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Liquid chromatographic determination of a new catechol-*O*-methyltransferase inhibitor, entacapone, and its *Z*-isomer in human plasma and urine

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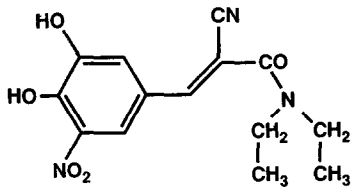
Abstract: Assay procedures for analysis of entacapone, (*E*)-2-cyano-*N*,*N*-diethyl-3-(3,4-dihydroxy-5-nitrophenyl)-propenamide, and its *Z*-isomer in human plasma and urine are described. The methods were based on reversed-phase liquid chromatography with amperometric detection. Entacapone and its *Z*-isomer were extracted with *n*-hexane–ethyl acetate mixtures after acidification with hydrochloric acid. From urine extracts the analytes were back-extracted into phosphate buffer (pH 7.2). During sample treatment 1–2% of entacapone was changed to the *Z*-isomer. With recoveries exceeding 75% the relative standard deviations for within-day precision were less than 11% for plasma and less than 6% for urine at the quantitation limit (10 ng ml^{-1}) and less than 6% for both methods at higher concentrations (20–2000 ng ml^{-1}). The assays were specific with respect to all known metabolites and selective, sensitive and precise enough for determination of entacapone and its *Z*-isomer in plasma and urine down to 10 ng ml^{-1}. The methods are thus suitable for the kind of pharmacokinetic studies exemplified in this paper.

Keywords: *Catechol-O-methyltransferase inhibitor; entacapone and Z-isomer; reversed-phase liquid chromatography; electrochemical detection; plasma; urine.*

Introduction

Entacapone (OR-611), (*E*)-2-cyano-*N*,*N*-diethyl-3-(3,4-dihydroxy-5-nitrophenyl)propenamide, is a novel inhibitor of the enzyme catechol-*O*-methyltransferase (COMT, EC 2.1.1.6).



Scheme
Structural formula of entacapone.

COMT catalyses the biotransformation of catechols to their methyl ethers. Levodopa, used for treatment of Parkinson's disease, is metabolized by COMT to 3-*O*-methyldopa (3-OMD) [1, 2]. 3-OMD is the main circulating metabolite in Parkinsonian patients receiving a combination of levodopa and an aromatic L-amino acid decarboxylase inhibitor such as carbidopa or benserazide. Because of the long elimination half-life of 3-OMD the plasma concentration of this potentially harmful metabolite is usually many times that of levodopa [3, 4]. The addition of a peripherally-acting COMT inhibitor to the levodopa and decarboxylase inhibitor medication could have several beneficial effects in the therapy of Parkinson's disease [5, 6]. A marked selective inhibition of COMT activity by entacapone has been demonstrated in the rat [7]. Entacapone has also been shown to reduce the circulating 3-OMD when coadministered with levodopa and carbidopa in cynomolgus monkeys [8] and in man [9].

This paper describes methods for the simultaneous determination of entacapone and its main non-conjugated metabolite (the *Z*-isomer) in human plasma and urine by reversed-phase liquid chromatography with amperometric detection. The assays have been applied to study the pharmacokinetics of these two stereoisomers in healthy volunteers [9].

Experimental

Chemicals and reagents

Entacapone (OR-611) and its *Z*-isomer ((*Z*)-OR-611) were synthesized by the Synthetic Department of Orion Research Center (Espoo, Finland). Ultrapure reagent-grade

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water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (HPLC grade) was obtained from Orion Pharmaceutica. Acetonitrile and tetrahydrofuran (both HPLC grade) were purchased from Rathburn (Walkerburn, UK). All other reagents and solvents were of analytical grade and used without further purification.

Separate stock solutions for entacapone and the *Z*-isomer, each containing 0.4 mg ml^{-1}, were prepared every week in 50 mM phosphate buffer (pH 7.2) and stored at 4°C protected from light. For spiking of plasma or urine, dilutions of the stock solutions were freshly prepared in the same buffer.

Chromatography

Plasma assay. The liquid chromatographic system consisted of a Model 2150 pump (LKB, Bromma, Sweden), a WISP 710B autosampler (Waters, Milford, MA, USA) and a Lichrosorb C-18 analytical column (250 mm × 4 mm i.d., 10 μm particle size; Merck, Darmstadt, Germany). The analytical column was fitted with a μBondapak C-18 Guard-PAC guard column (Waters). The temperature of the analytical column was controlled by a Temperature Control Module (Waters). The detector was a Model LC-4B amperometric detector with a glassy-carbon electrode assembly (Bioanalytical Systems Inc., West Lafayette, IN, USA). The electrode potential was 700 mV relative to an Ag–AgCl reference electrode and the sensitivity was set at 10 nA. The integrator used was a Model 3392A (Hewlett–Packard, Avondale, PA, USA). The mobile phase 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 (63:50:5, v/v/v). The mobile phase flow rate was 1.5 ml min^{-1}, the injection volume was 20 μl and the temperature of the column control module was 35°C.

Urine assay. The basic chromatographic equipment was identical to that used in the plasma assay. The mobile phase consisted of buffer (50 mM monosodium phosphate, 20 mM citric acid and 0.25 mM EDTA, pH adjusted to 3.0 using 10 M sodium hydroxide)–acetonitrile–tetrahydrofuran (125:50:5, v/v/v). The flow rate was 1.5 ml min^{-1} and the injection volume 30 μl. The temperature of the column control module was set at 28°C.

Sample preparation

Plasma. Before extraction, 50 μl of 50 mM phosphate buffer (pH 7.2) was added to 1.0 ml of human plasma. After vortex-mixing the sample was acidified with 100 μl of 2 M hydrochloric acid. The analytes were extracted into 6.0 ml *n*-hexane–ethyl acetate (1:1, v/v) by mixing for 2 min using a multitube vortexer. After centrifugation (5 min, 3500*g*), 5.0 ml of the organic phase was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 500 μl of dimethyl sulphoxide and an aliquot was injected into the chromatographic system. The samples were protected from light during the whole thawing and extraction procedure.

Calibration samples were made daily by spiking 1.0 ml of human plasma separately with various amounts of entacapone and its *Z*-isomer in 50 μl of the buffer. The concentrations of the calibration samples ranged from 10 to 4000 ng ml^{-1} for entacapone and from 10 to 500 ng ml^{-1} for the *Z*-isomer.

Urine. Before extraction, 50 μl of 50 mM phosphate buffer pH 7.2 was added to 1.0 ml of human urine and vortex-mixed. The sample was then acidified with 100 μl of 2 M hydrochloric acid. After vortexing, the analytes were extracted into 5.0 ml *n*-hexane–ethyl acetate (3:1, v/v) by mixing for 2 min using a multitube vortexer. The sample was then centrifuged (5 min, 3500*g*) and 4.0 ml of the organic phase was transferred into another tube and the analytes were back-extracted into 1.0 ml of 50 mM phosphate buffer pH 7.2 by vortexing for 2 min. After centrifugation (5 min, 3500*g*) the organic phase was discarded and the aqueous phase was transferred into an autosampler vial for chromatographic analysis. As in plasma analysis the samples were protected from light during the whole procedure.

Calibration samples were made daily by spiking 1.0 ml of human urine separately with various amounts of entacapone and the *Z*-isomer dissolved in 50 μl of the buffer. For both analytes the concentrations of the calibration samples ranged from 10 to 2000 ng ml^{-1}.

Quantitative determination

For both assays the equations for the calibration curves were calculated by linear regression of the peak heights of the calibration samples versus their analyte concentrations.

Separate calibration curves were calculated for the low and high concentration ranges using at least four calibration points per curve. The ranges of calibration for plasma were 10–200 and 200–4000 ng ml^{-1} for entacapone and 10–500 ng ml^{-1} for the Z-isomer. For urine, the ranges of calibration were 10–200 and 200–2000 ng ml^{-1} for both analytes.

Determination of recovery and reproducibility

The recoveries of entacapone and the Z-isomer were studied by analysing replicate spiked plasma and urine samples. The recoveries were calculated, after correcting for volume transfers, by comparing the peak heights obtained for the extracts of the spiked samples with the peak heights of solutions of the respective analyte in the same solvent as the extracted sample. Within-day reproducibility was studied by analysing replicate spiked plasma and urine samples at three different concentration levels. Between-day reproducibility and accuracy were determined by assaying control samples of three different concentrations. The control samples were prepared by adding appropriate amounts of both analytes in 50 mM phosphate buffer (pH 7.2) to pooled analyte-free human plasma or urine. The integrity of plasma and urine was maintained since the volume added was <2% of the total volume of the sample. After careful mixing the spiked pools were divided into aliquots of about 1.1 ml and stored at −70°C until analysed.

Stability and storage

To study the stability of the analytes in frozen samples, about 1.1 ml aliquots of spiked pooled plasma (conc. 200 ng ml^{-1}) and urine (200 ng ml^{-1}) were stored at −20°C in glass tubes and subsequently assayed by the methods described.

Results

Specificity

Typical chromatograms obtained from plasma and urine analyses are shown in Figs 1 and 2. The chromatograms of unspiked plasma and urine do not generally show any peak with retention time similar to that of entacapone or the Z-isomer. In certain plasma and urine chromatograms small, non-quantifiable, peaks were present close to the entacapone peak. In the plasma assay the retention times were 7.5 and 5.3 min for entacapone and the Z-isomer, respectively. In the urine assay the corresponding retention times were 10.7 and 9.1 min. No interfering metabolite peaks were observed in the chromatograms after administration of entacapone to volunteers. The main urinary metabolites (the glucuronides of entacapone and its Z-isomer [10]) were isolated from human urine and purified by semi-preparative liquid chromatography. The glucuronides were dissolved in water and the concentrations were determined after β-glucuronidase hydrolysis. When solutions containing the glucuronides (concentrations corresponding to 9.3 μg ml^{-1} of entacapone and 2.8 μg ml^{-1} of the Z-isomer) were chromatographed, no peaks could be detected. After addition of the glucuronides to blank plasma or urine (concentrations as above) and processing these samples, no peaks could be observed, either. The retention times of three known phase I-metabolites, isolated from urine after enzymatic hydrolysis, differed clearly from the retention times of both entacapone and the Z-isomer in urine [3,4-dihydroxy-5-nitrobenzaldehyde, 3.6 min; 2-cyano-*N*,*N*-diethyl-(3,4-dihydroxy-5-nitrophenyl)-propanamide, 7.6 min; and 2-cyano-*N*-ethyl-*N*-(2-hydroxyethyl)-3-(3,4-dihydroxy-5-nitrophenyl)propenamide, 12.4 min]. When a solution of the compounds carbidopa, levodopa and its metabolites (3-OMD, homovanillic acid and 3,4-dihydroxyphenylacetic acid) was injected, no disturbing peaks could be detected in either assay.

Recovery

The recoveries of entacapone and the Z-isomer from plasma and urine are given in Tables 1 and 2. At 10 ng ml^{-1} the apparent recovery of entacapone was rather high in both plasma and urine due to co-integration of the entacapone peak and some small peaks seen in the chromatograms of certain unspiked samples.

Linearity and limit of quantitation

For both analytes there was good linearity between the peak heights and the calibration sample concentrations over the calibrated ranges. The correlation coefficient of all calibration curves was greater than 0.99. In plasma the calibrated ranges were 10–200 and 200–4000 ng ml^{-1} for entacapone and 10–500 ng ml^{-1} for the Z-isomer. In urine the ranges were

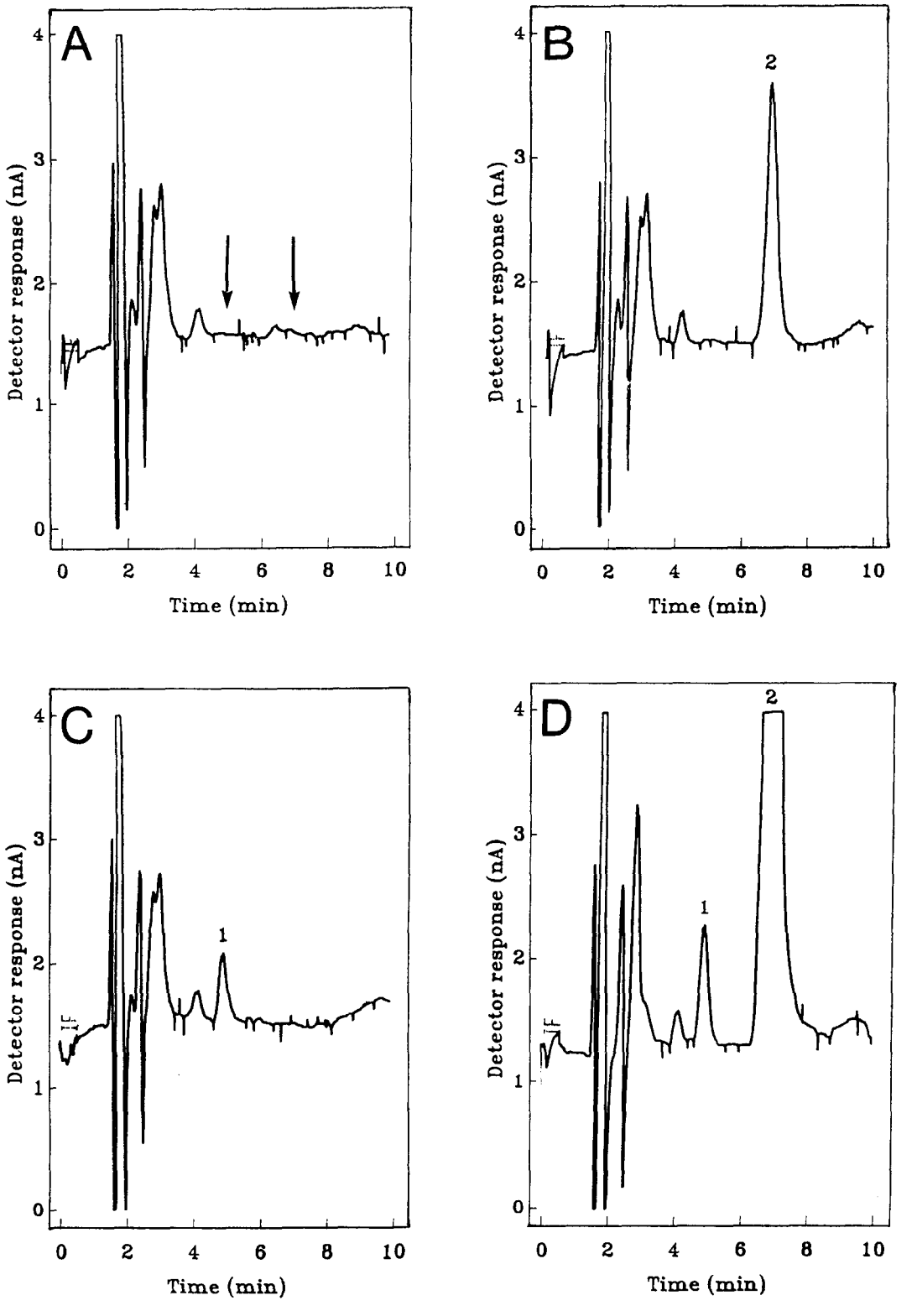

Figure 1
Chromatograms of (A) an unspiked plasma sample; (B) a 100 ng ml^{-1} calibration sample of entacapone; (C) a 20 ng ml^{-1} calibration sample of the *Z*-isomer; and (D) a plasma sample obtained from a healthy volunteer 30 min after oral administration of 200 mg of entacapone (peak heights correspond to 720 ng ml^{-1} of entacapone and 37 ng ml^{-1} of the *Z*-isomer). Peak 1 = *Z*-isomer of entacapone; peak 2 = entacapone.

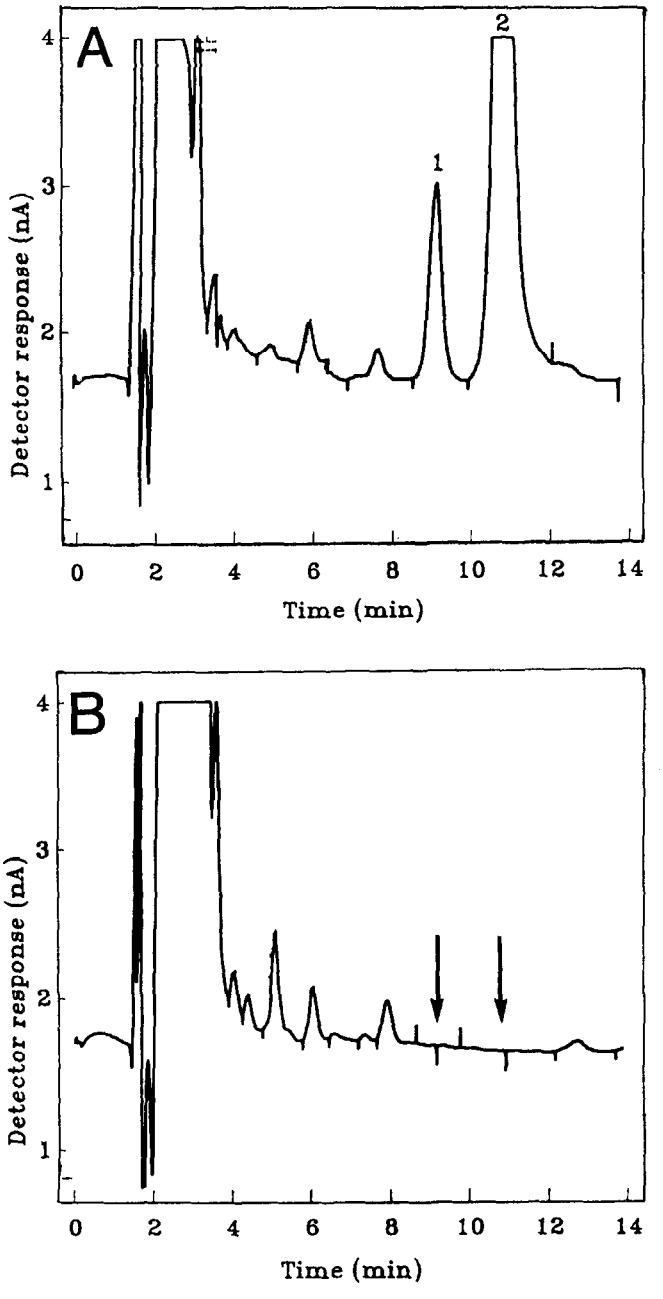

Figure 2
Chromatograms of urine collected (A) 0–2 h; and (B) 10–12 h after oral administration of 200 mg of entacapone to a healthy volunteer. Peak 1 = Z-isomer of entacapone; peak 2 = entacapone.

Table 1
Recovery of entacapone and its Z-isomer from plasma

	Entacapone		Z-isomer	
Conc. (ng ml^{-1})	Recovery (%) Mean (±SD)	n	Recovery (%) Mean (±SD)	n
10	97.1 (±20.0)	16	79.7 (±5.8)	16
200	88.2 (±2.0)	8	88.2 (±1.7)	8
500			85.9 (±5.0)	8
2000	89.0 (±2.2)	8		

Table 2
Recovery of entacapone and its Z-isomer from urine

	Entacapone	Z-isomer
Conc. (ng ml^{-1})	Recovery (%)* Mean (±SD)	Recovery (%)* Mean (±SD)
10	104.4 (±2.9)	74.9 (±4.4)
200	91.3 (±2.0)	86.8 (±1.1)
2000	86.6 (±1.9)	85.7 (±1.6)

*n = 8.

10–200 and 200–2000 ng ml^{-1} for both analytes. For the calibration lines the goodness-of-fit was evaluated by calculating the concentrations corresponding to the respective calibration samples using the equation of the appropriate calibration. For the plasma calibration samples the relative error was <12% and the relative standard deviation (RSD) was <15% (n = 43) for both analytes at the limit of quantitation (10 ng ml^{-1}). The RSD for all other calibration samples ranged from 0.1 to 6.9% with relative errors of 0–2.9%. For urine the limit of quantitation and the linearity were found to be almost the same as for plasma.

Precision and accuracy

The within-day precision data for the plasma and urine assays are presented in Table 3. Data on the accuracy and between-day precision of the methods are given in Tables 4 (plasma) and 5 (urine).

Stability of samples

No essential changes in the entacapone or Z-isomer concentrations were observed when plasma samples were stored for 14 weeks at −20°C. After 9 months of storage the concentrations had decreased 10–20% (Fig. 3). The stability of entacapone in human urine was followed for up to 17 weeks. The results of the

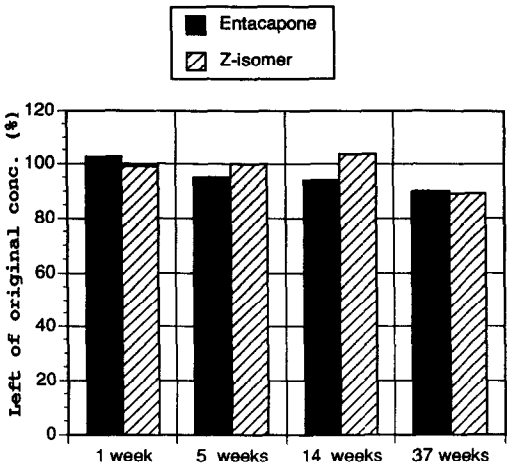

Figure 3
Stability of entacapone and its Z-isomer in spiked plasma samples stored at −20°C.

Table 3
Within-day precision data (n = 8) for entacapone and its Z-isomer in plasma and urine

	Relative standard deviation (%)			
	Plasma		Urine	
Conc. (ng ml^{-1})	Entacapone	Z-isomer	Entacapone	Z-isomer
10	10.7	8.3	2.8	5.9
200	2.2	1.9	2.1	1.3
500		5.9		
2000	2.4		2.2	1.9

Table 4
Between-day reproducibility and accuracy for entacapone and its Z-isomer in plasma

Entacapone				Z-isomer			
Nominal conc. (ng ml^{-1})	Mean (±SD) (ng ml^{-1})	% Error	n	Nominal conc. (ng ml^{-1})	Mean (±SD) (ng ml^{-1})	% Error	n
43.6	43 (±3)	−1.6	43	42	41 (±3)	−3.6	44
415	411 (±32)	−0.9	43	400	388 (±27)	−3.1	41
2072	1980 (±130)	−4.3	38				

Table 5
Between-day reproducibility and accuracy for entacapone and its Z-isomer in urine

Entacapone				Z-isomer			
Nominal conc. (ng ml^{-1})	Mean (±SD) (ng ml^{-1})	% Error	n	Nominal conc. (ng ml^{-1})	Mean (±SD) (ng ml^{-1})	% Error	n
20.7	21 (±3)	±0	24	20	20 (±3)	−1.0	24
207.2	204 (±20)	−1.6	23	200	197 (±19)	−1.3	24
2072	2020 (±160)	−2.5	22	2000	1990 (±110)	−0.4	12

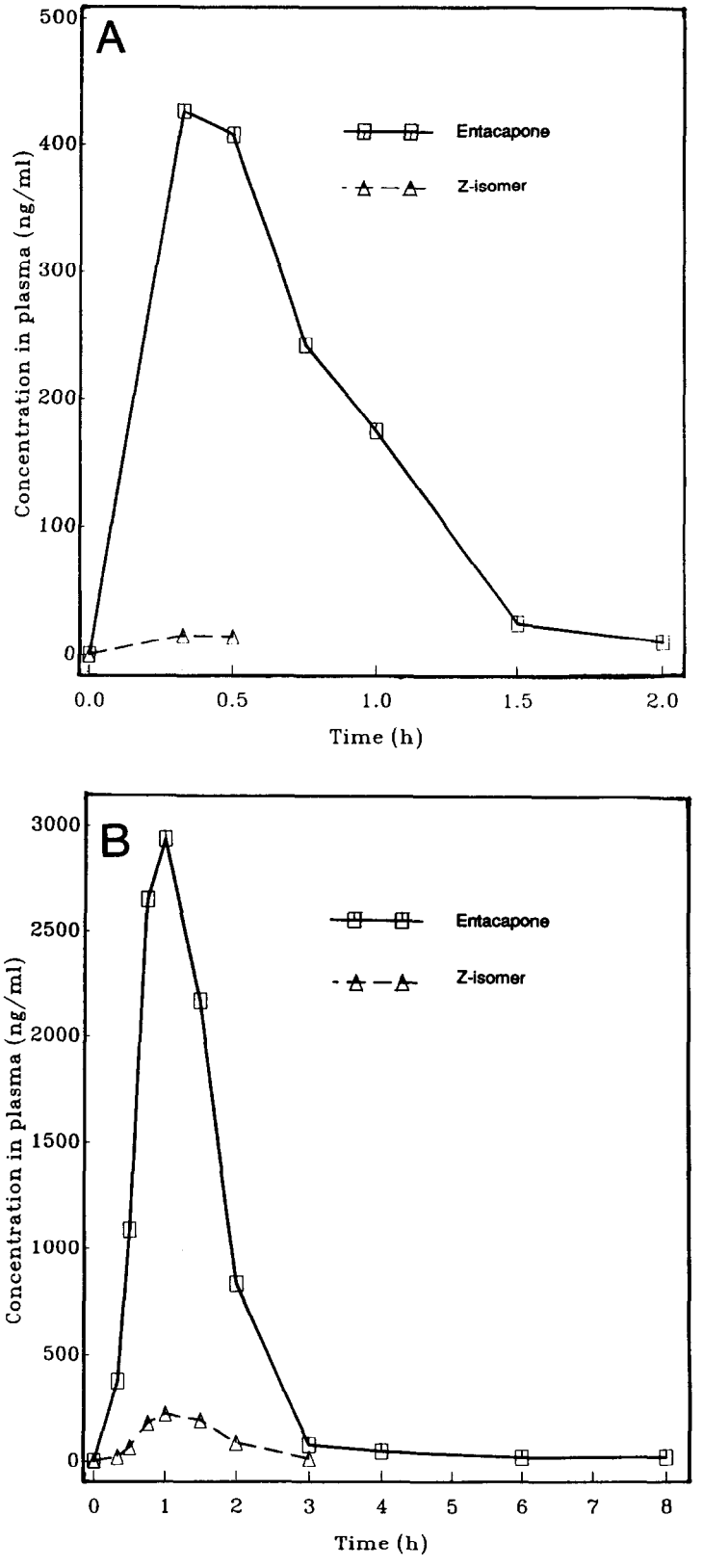

Figure 4
Plasma concentration–time curves for a healthy volunteer after a single (A) 25 mg and (B) 400 mg oral dose of entacapone.

analyses indicated a decrease of 5–10% of the concentrations during the storage time.

Application

Typical plasma level profiles for entacapone and its *Z*-isomer are shown in Fig. 4. The concentrations were obtained after oral administration of 25 and 400 mg of entacapone to one healthy volunteer. The assay enabled determination of plasma concentrations of entacapone over 2 h after the 25 mg dose and over 8 h after the 400 mg dose. The concentrations of the *Z*-isomer were typically <10% of the entacapone concentration in the same sample.

Discussion

The principal pK_a of entacapone is about 4.5. Extraction of it and its *Z*-isomer into an organic phase therefore requires acidification of the samples. A one-step extraction gave a recovery of over 80% for both analytes from plasma. The extraction was selective enough to allow the determination of the analytes down to 10 ng ml^{-1} without any significant interference of co-extracted compounds in the isocratic HPLC analysis. A one-step extraction from acidified urine gave, however, chromatograms with peaks which severely interfered with the integration of the peaks of interest. Back-extraction of the analytes from the organic phase into a pH 7.2 buffer, combined with a change of pH and the organic modifier of the mobile phase, resulted in a recovery of over 75% and chromatograms with no interfering peaks. The ion-pair extraction method of the diphenylborate derivative of the analytes employed for another nitrocatechol COMT-inhibitor to obtain selective extraction [11] was found to be unsuitable, as it resulted in rapid isomerization of entacapone.

Entacapone isomerizes to its *Z*-isomer during sample handling and preparation if precautionary measures are not taken. Daylight and plasma proteins catalyse the isomerization reaction. When the samples were handled as quickly as possible and protected from daylight during sampling and sample treatment, only 1–2% was converted to the *Z*-isomer. No correction for this was made in the quantitative determination.

The *Z*-isomer is the main extractable metabolite of entacapone in man. The chromatographic method of the urine assay separated all other known extractable meetabolites [10] from entacapone and the *Z*-isomer. The metabolite pattern in the rat is partly different from that of man [10]. It is thus necessary to check the selectivity of the separation with respect to the metabolites when applying the methods for plasma and urine of laboratory animals.

Entacapone and its *Z*-isomer are excreted in the urine mainly as their glucuronides. In this study the glucuronide conjugates isolated from urine were shown to be stable during the processing of biological fluids for analysis. This is in agreement with previous findings concerning the stability of ether glucuronides [12, 13].

Levodopa, its metabolites and carbidopa did not interfere with the determination of the analytes. The methods are specific for the analytes and linear regression was found suitable for calibrations down to 10 ng ml^{-1}. Additionally the methods are sufficiently precise and thus well suited for pharmacokinetic studies of the combination therapy of levodopa, carbidopa and entacapone in healthy volunteers and Parkinsonian patients.

References

[1] H.C. Guldberg and C.A. Marsden, *Pharm. Rev.* **27**, 135–206 (1975).
[2] J.G. Nutt and J.H. Fellman, *Clin. Neuropharmacol.* **7**, 35–49 (1984).
[3] J.M. Cedarbaum, *Clin. Pharmacokinet.* **13**, 141–178 (1987).
[4] R.J. Hardie, S.L. Malcolm, A.J. Lees, G.M. Stern and J.G. Allen, *Br. J. Clin. Pharmacol.* **22**, 429–436 (1986).
[5] P.T. Männistö and S. Kaakkola, *Pharmacol. Toxicol.* **66**, 317–323 (1990).
[6] P.T. Männistö and S. Kaakkola, *Trends Pharmacol. Sci.* **10**, 54–56 (1989).
[7] I.-B. Linde'n, E. Etemadzadeh, E. Schultz and P. Pohto, *Mov. Disord.* **5** (Suppl. 1), 49 (1990).
[8] J.M. Cedarbaum, G. Leger and M. Guttman, *Clin. Neuropharmacol.* **14**, 330–342 (1991).
[9] T. Keränen, A. Gordin, M. Karlsson, K. Korpela, P. Pentikäinen, E. Schultz and T. Wikberg, *Neurology* **41** (Suppl. 1), 213 (1991).
[10] T. Wikberg, A. Vuorela, P. Ottoila and J. Taskinen, *Drug Metab. Dispos.*, in press.
[11] T. Wikberg and J. Taskinen, *J. Pharm. Biomed. Anal.* **9**, 59–64 (1991).
[12] R.G. Dickinson and A.R. King, *Ther. Drug Monit.* **11**, 712–720 (1989).
[13] C.V. Eadsforth and P.C. Coveney, *Analyst* **109**, 175–176 (1984).

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