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A new radioimmunoassay for the determination of the angiotensin-converting enzyme inhibitor Perindopril and its active metabolite in plasma and urine: advantages of a lysine derivative as immunogen to improve the assay specificity

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Abstract: A new radioimmunoassay (RIA) was developed for the direct measurement of perindoprilat (PT), the active metabolite (diacid) of Perindopril (P), an angiotensin-converting enzyme (ACE) inhibitor. Antibodies were raised in rabbits against the lysine derivative of PT conjugated to bovine serum albumin. The *p*-hydroxyphenyl derivative of the lysine analogue was used for preparation of the radioligand by iodination (^{125}I). Cross-reactivities for the glucuronide metabolites of P and PT are low (0.25 and 3.5%, respectively). The theoretical limit of detection is 0.2 nM, the sensitivity attainable with random samples is about 0.5 nM. Within- and between-assay variabilities observed were 4.2–6.7 and 2.8–5.9%, respectively (concentration range 2.1–41.7 nM). Serial dilution of plasma and urine samples showed excellent parallelism ($r > 0.95$; $P < 0.001$). Recoveries of PT spiked to urine and plasma samples were 90–120%. The prodrug P can be measured in the same sample (plasma/urine) after chromatographic separation on a Dowex AG 1 × 2 anion-exchange column and quantitative alkaline hydrolysis of the P-containing fraction. It is concluded that the specificity and sensitivity of this assay are amply sufficient for pharmacokinetic studies and in patient monitoring.

Keywords: *Perindopril(at); angiotensin-converting enzyme inhibitor; radioimmunoassay (RIA); antihypertensive drug.*

Introduction

Perindopril (S-9490; tert-butylamine salt of 1-(2*S*)-2-[(1*S*)-1-carbethoxybutylamino]-1-oxopropyl (2*S*, 3a*S*, 7a*S*)-perhydroindole-2-carboxylic acid) is an antihypertensive agent whose de-esterified metabolite is an active inhibitor of angiotensin I-converting enzyme (ACE) [1]. The effectiveness of Perindopril as an antihypertensive drug has been demonstrated in various clinical trials [2, 3]. The prodrug is transformed *in vivo* by hydrolysis into the pharmacologically active diacid form (perindoprilat; S-9780) (see Fig. 1). Two metabolic pathways lead to inactive metabolites: glucuronide compounds and lactam derivatives [4, 5]. *In vivo* studies have shown that glucuronidation of S-9780 depends on a first-pass effect of S-9490 via the S-9490 glucuronide [6, 7]. For determination of both the prodrug and its active metabolite a gas chromatography–mass spectrometry (GC–MS) method has been reported [8] as well as, more recently, radioimmunoassay [9]. The GC–MS method has a rather low sensitivity, with a detection limit of about 3–6 nM (1–2 μg l^{-1}) in plasma, while no information is provided on the behaviour of the inactive metabolites in the assay. For pharmacokinetic studies a practical, more sensitive and specific assay is needed. The radioimmunoassay [9] has an acceptable sensitivity and a limit of detection of 1.6 nM (0.55 μg l^{-1}). However, due to the high cross-reactivity of the antiserum with the glucuronide metabolites, a chromatographic clean-up has to be included prior to the assay. For comparable compounds lysine derivatives have been successfully applied to produce specific antibodies [10, 11].

In this paper we report the development of a new, specific and sensitive radioimmunoassay, using the lysine derivative of perindoprilat as

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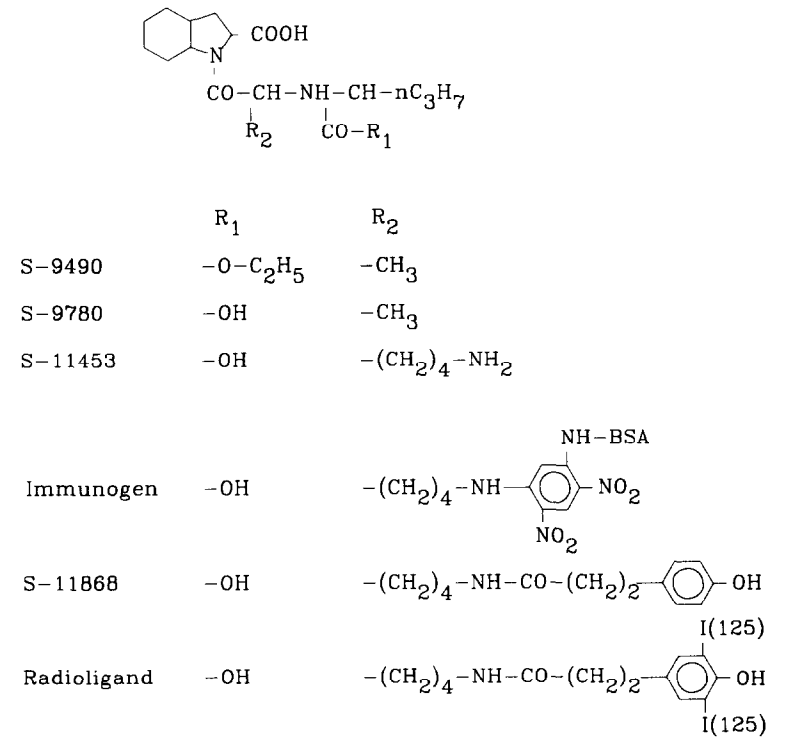


Figure 1
Structures of Perindopril and related compounds used for RIA development.

the immunogen enabling direct quantitation of the diacid (S-9780) and, after alkaline hydrolysis, of the ester S-9490.

Experimental

Materials/reagents

The parent drug (S-9490), the active metabolite and the lysine analogue of S-9780 (S-11453), used for preparation of the immunogen, as well as the compounds tested in the cross-reactivity studies were a gift from Servier Laboratories. All solvents and reagents used were of p.a. quality or the highest purity available.

Preparation of the immunogen

For antibody production the lysine analogue of S-9780 was coupled to bovine serum albumin (BSA) using difluoronitrobenzene (DFNB), by analogy with the procedure described by Eckert *et al.* [10]. In short, purified mono- or diformate of S-11453 (160 mg) was converted into the dihydrochloride by dissolving the compound in 10 ml 0.2 M HCl and subsequent freeze-drying (yield: 171 mg). To a solution of 94 mg (0.2 mmol) S-11453 in 2 ml methanol 129 mg (1 mmol) diisopropylethylamine (1 mmol) dissolved in 1 ml methanol was added. At −30°C 40.8 mg (0.2 mmol) difluoro-di-nitro-benzene (DFNB) in 2 ml methanol was added drop-wise over a period of 15 min. After stirring for 1 h at room temperature the methanol was evaporated. The reaction product S-11453-DFNB was purified over a silica column (Lobar B; Merck, Darmstadt, FRG) with acetone–water (85:15, v/v) as the eluent. After evaporation and subsequent freeze-drying the yield was 69 mg.

Purity of the DFNB conjugate was checked by TLC (Silica plastic plates; eluent: butanol–acetic acid–water, 4:1:1, v/v/v; detection with ninhydrin Reindel–Hoppe reagent) and by field desorption mass spectrometry (*m/z* values: 605, $[M + Na]^+$; 582, $[M]^+$; 564, $[M - H_2O]^+$; 536, $[M - H_2O - CO]^+$).

To 80 mg of BSA dissolved in 6 ml 0.2 M borate buffer pH 10, 50 mg S-11453-DFNB was added in 10 separate portions. After stirring for 16 h at room temperature the reaction mixture was filtered over a 0.45 μm filter and subsequently purified by ultrafiltration using a Pellicon PTGC 02510 filter (Millipore). The filter was washed with 5 ml borate buffer solution and four times with ultrapure water. The purified S-11453-DNB-

BSA immunogen was subsequently lyophilized (yield: 68 mg). Using UV spectroscopy (absorbance at 280 and 332 nm) the molar ratio of hapten molecules coupled to BSA was calculated to be 15:1.

Immunization procedure

Four rabbits were injected subcutaneously at 10 various sites of the skin with 1 mg immunogen suspended in 0.5 ml saline and 0.5 ml Freund's complete adjuvant (Difco). Another group of rabbits were immunized with 0.2 mg immunogen. Booster injections were given every 4 weeks using incomplete adjuvant (1:1) suspension with saline. At regular times blood samples were taken from the marginal ear vein for determination of the antibody titre. Twenty-four weeks after the immunization procedure had started, antiserum titres between 5000 and 120,000 were obtained. A typical curve showing titre development as well as the titre curve as shown in Fig. 2. Bleedings from one of the rabbits with a high antiserum titre and specificity (see below) were pooled and stored in small aliquots at −80°C (AS 4554).

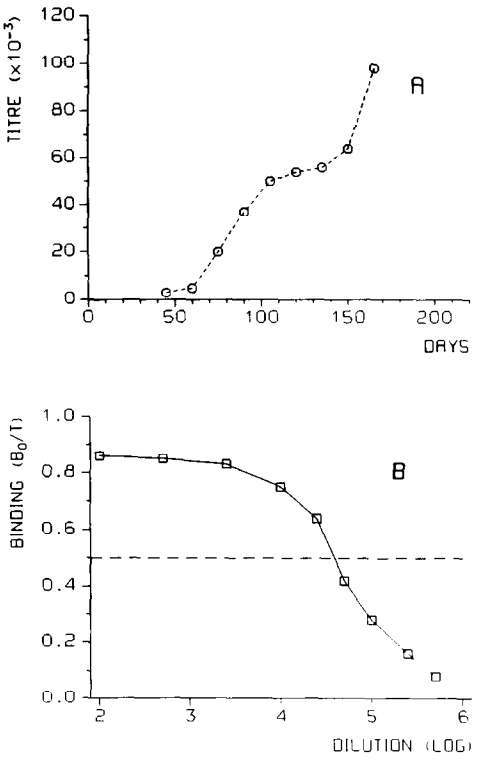


Figure 2
Typical titre development curve after immunization in rabbits (A) and titre dilution curve obtained with antiserum AS 4554 (B).

Preparation of the radioligand

The 3-(*p*-hydroxyphenyl)-1,propionyl derivative of S-11453 (i.e. S-11868) was prepared by reacting 59 mg S-11453 in 10 ml 1,2 dimethoxyethane and 5 ml 0.1 M NaOH with 37 mg 3-(*p*-hydroxyphenyl)-propionic acid-*N*-hydroxylsuccinamide (Bolton Hunter reagent) in 3.7 ml dimethoxyethane. A 50 µl volume (1 µg) of this compound dissolved in 0.05 M phosphate buffer pH 7.2 was iodinated using 10 µl [^{125}I]-NaI (SA 100 mCi ml^{-1}; Amersham, UK; Cat. No. IMS-30) and 10 µl Chloramine T (1 mg ml^{-1}). After 60 s 20 µl metabisulphite (1 mg ml^{-1}) was added to stop the reaction. The iodinated S-11868 was purified using chromatography on Dowex AG 1 × 2 (200–400 mesh; Biorad) as described by Doucet *et al.* [9]. After washing the column (130 × 11 mm; height 100 mm; flow 24 ml h^{-1}) with 40 ml 0.5 M citric acid and 20 ml 1 M citric acid, respectively, the iodinated compound was eluted from the column with 60 ml 1 M citric acid in 2 ml fractions. Aliquots of each fraction were used for counting the radioactivity (1272 CliniGamma counter; Pharmacia/LKB) and determination of the immunoreactivity [see Radioimmunoassay (RIA) procedure]. Peak fractions were collected and stored in 10 ml aliquots at −20°C.

Sample treatment/chromatography

Depending on the substances to be measured different protocols for sample treatment were established. For measurement of S-9780 no sample treatment other than dilution was necessary. In the 'direct' assay of S-9780, 20–100 µl sample (plasma, urine), depending on the expected concentration, was diluted with 0.25 M sodium chloride up to a volume of 1.00 ml and 100 µl of the diluted sample taken in the RIA. Samples with concentrations outside the standard range were appropriately diluted in 0.25 M sodium chloride. For determination of the ester (S-9490) a hydrolysis step was included in the procedure as the antiserum showed no affinity for the ester (see Results). Hydrolysis was performed by alkaline saponification after chromatographic separation of S-9780, glucuronide(s) and S-9490 using a similar Dowex AG 1 × 2 column as for purification of the radioligand (tracer).

To eliminate protein binding of S-9780, plasma samples were heat-treated before being applied onto the column: *ca* 750 µl plasma was diluted with an equal amount of 0.9% sodium

chloride and heated for 10 min at 70°C in a water bath. Urine samples (max. 500 µl) were applied directly onto the column. Columns were eluted with 0.2 M formic acid. S-9490 eluted in the first 8 ml, S-9780 in the next 16 ml of 0.2 M formic acid. Fractions were eluted batchwise using a multichannel peristaltic pump (Technicon) connected to 24 columns in parallel. After each run the columns were regenerated by washing with 1 M formic acid (*ca* 12 ml) followed by 12 ml 0.2 M formic acid. For each new batch of resin the exact flow and elution volumes were established.

An aliquot of each column fraction (both S-9490 and S-9780; maximum 1/10 volume) was transferred to a 16 mm × 100 mm glass tube and freeze-dried (overnight). Residues were dissolved in 250 µl distilled water and 125 µl 1 M NaOH. After hydrolysis (60 min at 37°C) 125 µl 1 M HCl was added to all tubes.

Radioimmunoassay (RIA) procedure

All samples and standards were analysed in 0.25 M NaCl so as to have a similar matrix for both the 'direct' and the hydrolysed samples (see Sample treatment). A chemically pure preparation of S-9780 (M = 340) was used as the standard (working standard range: 0.39–100 nM; absolute amount of standard added in the assay 0.039–10 pmol/100 µl). For incubation, the antiserum and radiotracer were diluted in 0.1 M Tris–HCl buffer pH 8.5, containing 0.1% BSA. The antiserum dilution was such that about 50% of the tracer (total count of *ca* 50,000 cpm/100 µl) added was bound (B_0/T = 0.5). A 100 µl sample or standard was incubated with 100 µl antiserum and 100 µl tracer solution for 48 h at 4°C. After cooling in an ice-water bath 50 µl of cold human citrate (pool) plasma was added as well as 1 ml of a 20% polyethylene glycol (PEG) solution. After mixing in a multivortex mixer all tubes were incubated for 5 min in an ice-water bath. After centrifugation (10 min at 4800g; 4°C) the supernatant was removed by decantation. The radioactivity of the pellets was counted using a gamma scintillation counter. For estimation of non-specific binding (NSB) the highest and lowest standard concentrations as well as two random patient samples were taken in the assay and incubated without antiserum (100 µl 0.1 M Tris–HCl buffer). For calculation of the data a commercially available automated data processing program (Multi-Calc LM; Pharmacia/LKB) was used. With this program concentrations of unknown samples are calculated with reference to the standard curve (four-parameter logistic function) after expressing the corrected counts as percentage of binding relative to the maximum bound counts ($B/B_0 \times 100$; B_0: corrected counts for '0' standard). The program corrects for the sample volume assayed as well as for relevant dilution factors depending on sample treatment.

Results and Discussion

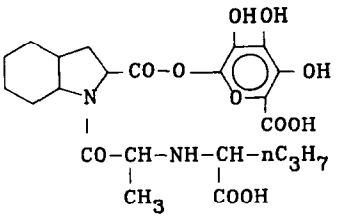
Antiserum titre and specificity

A typical titre development curve is shown in Fig. 2. As mentioned before, a working dilution of 1:40,000 was selected, resulting in about 50% tracer binding (B_0/T = 0.5) (final tube dilution 1:120,000). No significant differences in titre development and specificity were found between antisera obtained from rabbits immunized with a high (1 mg) or low (0.2 mg) dose of immunogen. To test the specificity of the antisera obtained binding studies with S-9780 and S-9490 were performed as well as with other relevant compounds. Chemical structures of the compounds tested are shown in Figs 1 and 3. Cross-reactivities, i.e. the amount needed to give 50% displacement of the tracer from the antibody relative to the standard S-9780, are summarized in Table 1. Relative to the diacid, the ester (S-9490) cross-reacts for only 0.25%, the glucuronide form of S-9780 for 3.5%. Antisera obtained using an immunogen prepared by coupling BSA to the 1-carboxyl group of the perhydroindol structure show an affinity for the glucuronidated compounds equal to, or even higher than the diacid [9]. The lactam forms showed no affinity for the antibody. These (limited) data suggest that the antigenic determinants are mainly located in the aliphatic side-chain of the perhydroindole molecule. The absence of binding of lysine and some related compounds indicated that the lysine bridge structure was not an important antigenic determinant. The few compounds tested with modifications in the perhydroindole ring, such as S-10814-1, showed no, or very low, cross-reactivities (<1%).

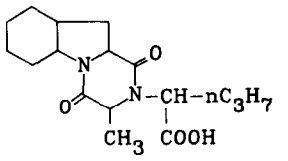
Development and optimization of RIA procedure

Binding studies at various temperatures between 4 and 37°C indicated that maximum

S-9780-glucuronide



BMD 5-1 (Lactam acid Y)



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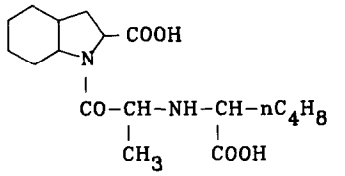

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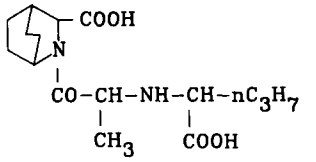

Figure 3
Structures of related compounds tested for cross-reactivity.

Table 1
Cross-reactivity of Perindopril and metabolites to AS 4554 of antiserum relative to perindoprilat (S-9780)

Compound*	Cross-reactivity (%) −	+
S-9490	0.25	100
S-9780-glucuronide	3.5	100
S-9490-glucuronide	0.37	110
BMD 5-1 (lactam)	0.02	0.04
S-11180-1	7.5	7.5
S-111868	88	88
S-11453	51	51
S-10814-1	0.7	0.7
Tryptophan, histidine, proline, lysine, phenylalanine	<0.001	<0.001

*Structures given in Figs 1 and 3.
−, Without hydrolysis; +, after hydrolysis.

binding occurred at the lowest temperature. At 4°C equilibrium conditions are reached after about 48 h. Therefore, this incubation time and temperature were adopted in the assay protocol. Good separation between the bound and free (tracer) fractions was obtained after precipitation with PEG after addition of pooled plasma to supply carrier proteins. A PEG concentration of 20% (w/v), with 50 µl plasma added, and incubation for 5 min in ice water gave the best results. For optimization of the antibody and tracer concentrations an empirical approach was followed by preparing standard curves at various stock tracer and antiserum dilutions. Which reactant concentrations are optimal depend on sensitivity, precision and detection limit, i.e. the analytical goals, wanted. An oral dose of 4–8 mg Per-

indopril results in plasma concentrations between 0.5 and 50 nM for S-9780, and up to 300 nM for S-9490, depending on the time of sample collection [6, 9]. Based on these expected ranges, a working standard range of 0.4–100 nM was adopted. With the reactant concentrations selected an optimum precision was obtained in the lower standard range, i.e. an ED-50 around 5 nM, especially relevant in pharmacokinetic studies. A typical standard curve and precision profile are shown in Fig. 4.

With reaction conditions as described the absolute detection limit was 0.02 pmol per tube, corresponding with a plasma concentration of about 0.2 nM (if 100 µl plasma is taken in the assay). The sensitivity of the assay, defined as the slope of the standard curve (12), was 0.5 nM, calculated from the mean count rate (±2 SD) at a concentration of about 5 nM.

Sample treatment

Due to the low cross-reactivity for the prodrug (S-9490), the glucuronide(s) and lactam(s) metabolites of the pharmacologically active diacid (S-9780) could be measured directly, i.e. without pre-treatment of the plasma or urine sample other than dilution in 0.25 M sodium chloride. The ester could be measured only after quantitative non-enzymatic hydrolysis by alkaline saponification. Enzymatic hydrolysis using a commercial esterase preparation from porcine liver (Sigma Chemical Company, St. Louis, MO, USA; Cat. no. E-3128) resulted in incomplete conversion.

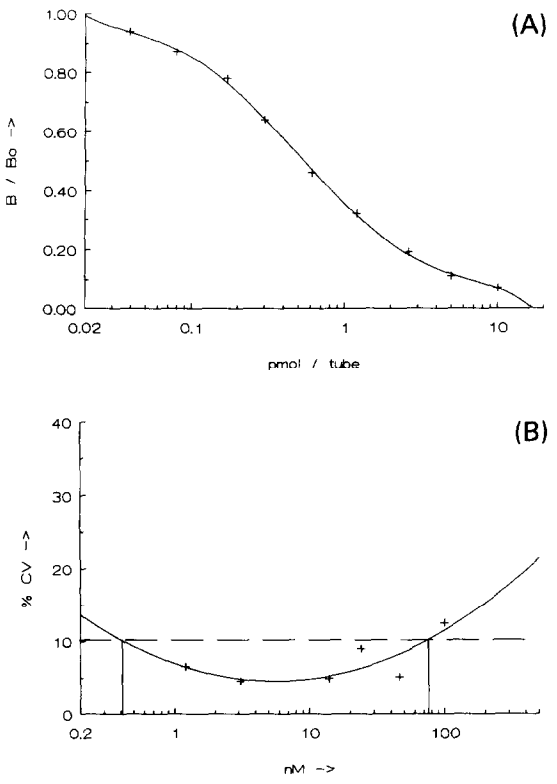

Figure 4
Typical standard curve (A) and precision profile (B) obtained with AS 4554 (dilution factor 1:40,000).

Theoretically, the ester content can be determined by subtracting the amount of S-9780 found before and after alkaline hydrolysis. However, with the direct assay after alkaline saponification inaccurate results (erratically high values and non-parallel dilutions) were obtained for the ester with some of the samples analysed, indicating the presence of matrix effects induced by saponification. Therefore, a chromatographic clean-up was included using ion-exchange chromatography on Dowex AG 1 × 2 to isolate the ester (and acid) fraction before hydrolysis. The glucuronide form of S-9780 can be eluted as a separate (third) fraction with 20 ml 0.5 M formic acid. Recovery studies indicated that a small fraction of S-9780 eluted in the ester fraction. This effect could be eliminated by heat treatment (10 min at 70°C) of the samples before applying them onto the column. Probably, this fraction represents protein-bound (ACE?) acid eluting in the ester fraction. Protein binding apparently had no effect in the direct assay. Preliminary experiments with solid-phase extraction, as an alternative for the chromatography on Dowex, indicated that a quantitative separation of the ester and acid fractions could be obtained using SAX quaternary amine cartridges (Amersham, UK) and batchwise elution with 0.005 and 0.1 M formic acid. However, recoveries from the cartridge varied among the batches of cartridges used.

Validation of assay

The intra- and inter-assay variation was calculated from data obtained with plasma pools spiked with different levels of S-9780, analysed in duplicate in 10 assays performed on 10 different days. The variation coefficients calculated from the data obtained are summarized in Table 2.

Plasma samples (n = 4) spiked with 50 nM S-9780 were assayed with the direct assay as well as after chromatography on Dowex. As shown in Fig. 5, linear dilution curves were obtained. Correlation coefficients were >0.95

Table 2
Assay precision

Pool	*N*	Mean (nM)	RSD-*O** (%)	RSD-*W*† (%)	RSD-*B*‡ (%)
P1	10	2.1	4.6	4.2	2.8
P2	10	10.3	6.3	6.0	2.4
P3	10	41.7	7.9	6.7	5.9

RSD = SD/mean value × 100; $(SD\text{-}O)^2 = (SD\text{-}W)^2 + (SD\text{-}B/2)^2$
*'Overall' RSD (i.e. variability of random sample in a random series, measured in duplicate).
†Within-assay RSD.
‡Between-assay RSD.

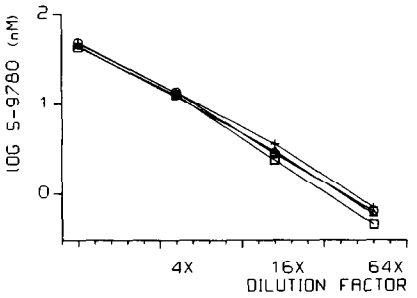

Figure 5
Dilution curves for perindoprilat in plasma after serial plasma dilution.

($P < 0.001$). Parallelism was also excellent for (spiked) urine samples (data not shown).

Recovery of S-9780 in the 'direct' assay (without column chromatography and/or saponification) was assessed by analysing different plasma and urine samples spiked with 0–50 nM S-9780 and/or 0–500 nM S-9490. Mean recovery of S-9780 varied between 95 and 119%. An excellent correlation was found between values measured in the direct assay as compared to those measured after chromatography and saponification ($y = 0.9037x + 2.9$; $r = 0.995$, $P < 0.001$). In some of the samples spiked with only S-9490, concentrations obtained with the direct assay were slightly higher than could be explained on the basis of cross-reactivity. Probably, some spontaneous hydrolysis had occurred during storage or sample treatment. Recovery of S-9490 in plasma and urine samples after chromatography and saponification varied between 85 and 127%.

As samples to be analysed with this assay usually will contain both compounds in different and varying ratios, accuracy studies were performed using eight spiked plasma and six spiked urine sample pools. All 14 samples were analysed in six-fold, in a random order with the sample content being unknown to the technician performing the assay. RIA was performed after chromatographic separation of the acid and ester fractions followed by saponification as described in Experimental.

Table 3
Recoveries of S-9490 and S-9780 spiked to the same plasma (P) or urine (U) sample*

	Amount added (nM)		Amount found [nM; $X \pm$ SD (% rec.)]			
	S-9490	S-9780	S-9490		S-9780	
P-1	0	0	0–0.5	(–)	0–0.5	(–)
P-2	0	2.9	0–1.2	(–)	2.8 ± 0.3	(96)
P-3	1.3	113	3.3 ± 0.6	(253)	135 ± 10.9	(119)
P-4	13.3	0	16.0 ± 2.9	(120)	0–0.6	(–)
P-5	39.7	43	38.9 ± 6.8	(98)	49 ± 10.9	(114)
P-6	107	29	98.5 ± 0.5	(92)	35 ± 4.7	(121)
P-7	267	1.4	230 ± 18.8	(86)	2.9 ± 0.6	(207)
P-8	536	14.4	453 ± 34.5	(85)	18.4 ± 1.5	(128)
U-1	27	29.5	20.1 ± 1.4	(74)	28.9 ± 3.5	(98)
U-2	2730	148	2255 ± 152	(83)	146 ± 7.8	(99)
U-3	136	1480	113 ± 12	(83)	1401 ± 81	(95)
U-4	547	593	571 ± 22	(104)	525 ± 38	(88)
U-5	0	2369		(–)	2233 ± 115	(94)
U-6	4096	0	3784 ± 130	(93)	10.9 ± 4.6	(–)

*Spiked samples were prepared in six-fold; each sample analysed separately.

From Table 3 it can be concluded that mean recoveries and reproducibilities obtained were satisfactory, correlation coefficients between the amounts 'added' and the amounts 'found' being >0.97. Recoveries of both the ester and the acid in the spiked plasma samples varied between 80 and 130%. However, in some of the plasma samples containing widely diverging amounts of both compounds apparent recoveries were too high (e.g. samples P-3 and P-7), probably due to cross-contamination of both fractions during chromatography. This seems to appear only by chance as recoveries obtained with plasma pool P-7, but also for the urine samples, were within the acceptable range. On the basis of these results the limit of quantitation, i.e. the minimum difference from zero that can be detected in a 'random' sample, was established at 1.5 nM.

In conclusion, the improved RIA described enables the direct quantification of the pharmacologically active compound (S-9780) by means of a specific antibody raised again the lysine derivative of perindopril. The prodrug (S-9490) can only be quantitated after chromatographic separation and saponification. The theoretical limit of detection is 0.2 nM, the sensitivity attainable with random samples is about 0.5–1.5 nM, amply sufficient for analysis of biological samples obtained in pharmacokinetic studies and in patient monitoring.

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References

[1] M. Laubie, M. Vincent and H. Schmitt, *J. Cardiovasc. Pharmacol.* **6**, 1076–1082 (1984).
[2] K.R. Lees and J.L. Reid, *Eur. J. Clin. Pharmacol.* **31**, 519–524 (1987).
[3] D. Herpin, J.P. Santoni, F. Pouyollon and J. Demange, *Curr. Therap. Res.* **45**, 576–584 (1989).
[4] D.H. Drummer, S. Kourtis and D. Iakovidis, *Arzneim. Forsch.* **5**, 647–650 (1988).
[5] L. Grislain, M.T. Mocquard, J.F. Dabe, M. Bertrand, W. Luyten, B. Marchant, G. Resplandy and M. Devissaguet, *Xenobiotica* **20**, 787–800 (1990).
[6] J.P. Desvissaguet, N. Ammoury, M. Devissaguet and L. Perret, *Fundam. Clin. Pharmacol.* **4**, 175–189 (1990).
[7] B. Lecocq, C. Funck-Brentano, V. Lecocq, A. Ferry, M.E. Gardin, M. Devissaguet and P. Jaillon, *Clin. Pharmacol. Ther.* **47**, 397–402 (1990).
[8] C. Tsaconas, M. Devissaguet and P. Padieu, *J. Chromatogr.* **488**, 249–265 (1989).
[9] L. Doucet, B. De Veyrac, M. Delaage, H. Cailla, C. Bernheim and M. Devissaguet, *J. Pharm. Sci.* **79**, 741–745 (1990).
[10] H.G. Eckert, G. Münscher, R. Oekonomopoulos, H. Strecker, H. Urbach and H. Wissmann, *Arzneim. Forsch.* **35**, 1251–1256 (1985).
[11] P.J. Worland and B. Jarrott, *Clin. Chem.* **35**, 509 (1989).

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