 500 ng ml^{-1}. For concentrations exceeding 750 ng ml^{-1} the calibration curve was no longer linear, probably because of non-linearity of the ion-pair extraction. Concentrations exceeding 500 ng ml^{-1} could, however, be determined by diluting the urine samples prior to extraction.

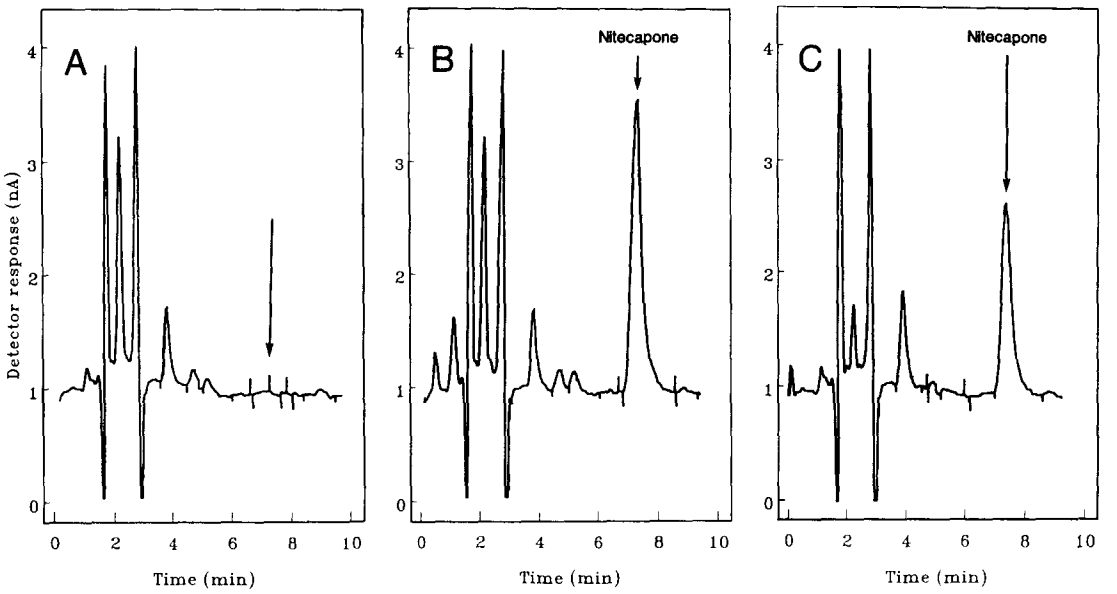

Figure 1
Chromatograms of extracts of (A) an unspiked plasma sample; (B) a 200 ng ml^{-1} calibration sample; and (C) a plasma sample obtained from a volunteer 1 h after oral administration of 10 mg of nitecapone (peak height corresponds to 133 ng ml^{-1}).

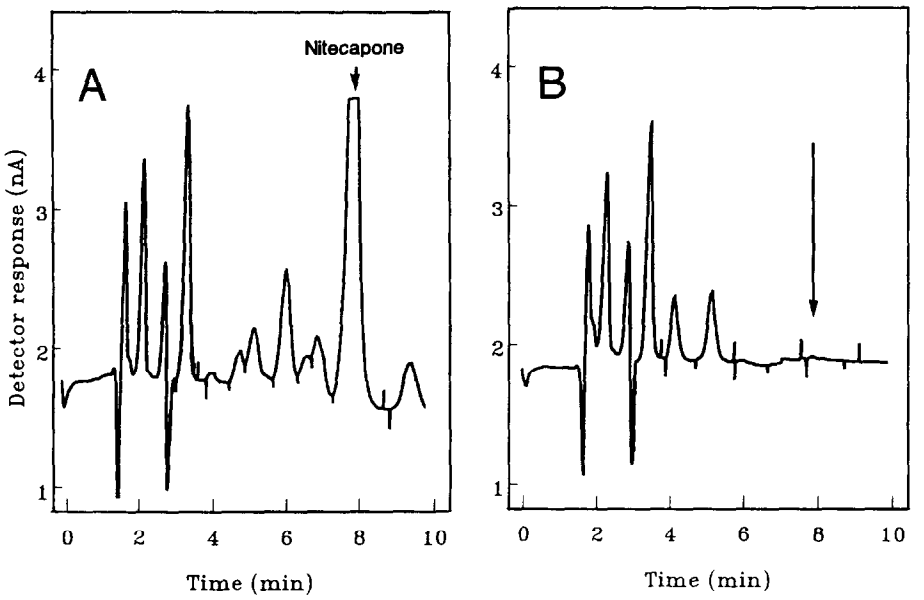

Figure 2
Chromatograms of extracts of (A) urine collected 0–2 h; and (B) 6–8 h after oral administration of 25 mg of nitecapone to a volunteer. The peak of nitecapone in the 0–2 h fraction corresponds to 135 ng ml^{-1}.

Table 1
Recovery of nitecapone from plasma and urine

Plasma		Urine	
Concentration (ng ml^{-1})	Recovery (%) mean ± SD (n = 5)	Concentration (ng ml^{-1})	Recovery (%) mean ± SD (n = 7)
10	87.7 ± 2.5	50	77.4 ± 2.4
100	74.4 ± 3.7	500	70.5 ± 2.4
1000	74.0 ± 0.5		

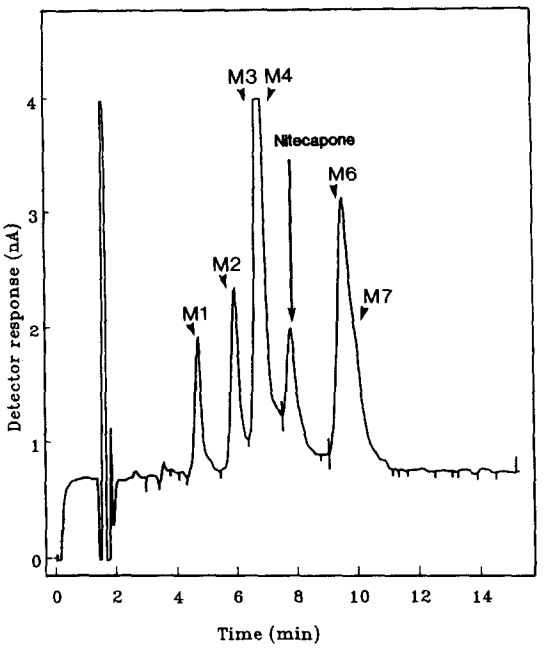

Figure 3
Chromatogram showing a mixture of nitecapone and its six extractable metabolites (M1–M4 and M6–M7) isolated from urine after β-glucuronidase hydrolysis.

Precision and accuracy

The within-day precision was studied by extracting and chromatographing replicate spiked samples at the concentration levels of 4, 10, 100 and 1000 ng ml^{-1} of plasma and 5, 50 and 500 ng ml^{-1} of urine. The relative standard deviations of the peak heights are given in Table 2.

The accuracy and between-day reproducibility data of the plasma method, given in Table 3, were estimated from the concentrations determined for homogeneous samples made by spiking human plasma with 20 or 100 ng ml^{-1} of nitecapone.

Stability of samples

Stability of nitecapone in the biological

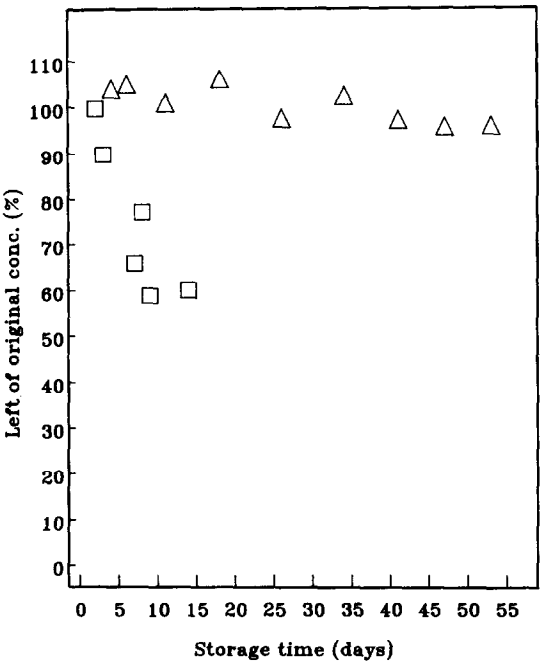

Figure 4
Nitecapone left in spiked plasma (△) and urine (□) samples stored at −20°C.

Table 2
Within-day precision data for nitecapone in plasma and urine

Plasma				Urine	
Concentration (ng ml^{-1})	Volume* (μl)	RSD (%)	n†	Concentration (ng ml^{-1})	RSD (n = 7) (%)
4	200	7.8	7	5	6.7
10	200	3.0	6	50	3.1
100	200	2.1	6	500	3.4
100	1000	3.8	6		
1000	1000	1.0	6		

*Volume of solvent used to dissolve the analyte prior to injection.
†Number of samples.

Table 3
Between-day reproducibility and accuracy for homogeneous spiked plasma samples

Concentration (ng ml^{-1})	Determined mean concentration (ng ml^{-1})	Inaccuracy	Variation SD (ng ml^{-1})	Days
20	20.1	+0.5%	0.7	10
100	98.4	−1.6%	3.3	16

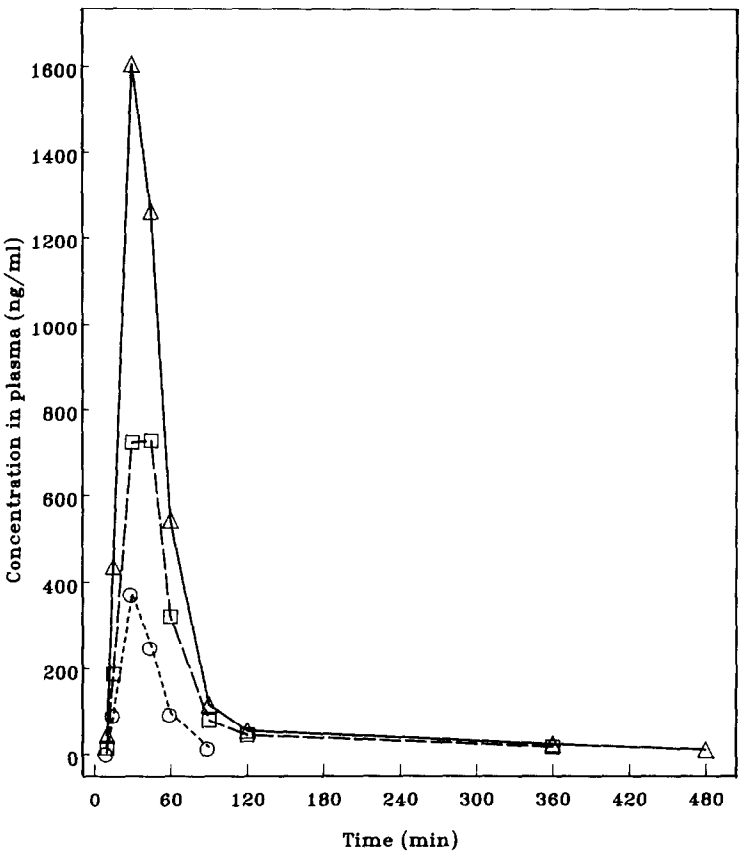

Figure 5
Plasma concentration–time curve for a healthy subject given a single 10 (○), 25 (□) or 50 mg (△) oral dose of nitecapone.

samples stored at −20°C was studied using spiked plasma and urine samples. The results are shown in Fig. 4. Nitecapone was stable in plasma samples for at least three weeks. In urine samples, some decrease in the drug concentration occurred in two days and in two weeks the concentration decreased almost to one-half of the original level.

Application

An example of the plasma pharmacokinetic profiles obtained after oral administration of 10, 25 and 50 mg of nitecapone to one healthy volunteer is presented in Fig. 5. The assay enabled determination of plasma concentrations of nitecapone over 8 h following oral administration of a 50 mg dose. Less than 1% of the administered dose of nitecapone was excreted in 8 h as unchanged drug into the urine. Thus, the range of nitecapone concentration in the collected urine samples was typically from *ca* 5–500 ng ml^{-1}.

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[*Received for review 4 April 1990*]