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Determination of the monoamine oxidase B inhibitor Ro 19-6327 in plasma by high-performance liquid chromatography using precolumn derivatization with fluorescamine and fluorescence detection

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SUMMARY

A specific high-performance liquid chromatographic (HPLC) method using precolumn derivatization and fluorescence detection was developed for the determination of the monoamine oxidase B inhibitor Ro 19-6327 in human plasma. After extraction of the basified plasma with *tert.*-butyl methyl ether–1-butanol (8:2, v/v) and back-extraction into dilute phosphoric acid, the solution was neutralized with phosphate buffer and the drug derivatized with fluorescamine. The derivative was chromatographed on a reversed-phase C_8 column, using phosphate buffer–acetonitrile (68:32, v/v) as mobile phase, and fluorescence detection (excitation 370 nm, emission 485 nm). The limit of quantification was 1 ng/ml using 1 ml of plasma. The recovery was 79% in the range 5–200 ng/ml and the inter-assay precision was 3.1–7.9% in the range 2–500 ng/ml. The compound proved to be stable in human plasma. Moderate instability was found in rat plasma and, surprisingly, severe instability in dog plasma. Measures for handling unstable dog plasma samples are described. An HPLC method with UV detection was used for the analysis of dog and rat plasma samples, which is also described briefly. The fluorescence method, which was five times more sensitive than the UV method, was successfully applied to a human tolerance study.

INTRODUCTION

Ro 19-6327, a pyridine carboxamide derivative with a primary amino group in the side-chain (**1**, Fig. 1), is the most potent and selective inhibitor of monoamine oxidase type B (MAO B) reported so far[1], and is very well tolerated at doses that inhibit MAO B 24 h per day[2]. The drug, which has no structural analogy to known MAO B inhibitors, is currently under clinical development as a therapeutic agent in Parkinson's disease. Because of its polar structure (*n*-octanol–water partition coefficient *ca.* 0.1, pK_a of the amino group 8.9), **1** is difficult to extract from plasma with

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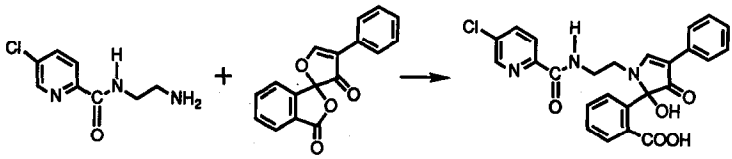

1: Ro 19-6327 2: Fluorescamine 3

Fig. 1. Structures of (1) Ro 19-6327, (2) fluorescamine and (3) their reaction product.

organic solvents, and also shows the typical chromatographic problems with amines in high-performance liquid chromatography (HPLC), as reported, *e.g.*, for moclobemide[3].

Initially, a reversed-phase ion-pair HPLC method with UV detection was developed for the determination of **1** in human, dog and rat plasma[4]. This method, with a limit of quantification of 5 ng/ml, proved to be sensitive enough for use in animal pharmacokinetic and toxicokinetic studies. However, a more sensitive method was needed for pharmacokinetic studies in man after the administration of therapeutic doses. Therefore, precolumn derivatization of the primary amino group was tried.

This paper describes this method, which consists of extraction of the basified plasma with *tert.*-butyl methyl ether–1-butanol (8:2, v/v) and back-extraction into dilute phosphoric acid followed, after neutralization with phosphate buffer, by precolumn derivatization with fluorescamine, HPLC separation and fluorescence detection.

EXPERIMENTAL

Materials and reagents

tert.-Butyl methyl ether (distilled in glass, Burdick and Jackson), orthophosphoric acid (*ca.* 85%) and sodium decanesulphonate monohydrate (both puriss. p.a. grade) were obtained from Fluka (Buchs, Switzerland), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). 1-Butanol (Uvasol), 1-hexanol (for synthesis), disodium hydrogenphosphate dihydrate and sodium dihydrogenphosphate monohydrate (both p.a. analytical-reagent grade) and sodium hydroxide (Titrisol) were purchased from E. Merck (Darmstadt, F.R.G.). Water was distilled twice from an all-glass apparatus. Helium was obtained from PanGas (Lucerne, Switzerland). Compound **1** (as its hydrochloride) and fluorescamine (Fluram) were provided by Hoffmann-La Roche (Basle, Switzerland). Disposable 14-ml polypropylene tubes (100 × 17 mm I.D.; Huber, Reinach BL, Switzerland) were first washed with 5 ml of *tert.*-butyl methyl ether–1-butanol (8:2, v/v) for 20 min on a rotating shaker (Heidolph, Keilheim, F.R.G.), and 5-ml polypropylene tubes (75 × 12 mm I.D.; Semadeni, Ostermundingen, Switzerland) were rinsed beforehand with 1 ml of acetonitrile for 5 s on a Vortex mixer.

Plasma standards were prepared using fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

Solutions and standards

Dilute phosphoric acid was prepared by diluting 1 ml of 85% orthophosphoric acid to 500 ml with water, and neutralization buffer by making up 30 g of disodium hydrogenphosphate and 24 ml of 1 *M* sodium hydroxide to 1000 ml with doubly distilled water. A 0.5-mg amount of fluorescamine was dissolved in 10 ml of acetonitrile (this solution was freshly prepared every day).

A stock solution of **1** was prepared by dissolving 5.92 mg of the hydrochloride in 100 ml of doubly distilled water (= 0.05 mg/ml free base). Appropriate amounts of the stock solution were diluted with water to give solutions in the range 50–0.1 μg/ml. These solutions were used as plasma standards by diluting 0.1 ml with blank plasma to 10 ml. For greater accuracy, volumes greater than 4 ml were weighed, taking into account the density of plasma (d_{20} = 1.027 g/cm^3). The plasma standards, containing 500, 200, 50, 20, 5, 2 and 1 ng/ml, were divided into aliquots of 1.3 ml and stored at −20°C (for no more than 2 months). The aqueous solutions were freshly prepared prior to use.

Sample preparation and derivatization

A 1-ml volume of plasma and 50 μl of sodium hydroxide (0.4 *M*) were vortex mixed in a polypropylene tube (14 ml) and 10 ml of *tert.*-butyl methyl ether–1-butanol (8:2, v/v) were added. After extraction for 20 min at 30 rpm on a rotating shaker and centrifugation (5 min, 2000 *g*), 9 ml of the organic phase were transferred to a polypropylene tube (14 ml) containing 0.5 ml of dilute phosphoric acid. After back-extraction (20 min) and centrifugation (5 min), the acidic phase was transferred quantitatively, but without organic phase, to a polypropylene tube (5 ml). After addition of 0.5 ml of neutralization buffer (final pH 7.5), 0.5 ml of fluorescamine solution were slowly added under constant vortex mixing. Subsequently, the acetonitrile portion was evaporated *in vacuo* (Speed Vac Concentrator SVC 200H; Savant Instruments, Farmingdale, NY, U.S.A.; evaporation time exactly 10 min, pre-heating time 10 min). All samples produced in one day (calibration standards, quality control samples and unknowns) must undergo this evaporation step simultaneously. After vortex mixing to dissolve ice and salt crystals, which were sometimes formed, the solution was transferred to the autosampler vial, and 100 μl were injected.

Chromatographic system

A modular HPLC system was used, consisting of a Model 420 HPLC pump and Model 460 autosampler (run time 9 min) (both from Kontron, Zurich, Switzerland), a Model F 1000 fluorescence detector (Merck) (excitation wavelength 370 nm, emission 485 nm), a Model SP 4200 integrator with Kerr minifile 4100D (Spectra-Physics, San Jose, CA, U.S.A.) (sensitivity 8 mV, chart speed 0.5 cm/min) working with a modified version of a BASIC program described earlier for the SP 4100 integrator.

A LiChroCART Superspher 60 RP-8e cartridge (125 × 4 mm I.D.) (Merck) was used as an analytical column. The mobile phase was water–acetonitrile (68:32, v/v), containing 100 m*M* sodium dihydrogenphosphate and 5 m*M* disodium hydrogenphosphate (pH 5.9 ± 0.1, not adjusted), and was degassed with helium prior to use. The flow-rate was 1.0 ml/min.

HPLC method with UV detection

This previously developed method consisted of addition of 50 μl of sodium hydroxide (4 *M*) to 1 ml of plasma. After vortex mixing, the mixture was applied to an Extrelut 1 column (Merck) and, after 15 min, eluted twice with 5 ml of *tert.*-butyl methyl ether–1-hexanol (1:1, v/v). The eluate was collected in a centrifuge tube containing 0.4 ml of dilute phosphoric acid. After extraction (15 min) and centrifugation (5 min, 2000 *g*), the acidic phase was directly injected (40 or 100 μl). The analytical column (125 × 4 mm I.D.) (Merck) contained Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, F.R.G.), and the mobile phase was water–acetonitrile (705:295, v/v), containing 3 g/l sodium dihydrogenphosphate and 2 g/l sodium decylsulphonate. The flow-rate was 1.0 ml/min, and the detection wavelength was 233 nm, using a Spectroflow 773 UV detector (Kratos, Ramsey, NJ, U.S.A.).

Calibration and calculation

Together with the unknown and quality control samples, seven plasma standards were processed as described above. The calibration graph ($y = a + bx$) was obtained by a weighted linear least-squares regression (weighting factor $1/y^2$) of the peak height y *versus* the concentration x. The calibration graph was used to interpolate unknown concentrations in the biological samples from measured peak heights.

RESULTS AND DISCUSSION

Extraction

Compound **1**, a primary amine with a polar structure, is difficult to extract from plasma with common solvents. *n*-Butyl chloride, chloroform, diethyl ether or ethyl or butyl acetate gave low recoveries even after the addition of alcohol. Many interferences, due to various biogenic amines which occur in plasma, prevented a specific determination using these solvents. The use of liquid–liquid extraction in combination with solid-phase extraction with a strong cation exchanger gave no improvement. Therefore, back-extraction into dilute phosphoric acid was considered for the HPLC method with UV detection, allowing the possibility of direct injection of the extract onto the column. In this way, time-consuming evaporation of the extraction solvent, which would be difficult with polar solvents and could lead to loss of the drug in the case of amines, could be avoided. Several extraction solvent mixtures were tested. Finally, *tert.*-butyl methyl ether–1-hexanol (1:1, v/v), in combination with Extrelut columns, and dilute phosphoric acid for back-extraction were selected.

However, when these extraction conditions were tried for the method using precolumn derivatization, many interfering substances prevented the determination of **1**. Therefore, cleaner reagents and solvents had to be used in order to avoid impurities from these sources. 1-Butanol (Uvasol quality) gave rise to far less interference than 1-hexanol (for synthesis grade). The latter was preferred during the development of the UV method because it was much less soluble in the phosphoric acid used for back-extraction and gave better chromatograms. The injection of traces of butanol was avoided by using a vacuum evaporation step in the precolumn derivatization method.

Another source of problems was the 4 *M* sodium hydroxide used for adjusting

the pH before extraction, which sometimes resulted in a large tailing front peak in the chromatograms; 0.4 *M* sodium hydroxide gave the same recovery without this problem. Instead of glass tubes, which lead to memory effects, disposable polypropylene tubes were used for the extraction. However, these tubes had to be prewashed with the extraction solvent to prevent interferences.

Derivatization

In a first attempt, 9-fluorenylmethyl chloroformate (FMOC-Cl) was tried as a derivatization reagent. FMOC-Cl reacts with both primary and secondary amino acids or amines to form highly fluorescent and stable derivatives[6]. However, this approach did not provide sufficient sensitivity. Using an excitation wavelength of 220 nm (emission 315 nm), relatively small peaks were obtained, and at 260 nm the high background noise prevented sensitive detection. The frequently used reagent *o*-phthalaldehyde (OPA)–mercaptoethanol[7,8] was also investigated. Surprisingly, the OPA derivative of **1** was about 1000 times less fluorescent than that of glycine or octylamine. The reason for this phenomenon is not known and was not investigated further.

Fluorescamine (Fluram), which is non-fluorescent, reacts rapidly with primary amines to give highly fluorescent pyrrolinone derivatives[9]. Excess of the reagent is rapidly hydrolysed to non-fluorescent products. Precolumn derivatization of **1** with fluorescamine resulted in a sensitive method. The reaction scheme is shown in Fig. 1. However, the exact conditions of the reaction and the detection had to be optimized. First, the pH of the substance solution, which is normally in the range 8–9.5[9], had to be established; pH 7.5 was found to be most suitable for **1**. Other parameters that had to be optimized were the concentration and the solvent of the reagent solution. An excess of the reagent was required owing to hydrolysis in aqueous solution. Fig. 2 shows the dependence of the formation of the derivative **3** on the pH of the reaction solution and the fluorescamine concentration. At concentrations significantly above 0.3 mg/ml, De Bernardo *et al.*[9] observed fluorescence quenching. This effect was only observed at pH 8 using a 0.8 mg/ml concentration. On the other hand, a fluorescamine concentration of 0.8 mg/ml or a pH higher than 7.5 resulted in an increase in plasma interferences. Therefore, a concentration of 0.05 mg/ml at pH 7.5 was found to be optimum. Fluorescamine has to be added in a water-miscible, non-hydroxylic solvent which should contain no fluorogenic impurities. Acetonitrile, acetone, tetrahydrofuran and dioxane were described by De Bernardo *et al.*[9]. Acetonitrile was used in this study, although acetone also gave good results. The instability of the derivative was not investigated in detail, because less than 5% degradation occurred over 12 h at 22°C, or over 24 h at 4°C, and, therefore, was considered unproblematic for overnight automatic injections.

After derivatization, the solution was concentrated in a vacuum concentrator by removing the acetonitrile and a minor part of the aqueous portion. An injection solution without acetonitrile resulted in on-column concentration and, therefore, sharper peaks. However, as no internal standard was used, all samples from one working day had to be treated in one batch, and the evaporation time had to be controlled closely. Interestingly, this procedure appeared to have no negative influence on the precision of the method, provided that the inner diameter of the evaporation tubes was larger than 11 mm.

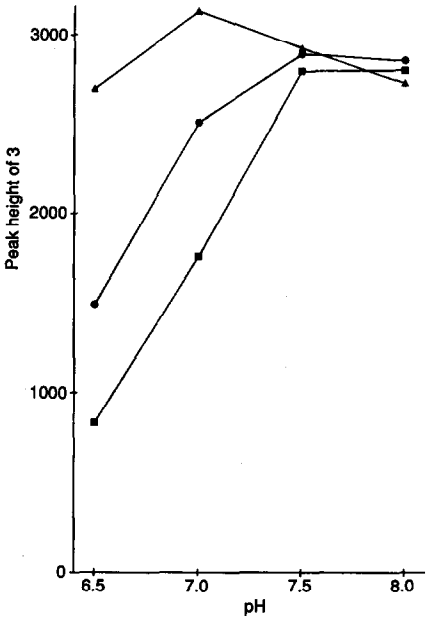

Fig. 2. Dependence of the formation of derivative **3** on the pH of the reaction solution and the fluorescamine concentration (■ = 0.05; ● = 0.2; ▲ = 0.8 mg/ml).

Chromatographic system

The fluorescamine derivative of **1** was strongly retained. Therefore, an ion-pairing agent, as used in the method with UV detection, was no longer necessary. At first, Nucleosil C_{18}, which had proved suitable for **1** in the HPLC method with UV detection, was tried as the stationary phase. However, as with Nucleosil C_8 and Spherisorb C_6, the separation of the fluorescamine derivative **3** from interferences was inadequate. Finally, Superspher C_8 proved to be the best material under these conditions. The composition of the mobile phase also had to be optimized, not only to obtain a good separation, but also to optimize the fluorescence intensity. Concerning the latter, De Bernardo *et al.*[9] mentioned pH, organic modifier content and concentration of reagent and analyte as important factors.

Under the conditions used, the retention time was about 3.7 min. Chromatograms of a blank and a spiked plasma sample are shown in Fig. 3. Investigations with an internal standard (bromine instead of chlorine in the pyridine ring) led to a decrease in the precision of the assay; therefore, the external standard method was preferred[10].

Selectivity

As already mentioned, selectivity was the main problem after extraction and derivatization of plasma samples, because of the many biogenic amines with structures similar to **1**. After derivatization with fluorescamine, separation became even more difficult. However, more than 30 human blank plasma samples have been analysed and more than 90% showed either no interference or a peak corresponding to less than 0.25 ng/ml of **1**. One of these blank plasma samples is shown in Fig. 3.

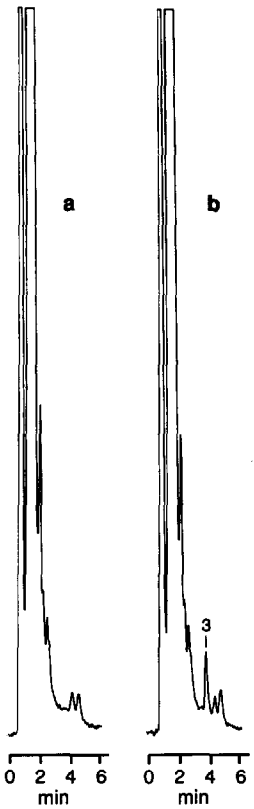

Fig. 3. Chromatograms of human plasma samples. (a) Blank plasma sample; (b) blank plasma sample spiked with 5 ng/ml of **1**. **3** is the fluorescamine derivative of **1**.

Levodopa, benserazide and other decarboxylase inhibitors, which could appear in the plasma of patients with Parkinson's disease, did not interfere with **1**. Dopamine at endogenous levels was well separated. After the administration of levodopa, higher levels of dopamine are expected because this substance is a metabolite of levodopa. Up to a few hundred ng/ml, no interference was observed. However, very high plasma levels of dopamine (μg/ml) would disturb the baseline during chromatography and prevent the determination of **1**.

For dog and rat plasma, the endogenous component eluting directly after **3** caused greater interference than for human plasma. Even though this interference affected quantification adversely only at concentrations below 5 ng/ml, the use of the HPLC method with UV detection for animal plasma determinations is recommended.

Care must be taken to follow the experimental conditions described exactly. Interfering peaks could also be generated by the use of impure solvents, reagents, tubes or by rubber or plastic stoppers.

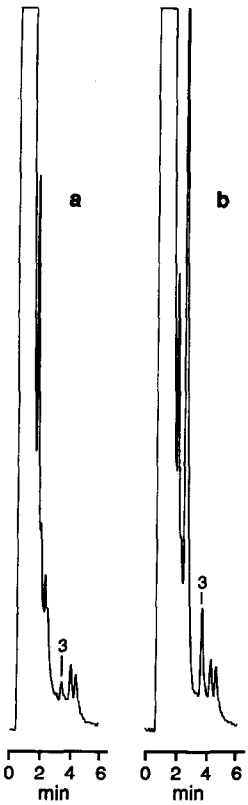

Fig. 4. (a) Chromatogram showing the quantification limit: human blank plasma sample, spiked with 1 ng/ml of **1**. (b) Chromatogram of a volunteer's plasma sample taken 4 h after a single dose of 10 mg of **1**. Measured concentration: 4.71 ng/ml.

Limit of quantification

The limit of quantification of **1** in plasma was 1 ng/ml using a 1-ml sample. A chromatogram of a spiked plasma sample at this concentration is shown in Fig. 4a. The inter-assay (n = 8) relative standard deviation (R.S.D.) at this concentration was 17.7% (see Table II). The detection limit, defined by a signal-to-noise ratio of 3:1, was *ca.* 0.5 ng/ml. However, at this concentration the calibration intercept and the precision of the method were found to be unacceptable.

Linearity

The correlation between peak height and concentration of **1** was linear over at least the range 0.5–500 ng/ml. The coefficients of determination (r^2) were better than 0.99, using the weighting factor $1/y^2$.

Recovery

The recovery (extraction yield) of **1** from human plasma was established as follows. Three concentrations of spiked plasma samples were analysed as described

TABLE I

RECOVERY OF 1 FROM HUMAN PLASMA (n = 5)

Concentration (ng/ml)	*Recovery (%)*	*R.S.D. (%)*
5	79.9	2.3
20	78.6	4.7
200	78.0	2.7

above, each in quintuplicate. A second series of samples was analysed simultaneously by extraction and back-extraction of blank plasma, and then adding **1** to the final extract. Recovery was calculated by comparing the peak heights for back-extracted spiked samples with those for the samples to which **1** had been added after back-extraction. The results are presented in Table I.

Reproducibility

The precision (defined as R.S.D. of replicate analyses) and the accuracy (defined as the difference between found and added concentrations) of the method were evaluated in inter-assay studies. In a first study, using calibration standards measured against an independent calibration set, one specimen was analysed on eight days over a period of 3 weeks. The results are given in Table II. Using quality control samples over 2–3 months, similar results were obtained, as shown in Table III. A precision of 3–8% in the concentration range 2–500 ng/ml is acceptable considering the rather extensive extraction and derivatization procedure.

Stability

The stability of **1** in human plasma was investigated by adding the drug to blank plasma at three different concentrations and storing it for 3 and 9 months at −20°C. The results of these stability tests, which were carried out according to our established method[11], are presented in Table IV. The data indicate that **1** is stable in human plasma for 9 months at −20°C, with the exception of the 500 ng/ml concentration, where a decrease of 10.2% after storage for 9 months was found. This corre-

TABLE II

INTER-ASSAY REPRODUCIBILITY FROM PLASMA STANDARDS (n = 8)

Concentration (ng/ml)		*R.S.D. (%)*	*Difference between found and added (%)*
Added	*Found*		
1	1.00	17.7	0.0
2	2.06	7.9	+3.0
5	5.00	7.7	0.0
20	19.6	4.7	−2.0
50	48.5	3.1	−3.0
200	199	3.5	−0.5
500	496	3.1	−0.8

TABLE III

INTER-ASSAY REPRODUCIBILITY FROM QUALITY CONTROL SAMPLES

Replicates (n)	*Concentration (ng/ml)*		*R.S.D. (%)*	*Difference between found and added (%)*
	Added	*Found*		
18	4.14	4.15	7.1	+0.2
21	4.16	4.06	7.2	−2.4
21	42.4	41.7	4.4	−1.7
22	42.7	42.1	6.4	−1.4
20	422	425	5.0	+0.7
21	423	418	5.7	−1.2

sponds exactly to the limit of the relevant instability (−10%)[11]. No instability could be observed when the drug was stored in human plasma at 22°C for 24 h (data not shown).

Surprisingly, severe instability was observed with dog plasma, amounting to −99.8% at 22°C after 24 h, and about −15% per month at −20°C. This decrease, which was not found with dog urine, was confirmed in metabolic studies[12], and could be explained by the presence of a plasma amine oxidase, which only exists in dog plasma[13]. The stability of **1** in rat plasma lay between those in human and dog plasma. In rat plasma, a decrease of nearly 10% after storage at 22°C for 25 h was found, whereas at −20°C no instability was observed after 5 weeks. Dog plasma could be stabilized by immediate addition of 1 *M* sodium hydroxide solution (50 µl/ml) to a plasma sample. In this way, the drug remained stable for 2 months at −20°C.

Application of the method to biological samples

The method was successfully applied to the analysis of more than 360 plasma samples from a tolerance study performed on volunteers. Fig. 4b shows a typical chromatogram, and Fig. 5 shows the good correlation between the concentrations of **1** from this study, as determined by the HPLC method with UV detection, and the precolumn derivatization method.

TABLE IV

STABILITY OF 1 IN HUMAN PLASMA (n = 5)

Storage conditions	*Concentration (ng/ml)*	*Change in concentration after storage (%)*	*90% confidence interval (%)*
3 months at −20°C	5	−4.0	−9.5 to +1.7
	50	−3.4	−7.7 to +1.1
	500	−0.9	−5.2 to +3.6
9 months at −20°C	5	−7.5	−9.9 to −5.0
	50	−6.1	−7.9 to −4.2
	500	−10.2	−12.1 to −8.2

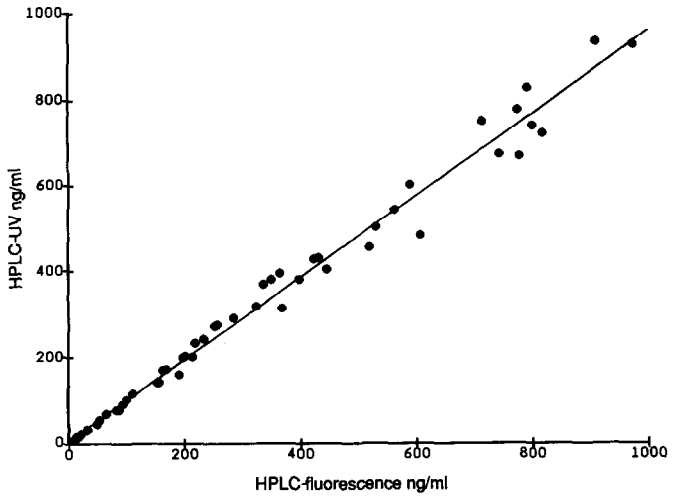

Fig. 5. Correlation between plasma concentrations of **1** as determined by HPLC with UV detection and HPLC with precolumn derivatization and fluorescence detection ($n = 53$, $r^2 = 0.9860$, $y = 6.9731 + 0.9510x$).

CONCLUSION

A specific HPLC method for the determination of **1** in human plasma was developed using precolumn derivatization with fluorescamine and fluorescence detection. The limit of quantification (1 ng/ml) was improved by a factor of five compared with the HPLC method with UV detection[4]. This should be sufficient to study the pharmacokinetic profile after the administration of therapeutic doses, and is much better than other methods using precolumn derivatization with fluorescamine for the determination of drugs in plasma, for which a quantification limit 10 ng/ml[14,15] or greater[16] has been reported. Whereas **1** proved to be stable in human plasma, severe instability was observed in dog plasma. Measures to overcome this instability have been presented.

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