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# Assay of *R*-apomorphine, *S*-apomorphine, apocodeine, isoapocodeine and their glucuronide and sulfate conjugates in plasma and urine of patients with Parkinson's disease

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**Abstract**

Analytical methods are described for the selective, rapid and sensitive determination of *R*- and *S*-apomorphine, apocodeine and isoapocodeine and the glucuronic acid and sulfate conjugates in plasma and urine. The methods involve liquid–liquid extraction followed by high-performance liquid chromatography with electrochemical detection. The glucuronide and sulfate conjugates are determined after enzymatic hydrolysis. For the assay of *R*- and *S*-apomorphine a 10 μm Chiralcel OD-R column is used and the voltage of the detector is set at 0.7 V. The mobile phase is a mixture of aqueous phase (pH 4.0)–acetonitrile (65:35, v/v). At a flow-rate of 0.9 ml $min^{-1}$ the total run time is ca. 15 min. The detection limits are 0.3 and 0.6 ng $ml^{-1}$ for *R*- and *S*- apomorphine, respectively (signal-to-noise ratio 3). The intra- and inter-assay variations are <5% in the concentration range of 2.5–25 ng $ml^{-1}$ for plasma samples, and <4% in the concentration range of 40–400 ng $ml^{-1}$ for urine samples. For the assay of apomorphine, apocodeine and isoapocodeine, a 5 μm $C_{18}$ column was used and the voltage of the detector set at 0.825 V. Ion-pairing chromatography was used. The mobile phase is a mixture of aqueous phase (pH 3.0)–acetonitrile (75:25, v/v). At a flow-rate of 0.8 ml $min^{-1}$ the total run time is ca. 14 min. The detection limits of this assay are 1.0 ng $ml^{-1}$ for apomorphine and 2.5 ng $ml^{-1}$ for both apocodeine and isoapocodeine (signal-to-noise ratio 3). The inter-assay variations are 5% in the concentration range of 5–40 ng $ml^{-1}$ for plasma samples and 7% in the concentration range of 50–500 ng $ml^{-1}$ for urine samples. The glucuronic acid and sulfate conjugates of the various compounds are hydrolysed by incubation of the samples with β-glucuronidase and sulfatase type H-1, respectively. Hydrolysis was complete after 5 h of incubation. No measurable degradation of apomorphine, apocodeine and isoapocodeine occurred during the incubation. A pharmacokinetic study of apomorphine, following the intravenous infusion of 30 μg $kg^{-1}$ for 15 min in a patient with Parkinson's disease, demonstrates the utility of the methods: both the pharmacokinetic parameters of the parent drug and the appearance of apomorphine plus metabolites in urine could be determined. 

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## 1. Introduction

*R*-Apomorphine (APO) is a mixed $D_1/D_2$ receptor agonist which is used in the treatment of Parkinson's disease [1]. The drug has proven to be of great value, particularly in the treatment of refractory "on–off" oscillations. Due to its unfavourable pharmacokinetic properties APO is usually administered by repeated subcutaneous injections. In addition, continuous subcutaneous infusion of APO has shown to be an effective therapy [2]. At present several alternative modes and routes of administration are under development. Optimization of the mode and route of administration requires detailed knowledge of both the delivery system under various conditions and the pharmacokinetic/pharmacodynamic (PK/PD) relationship of the drug [3]. In this respect, factors related to both the pharmacokinetics (i.e., tissue distribution, metabolite formation) and pharmacodynamics (i.e., tolerance development) must be taken into consideration.

The metabolic breakdown of APO is potentially quite complex (Fig. 1). APO is administered as the *R*-(+)-enantiomer. It is therefore possible that in vivo conversion of the *R*-(+)-enantiomer into the *S*-(−)-enantiomer occurs. Very little information is available on the possible racemization of APO. Especially in the in vivo situation also the possibility of metabolic interconversion should be considered. It has been demonstrated that *S*-APO acts as an antagonist at the dopamine receptors [4]. With respect to metabolism of APO, there is evidence that the catechol-O-methyl transferase system may be involved [5], resulting in the formation of the metabolites apocodeine (AC) and isoapocodeine (IAC). These metabolites are known to be dopamine receptor agonists [6]. Also, glucuronidation and sulfation by liver enzymes are likely conjugation reactions preceding excretion into the urine. The involvement of the glucuronidation pathway was first suggested by Kaul et al. [7]. Non-enzymatic oxidation at the double –OH catechol group can readily occur in plasma at physiological pH [8] and is likely to contribute to the overall elimination of the drug. However, due to ongoing formation of oxidative breakdown products [9], quantification of this pathway in vivo is a difficult task.

Several reversed-phase high-performance liquid chromatography (HPLC) methods combined with an extraction procedure have been described for the assessment of the pharmacokinetic characteristics of APO. Varying degrees of sensitivity were found in these studies. Sensitivity largely depends on the employed detection method. Relatively low sensitivi-

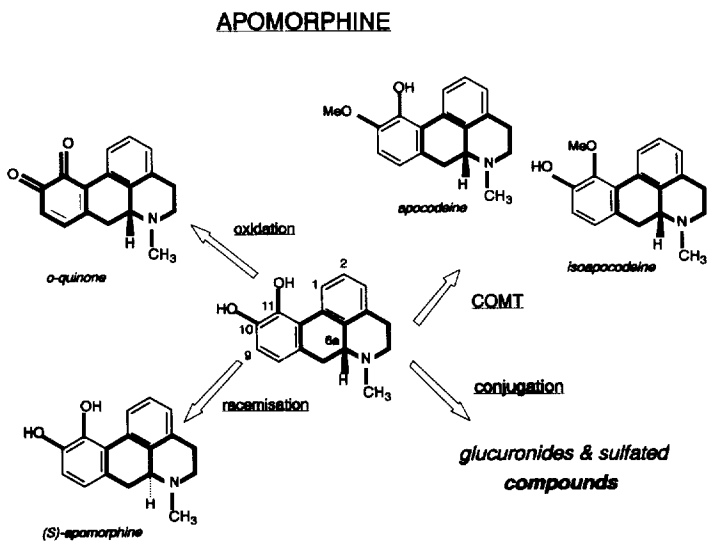


Fig. 1. Chemical structures of *R*-APO and its potential metabolites. In particular the interconversion into *S*-APO, the formation of the methylated metabolites AC and IAC, conjugation to sulphates and glucuronides and oxidation.

ty was found when UV detection was used [10]. Higher sensitivities were found with fluorescence detection [11,12] and especially electrochemical detection (ED) [8,13–16]. HPLC combined with fluorescence detection has been used for the combined analysis of APO, AC and IAC solutions in methanol [17]. The sensitivity of the assay can be further improved when a catecholamine-selective extraction procedure is employed, resulting in recoveries that are close to 100% [13].

In order to fully understand the PK/PD correlation of APO in patients with Parkinson's disease, it is essential to have detailed information on the disposition of the drug, in particular the interconversion between the enantiomers and the formation of potentially active metabolites. The purpose of the present investigation was to develop a rapid, specific and sensitive assay for the determination of the concentrations of APO enantiomers and metabolites in plasma and urine.

## 2. Experimental

### 2.1. Chemicals

*R*-APO hydrochloride was obtained from OPG (Utrecht, Netherlands); (6aS)-10,11-dihydroxyaporphine (*S*-APO), N-propylnorapomorphine (NAM) and AC were obtained from RBI (Natick, MA, USA). IAC was obtained by organic synthesis. Sulfatase type H-1 and β-glucuronidase were obtained from Sigma (Bornem, Belgium). D-Saccharic 1,4-lactone monohydrate, tetraoctylammoniumbromide (TOABr) and diphenylborinic acid ethanolamine ester (DPBEA) were obtained from Aldrich (Bornem, Belgium).

### 2.2. Synthesis of IAC

IAC was synthesized according to the method described by Cannon et al. [5]. Briefly, *R*-APO hydrochloride (10 g, 0.032 mol) in 150 ml of anhydrous DMSO was added slowly to 1.92 g (0.08 mol) of NaH at 0°C and stirred for 1 h. A solution of 7.4 g (0.039 mol) of MeOTs in 20 ml of $Et_2O$ was added in 15 min and then stirred for 18 h. This mixture was subsequently treated with 150 g of ice and extracted four times with ethanol. The combined extracts were washed with water, dried ($Na_2SO_4$) and filtered. Volatiles were removed from the filtrate under reduced pressure. The residue was dissolved in 3.5 ml petroleum ether and 0.5 ml dichloromethane and purified on a silica column [100 cm×6 cm I.D., eluent: petroleum ether–dichloromethane (1:1)+4% triethylamine+1% methanol]. Eluted fractions were pooled in three groups, based on thin-layer chromatography (TLC) and HPLC analysis. Silica TLC plates pre-treated with triethylamine were used and eluted with petroleum ether–dichloromethane (1:1). All fractions were dried under reduced pressure and dissolved in $Et_2O$. The HCl salt was formed by boiling with ethereal HCl. IAC was identified by nuclear magnetic resonance (NMR) combined with a nuclear overhouser analysis. The melting point was 243°C.

### 2.3. Instrumentation

The HPLC-ED system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA), a WISP 710 B autosampler (Millipore–Waters, Milford, MA, USA) and an Antec ED system (Leiden, Netherlands). The chromatograms were recorded on a Chromatopack C-R3A reporting integrator (Shimadzu, Kyoto, Japan).

A 10 μm Chiralcel OD-R chiral column (200 mm×4.6 mm I.D.) (Diacel Chemical Industries, Tokyo, Japan) was used for the quantification of *R*- and *S*-APO. A mobile phase of acetonitrile–aqueous phase (35:65) was used. The aqueous phase consisted of 0.1 *M* $NaH_2PO_4$, 0.1 *M* $NaClO_4 \cdot H_2O$ and 10 mg $l^{-1}$ EDTA, adjusted to pH 4 with 98% phosphoric acid. The flow-rate was 0.9 ml $min^{-1}$. Chromatography was performed at room temperature. A 50 μl volume of the aqueous phase was injected into the column directly after the extraction procedure. The voltage of the detector was 0.7 V.

For the quantification of APO, AC and IAC a 5 μm $C_{18}$ column was used (200 mm×5 mm I.D.) (YMC, Morris Planes, NJ, USA). The mobile phase consisted of acetonitrile–0.1 *M* phosphate buffer (25:75). In the buffer (0.1 *M* $NaH_2PO_4$ adjusted to pH 3 with 98% phosphoric acid), 20 mg $ml^{-1}$ octanedisulphonic acid and 10 mg $ml^{-1}$ EDTA were dissolved. The flow-rate of the mobile phase was

adjusted to 0.8 ml $min^{-1}$. Chromatography was performed at room temperature. An electrochemical potential of 0.825 V was used for detection of the metabolites. 50 μl of the aqueous phase was injected into the column.

### 2.4. Standards

In order to prevent auto-oxidative breakdown, stock solutions of APO, metabolites and internal standard (NAM) were prepared in a 5 m*M* citrate buffer (2.1 m*M* $Na_3C_6H_5O_7 \cdot 2H_2O$, 2.9 m*M* $C_6H_8O_7$) of pH 4, to which 0.1% sodium-metabisulphite and 0.01% EDTA were added. All stock solutions of APO and metabolites were diluted with the citrate buffer to concentrations in the range of 6–600 ng $ml^{-1}$ for plasma analysis and to 10–4000 ng $ml^{-1}$ for urine analysis. A stock solution of 10 μg $ml^{-1}$ of NAM in citrate buffer was prepared. All stock solutions were stored at −20°C. 0.2% DPBEA was dissolved in bidistilled water containing 14 ml $l^{-1}$ 25% $NH_4OH$ and 0.5% EDTA, adjusted to the appropriate pH with 10 *M* HCl. 0.165% TOABr was dissolved in octanol–hexane (1:10), respectively.

### 2.5. Extraction procedure

A 30 μl volume of NAM (2 μg $ml^{-1}$), 0.5 ml DPBEA buffer pH 8.45 and 1.5 ml TOABr organic phase were added to 1 ml of plasma. After 2 min of shaking and 15 min of centrifugation at 5°C, the organic phase was taken off and 3 ml of octanol and 0.5 ml of aqueous phase (0.05 *M* $H_3PO_4$), containing 0.1% sodium-metabisulphite and 0.01% of EDTA were added. After 2 min of shaking and 15 min of centrifugation at 5°C, the aqueous phase was separated and used for injection into the HPLC system.

The extraction of the urine samples was as follows: the total volume to be extracted was 2.2 ml (1 ml urine+1.2 ml enzyme solution). To compensate for the buffering capacity of the enzyme solution the pH of the DPBEA buffer was increased to 9 and the pH of each sample was checked after adding the buffer. If the pH was <8.5, it was adjusted with an 0.1 *M* NaOH solution. Thereafter the same extraction procedure as in plasma was performed.

### 2.6. Enzymatic hydrolysis

Prior to extraction and quantification of APO in the urine samples, enzyme incubations with β-glucuronidase and sulfatase [18] were used to hydrolyse the glucuronidated and sulfated conjugate of APO. All incubations were performed at 37°C. Three solutions in 0.05 *M* acetate buffer pH 4.5 were prepared for this purpose: 44 mg $ml^{-1}$ sulfatase, 6.8 mg $ml^{-1}$ β-glucuronidase and 50 mg $ml^{-1}$ D-saccharic 1,4-lactone monohydrate. Three different protocols were used for the quantification of APO and its glucuronidated and sulfated conjugates in the patients' urine samples: (1) for the quantification of free APO 1 ml of urine was incubated with 1.2 ml 0.05 *M* acetic acid buffer pH 4.5; (2) the sulfated metabolite was quantified by incubation of 1 ml of urine with a mixture of 0.7 ml 0.05 *M* acetic acid buffer, 0.2 ml D-saccharic 1,4-lactone monohydrate solution and 0.3 ml sulfatase solution; (3) the glucuronidated metabolite was quantified by incubation of 1 ml of urine with a mixture of 0.9 ml 0.05 *M* acetic acid pH 4.5 and 0.3 ml β-glucuronidase. The amounts of conjugated APO were calculated by subtracting the amount of free drug from the total amount of APO after incubation.

Two experiments were performed to determine the optimal incubation time with each enzyme individually. Urine samples containing conjugated metabolites were incubated with either sulfatase or β-glucuronidase. Two control experiments were performed as well: (1) non-specific hydrolysis was determined for up to 6 h by incubating a sample with the 0.05 *M* acetic acid buffer pH 4.5 without enzymes; (2) stability of APO in urine+0.05 *M* acetic acid buffer was determined for 6 h by spiking a blank urine sample with APO. During these experiments 1 ml samples were taken each hour and APO formation was analysed by the method described previously.

### 2.7. Calibration and validation

Eight-point calibration curves were prepared on different days by spiking 900 μl of plasma or urine with 100 μl of a solution of APO or one of its metabolites. Quality control samples of fixed concentrations (see Tables 1 and 2) were prepared to

Table 1
Assay validations of *R*-APO, *S*-APO, AC and IAC in plasma at the used concentrations (in parentheses if different concentrations are used)

| Compound | Concentration (ng ml$^{-1}$) | Recovery (%) | Found (ng ml$^{-1}$) | C.V. (%) | Accuracy (%) |
|---|---|---|---|---|---|
| *Intra-assay* | | | | | |
| *R*-APO | 2.5 | 85.4 (6.3)[a] | 2.44±0.10 | 4.1 | 97.6 |
| | 12.6 | 81.3 | 11.98±0.42 | 3.5 | 95.1 |
| | 25.1 | 78.6 | 25.50±1.29 | 5.1 | 101.6 |
| *S*-APO | 2.5 | 85.2 (6.2) | 2.44±0.16 | 6.6 | 97.6 |
| | 12.3 | 82.3 (12.6) | 12.82±0.43 | 3.4 | 104.2 |
| | 24.6 | 86.1 (25.1) | 27.46±1.37 | 5.0 | 111.6 |
| *Inter-assay* | | | | | |
| *R*-APO | 2.5 | | 2.37±0.09 | 3.8 | 94.7 |
| | 12.6 | | 12.60±0.22 | 1.7 | 100.0 |
| | 25.1 | | 26.67±0.09 | 0.3 | 106.2 |
| *S*-APO | 2.5 | | 2.43±0.10 | 4.1 | 98.8 |
| | 12.3 | | 12.39±0.30 | 2.4 | 100.6 |
| | 24.6 | | 25.32±0.53 | 2.1 | 102.8 |
| AC | 5.0 | | 5.23±0.09 | 4.7 | 104.6 |
| | 20.2 | | 20.24±0.76 | 0.2 | 100.2 |
| | 40.2 | | 40.14±0.91 | 0.1 | 99.9 |
| IAC | 5.0 | | 5.18±0.46 | 3.6 | 103.6 |
| | 20.2 | | 19.18±0.71 | 3.6 | 95.0 |
| | 40.2 | | 40.92±1.32 | 1.8 | 101.8 |

[a] When the recovery is determined at a different concentration this value is given in parentheses in ng ml$^{-1}$.

determine intra- and inter-assay variability and recoveries. Peak areas were recorded as described and the area ratios of APO to NAM were calculated. Calibration curves were constructed by weighted linear regression [weight factor: 1/(peak area ratio)].

### 2.8. Study in a patient with Parkinson's disease

To test the utility of the analytical procedures, a pharmacokinetic study was conducted in a male patient with idiopathic Parkinson's disease. One intravenous (i.v.) catheter was inserted in each forearm of the patient: one was used for blood sampling and the infusion of 500 ml saline per hour (to maintain ongoing urine production). The second catheter was used for a 15 min zero-order infusion of APO (total dose: 30 μg kg$^{-1}$). 5 ml blood samples were obtained and urine was collected over specific time intervals. Blood samples were collected in tubes containing 5 mg of sodium metabisulphite and 15 mg of EDTA. All samples were closed, mixed and put on ice immediately after collection. Plasma was obtained after centrifugation of the blood at 3000 *g* for 5 min. Urine samples were mixed with 1 g sodium metabisulfite and 0.1 g EDTA per litre of urine. Samples were stored immediately at −20°C and at −70°C at the end of the study day.

The plasma concentration–time profile was fitted to a poly-exponential equation for i.v. infusion [19] using the non-linear least squares-regression program Siphar (Simed, Creteil, France).

## 3. Results and discussion

### 3.1. Sample analysis

A good sample clean-up and a high recovery are obtained for both urine and plasma samples through a selective extraction of the catechol functional group, using DPBEA. This method has also been applied successfully by Essink et al. [13] in the assay of racemic APO in plasma. DPBEA specifically binds to the diol group in alkaline medium. This

Table 2
Assay validations of *R*-APO, *S*-APO, in urine at the used concentrations, treated with β-glucuronidase, sulfatase and the control (acetate buffer)

| Compound | Enzyme | Concentration (ng ml$^{-1}$) | Recovery (%) | Found (ng ml$^{-1}$) | C.V. (%) | Accuracy (%) |
|---|---|---|---|---|---|---|
| *Intra-assay* | | | | | | |
| *R*-APO | Acetate | 49.1 | 89±6 | 48.5±0.2 | 0.4 | 98.8 |
| | | 196.4 | | 192.8±1.1 | 0.6 | 98.1 |
| | | 392.9 | 91±5 | | | |
| *R*-APO | β-Glucuronidase | 49.1 | 70±11 | 48.6±0.5 | 1.0 | 99.0 |
| | | 196.4 | | 193.5±2.9 | 1.5 | 98.5 |
| | | 392.9 | 79±3 | | | |
| *R*-APO | Sulfatase | 49.1 | 68±6 | 48.0±1.7 | 3.6 | 97.8 |
| | | 196.4 | | 193.7±5.4 | 2.8 | 98.6 |
| | | 392.9 | 73±1 | | | |
| *S*-APO | Acetate | 3.9 | 95±5 | 3.5±0.1 | 2.8 | 92.0 |
| | | 15.4 | 93±5 | 15.4±0.1 | 0.9 | 100.2 |
| *S*-APO | β-Glucuronidase | 3.9 | 66±3 | 3.8±0.1 | 2.6 | 99.5 |
| | | 15.4 | 80±5 | 15.6±0.2 | 1.2 | 99.7 |
| *S*-APO | Sulfatase | 3.9 | 65±8 | 3.9±0.2 | 3.8 | 102.1 |
| | | 15.4 | 69±5 | 15.2±0.5 | 3.4 | 98.8 |
| *Inter-assay* | | | | | | |
| *R*-APO | Acetate | 24.6 | | 23.6±1.9 | 7.9 | 96.3 |
| | | 147.3 | | 145.1±1.9 | 1.3 | 98.5 |
| *R*-APO | β-Glucuronidase | 49.1 | | 47.9±3.7 | 7.8 | 97.6 |
| | | 196.4 | | 195.4±12.2 | 6.3 | 99.5 |
| *R*-APO | Sulfatase | 49.1 | | 50.5±0.9 | 1.7 | 102.7 |
| | | 196.4 | | 201.2±8.9 | 4.4 | 102.4 |
| *S*-APO | Acetate | 7.7 | | 7.7±0.1 | 1.6 | 99.7 |
| | | 61.6 | | 64.5±0.5 | 0.7 | 104.7 |
| *S*-APO | β-Glucuronidase | 7.7 | | 7.6±0.2 | 2.2 | 102.3 |
| | | 61.6 | | 61.4±1.0 | 1.6 | 101.6 |
| *S*-APO | Sulfatase | 7.7 | | 7.8±0.6 | 7.3 | 107.3 |
| | | 61.6 | | 60.6±1.3 | 2.2 | 102.2 |
| AC | – | 47.5 | | 48.9±2.3 | 4.8 | 102.9 |
| | | 237.5 | | 245.4±5.2 | 2.1 | 103.3 |
| | | 475 | | 471.6±9.0 | 1.9 | 99.3 |
| IAC | – | 50 | | 53.2±3.7 | 7.0 | 106.4 |
| | | 250 | | 252.5±3.8 | 1.5 | 101.0 |
| | | 500 | | 501.4±7.5 | 1.5 | 100.3 |

complex can form an ion pair with TOABr in the organic phase. At low pH (0.05 *M* $H_3PO_4$) APO subsequently dissociates from the borate group of DPBEA. In the original publication on this extraction technique by Smedes et al. [20], it was demonstrated that the extraction efficiency can differ substantially between compounds. Within the context of the present investigation it is therefore important that the

recoveries of the separate enantiomers of APO are similar and furthermore that the extraction with DPBEA can also be applied in the assay of the metabolites AC and IAC. The recoveries in plasma and in urine are given in Tables 1 and 2. Chromatography was performed at low pH to guarantee the stability of the catecholamines. EDTA was added to further increase stability of the compounds and to minimise the background current of the ED detector, caused by solvated divalent cations. Representative chromatograms are given in Figs. 2 and 3.

Fig. 2 shows the chromatograms for the analysis of *R*-APO in plasma and urine of a patient with Parkinson's disease. Fig. 2a shows a blank sample spiked with the internal standard (NAM) (3). Fig. 2b shows a sample obtained after a zero-order infusion of *R*-APO (1). Fig. 2c shows a blank sample spiked with 2.5 ng $ml^{-1}$ *R*-and *S*-APO (1), (2). Figs. 2d and 2e show results that are analogous to those in Figs. 2a and 2b but obtained in the urine of the patient. The plasma peak is shown not to interfere with the *R*-APO peak. Reasonable separation is obtained between the plasma peak and *S*-APO. No *S*-APO was formed in this patient.

As shown in Table 3, the plasma calibration curves for both enantiomers were linear ($r>0.995$). From Table 1 it can be deduced that the intra- and inter-assay variations were <5% for all but one (6.6%) of the concentrations tested ($n=5$). The detection limit was 0.3 ng $ml^{-1}$ for *R*-APO and 0.6 ng $ml^{-1}$ for *S*-APO at a signal-to-noise ratio of 3 and an injected volume of 50 μl.

Fig. 3 shows the chromatograms for the analysis of APO, AC and IAC in plasma and urine of a patient with Parkinson's disease. Fig. 3a shows a blank plasma sample spiked with the internal standard (NAM) (3). Fig. 3b shows a plasma sample obtained after a zero order infusion of *R*-APO (1). Fig. 3c shows a blank sample spiked with 41.4 ng $ml^{-1}$ *R*-APO (1), 40.2 ng $ml^{-1}$ AC (4) and 40.2 ng $ml^{-1}$ IAC (5). Figs. 3d–3f show the same figures as Figs. 3a–3c for the analysis in urine. The spiked concentrations in Fig. 3f are 103.5 ng $ml^{-1}$ *R*-APO (1), 50.1 ng $ml^{-1}$ AC (4) and 50.3 ng $ml^{-1}$ IAC (5).

AC and IAC do not posses the reactive catechol group. Therefore, the sensitivity of detection at 700 mV is low. However, when the voltage is increased to 825 mV, oxidation of the backbone of these

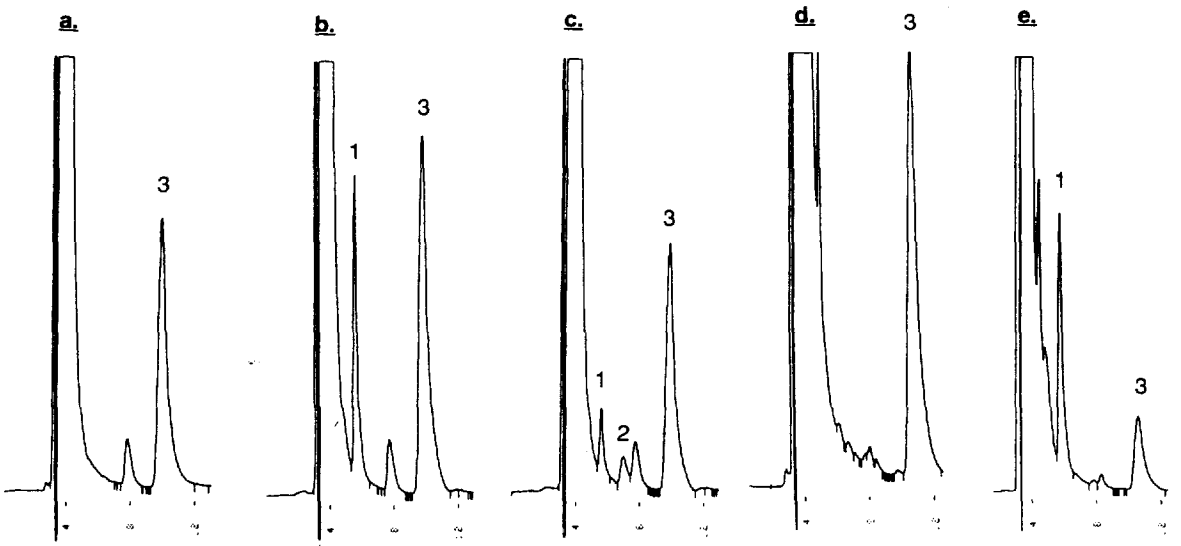


Fig. 2. Chromatograms for the analysis of *R*-APO in plasma and urine of a patient with Parkinson's disease: (a) Blank sample spiked with the internal standard (NAM) (3). (b) Sample obtained after a zero-order infusion of *R*-APO (1). (c) Blank sample spiked with 2.5 ng $ml^{-1}$ *R*- and *S*-APO (1), (2). (d) And (e) analogous results as (a) and (b) but obtained in the urine of the patient.

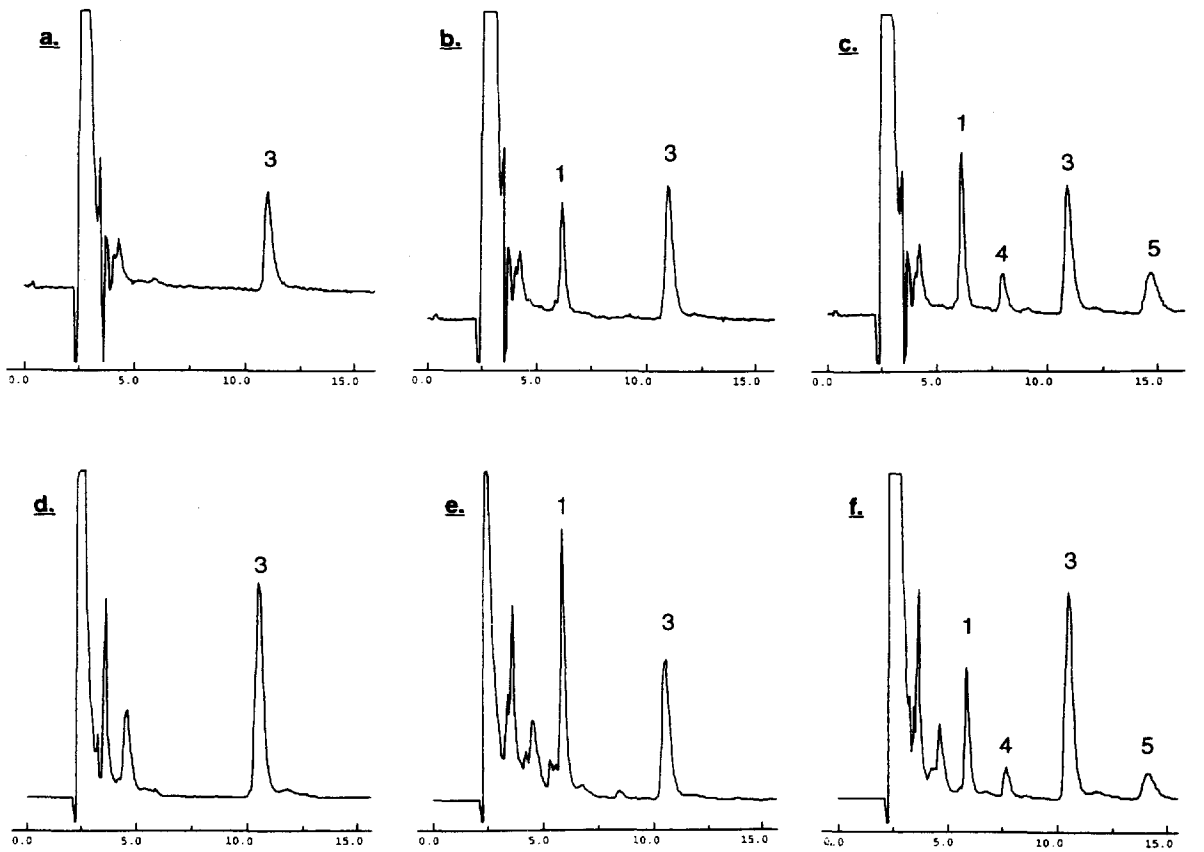


Fig. 3. Chromatograms for the analysis of APO, AC and IAC in plasma and urine of a patient with Parkinson's disease. (a) Blank plasma sample spiked with the internal standard (NAM) (3). (b) Plasma sample obtained after a zero-order infusion of *R*-APO (1). (c) Blank sample spiked with 41.4 ng $ml^{-1}$ *R*-APO (1), 40.2 ng $ml^{-1}$ AC (4) and 40.2 ng $ml^{-1}$ IAC (5). (d) To (f) show the same figures as (a) to (c) for the analysis in urine. Spiked concentrations in (f) are 103.5 ng $ml^{-1}$ *R*-APO (1), 50.1 ng $ml^{-1}$ AC (4) and 50.3 ng $ml^{-1}$ IAC (5).

molecules occurs and a good signal is obtained for both metabolites. Further increment of the voltage did not improve the sensitivity since background noise increases at the same time. The plasma calibration curves for both AC and IAC were linear (Table 3) and the inter-assay variabilities were <5% for all concentrations tested (5–40 ng $ml^{-1}$) ($n$=5) (Table 1). Urine calibration plots were also linear ($r$>0.98). The inter-assay variability was ≤5% except for the 50 ng $ml^{-1}$ IAC sample (7.0%) (Table 2). The detection limit was 2.5 ng $ml^{-1}$ for both metabolites in both plasma and urine. The detection limit of AC and IAC obtained with electrochemical detection was a factor of 2 lower than the detection limit with the most sensitive fluorescence detector that was available (Jasco 821-FP, H.I. Ambacht, Netherlands or RF-530, Shimadzu Corporation, Kyoto, Japan). Due to the increased baseline noise at 825 mV the sensitivity for the detection of APO worsened (detection limit: 1.0 ng $ml^{-1}$). Therefore

Table 3
Linear regression analysis of *R*-APO, *S*-APO, AC and IAC in plasma

| | $a_0$ | $a_1$ | $r^2$ |
|---|---|---|---|
| *R*-APO | −0.0115 | 0.0481 | 0.9993 |
| *S*-APO | −0.0023 | 0.0217 | 0.9996 |
| AC | −0.0064 | 0.0122 | 0.9990 |
| IAC | −0.0030 | 0.0057 | 0.9983 |

$y=a_0+a_1x$, $y$=peak area analyte/peak area internal standard, $a_0$=intercept, $a_1$=slope, $x$=concentration of analyte (ng $ml^{-1}$), $r^2$=correlation coefficient.

the enantioselective assay was used for determination of the pharmacokinetic parameters of APO in a patient with Parkinson's disease.

### 3.2. Enzymatic hydrolysis

In urine the extraction procedure is preceded by an enzyme incubation to dissociate conjugated groups from APO. The procedure is validated by incubating a collected urine sample that is known to contain the conjugated metabolites. The hydrolysis of the metabolites is followed by taking hourly samples during the enzyme incubation. APO is then extracted and analysed by HPLC. Fig. 4a and Fig. 4b show the hourly APO formation during the incubation with β-glucuronidase and sulfatase, respectively. Despite the fact that the hydrolysis reached a maximum after 5 h of incubation, there is still a slight chance of incomplete deconjugation since we did not have the pure conjugated compounds available to check this. Based on these results incubation periods of 5 h were used for all urine samples.

Control experiments are shown in Fig. 5 and Fig. 5a shows the incubation with acetic acid buffer, but without the enzymes to control for non-selective hydrolysis of conjugated APO. Minimal conjugate hydrolysis was observed during 6 h of a urine sample containing conjugated metabolites. Fig. 5b shows the incubation with acetic acid buffer of a blank urine sample spiked with a known concentration (680 ng $ml^{-1}$) of APO: no APO degradation in acetic acid buffer was observed for 6 h.

There seems to be an additional influence of the enzyme pre-treatment on the recovery in urine

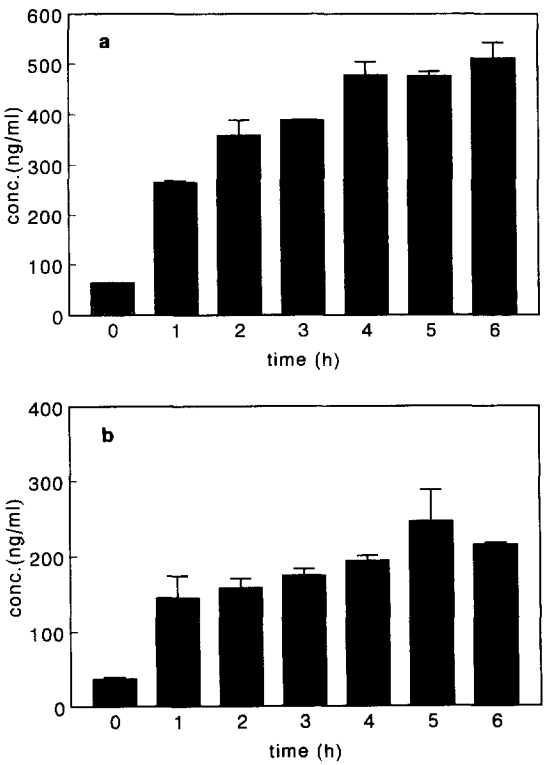


Fig. 4. Optimization of the incubation time: urine of a patient that had received 30 μg $kg^{-1}$ *R*-APO intravenously was incubated at 37°C. 1 ml samples were taken every hour. After extraction they were analysed by HPLC. (a) Incubation with β-glucuronidase in acetic acid buffer. (b) Incubation with sulfatase in acetic acid buffer.

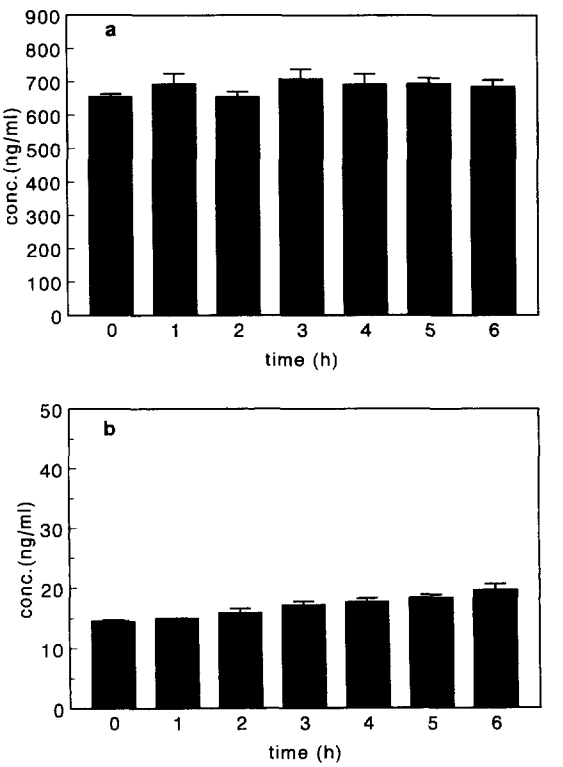


Fig. 5. Control experiments for the enzyme incubations: (a) incubation in acetic acid buffer without the enzymes of a urine sample of a patient that had received 30 μg $kg^{-1}$ *R*-APO intravenously. (b) Incubation in acetic acid buffer of a blank urine sample spiked with 680 ng $ml^{-1}$ APO.

(Table 2). Addition of both enzyme suspensions to the buffer lowers the recovery from about 90% or higher to about 70% for the pre-treatment with enzyme.

### 3.3. Study in a patient with Parkinson's disease

Fig. 6 shows a representative plasma concentration–time profile of *R*-APO in a patient with idiopathic Parkinson's disease, following a 15 min zero-order infusion of 30 μg $kg^{-1}$. The plasma concentrations were determined with the stereoselective HPLC-ED method as described previously. The data were fitted to a bi-exponential equation representing a two-compartment pharmacokinetic model. From the fitted data the pharmacokinetic parameters were obtained: the clearance was 45.7 ml $min^{-1}$ $kg^{-1}$, the volume of distribution at steady state 1.15 l $kg^{-1}$ and the terminal half life was 39.2 min. No *S*-enantiomer, no AC or IAC were detected in the patient's plasma. Fig. 7 shows a histogram of the excreted amounts of *R*-APO (urine concentration times the collected volume) and its sulfated and glucuronidated conjugates for each time interval in a patient with idiopathic Parkinson's disease. Only a small fraction of the dose (0.3%) is excreted as free APO. The main part is excreted as conjugated metabolite: 9.3 and 6.4% of the dose for glucuronidated and sulfated APO, respectively. The concentrations of APO and its metabolites rapidly decline during the first two time intervals, which is in accordance with the short elimination half-life of the drug. However, a significant amount is found at later time intervals even up to 12 h after the infusion was stopped. No *S*-enantiomer and no COMT metabolites were found in the urine of this patient. The percentage of the dose that was recovered either as metabolite or unchanged APO in the urine was only about 16%.

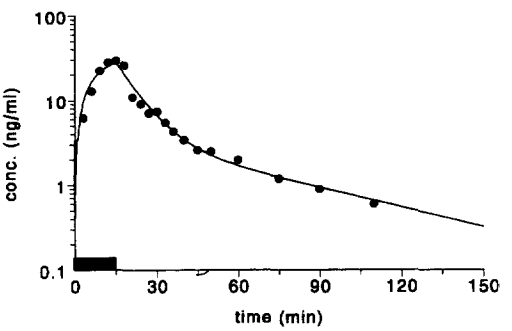


Fig. 6. Plasma concentration–time profile and bi-exponential fit of *R*-APO following a zero-order infusion of 30 μg $kg^{-1}$ in 15 min in a patient with idiopathic Parkinson's disease.

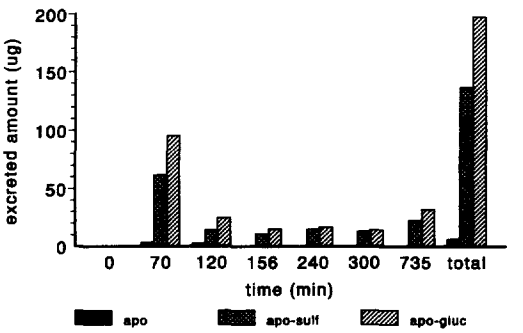


Fig. 7. Histogram of the excreted amounts of *R*-APO, APO-sulfate and APO-glucuronide at consecutive time intervals, following a zero-order infusion of 30 μg $kg^{-1}$ *R*-APO in 15 min in a patient with idiopathic Parkinson's disease.

These findings show that the developed assay methods can be used to characterize the time course of the concentration of *R*-APO in plasma and the urinary excretion of APO and the metabolites AC and IAC following the administration of a therapeutic dose in patients with Parkinson's disease. In this patient, who had a normal renal function, no measurable concentrations of *S*-APO and the metabolites AC and ICA were observed in plasma. In patients with an impaired renal function, however, this may be quite different.

## 4. Conclusions

Rapid, selective and sensitive methods were developed for the determination of the pharmacokinetics and possible conversions of *R*-APO in vivo, including enantiomeric interconversion and metabolite formation. This opens up the possibility to investigate the PK/PD relationship of *R*-APO in patients with Parkinson's disease and to relate this to the possible formation of interacting metabolites.

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