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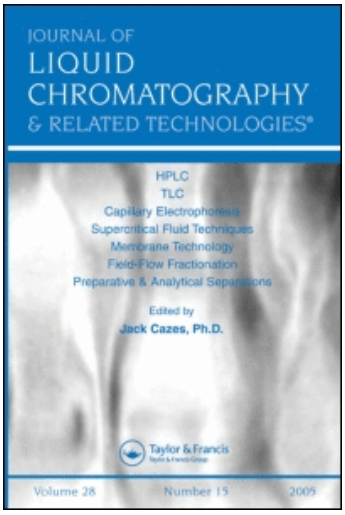

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DETERMINATION OF A NON-PEPTIDE OXYTOCIN RECEPTOR ANTAGONIST IN HUMAN PLASMA BY AUTOMATED PRE-COLUMN DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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DETERMINATION OF A NON-PEPTIDE OXYTOCIN RECEPTOR ANTAGONIST IN HUMAN PLASMA BY AUTOMATED PRE-COLUMN DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A sensitive and selective method for the determination of 1-(((7,7-dimethyl-2(*S*)-(2(*S*)-amino-4-(methylsulfonyl)butyramido)-bicyclo[2.2.1]-heptan-1(*S*)-yl) methyl) sulfonyl) - 4 - (2 - methyl-phenyl)-piperazine (L-368,899) in human plasma is described. The method is based on liquid-liquid extraction followed by automated pre-column chemical derivatization of the primary amino group of the drug and an internal standard with 2,3-naphthalene dicarboxaldehyde and N-acetylcysteamine to form fluorescent benzo[*f*]isoindole derivatives.

The derivatives are separated from endogenous interferences using column-switching high-performance liquid chromatography (HPLC) and quantified with fluorescence detection (FD). The derivatives were initially injected onto a Zorbax SB-CN column and "heart cut" onto a Waters Symmetry™ C8 column for final separation prior to fluorescence detection.

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The assay in human plasma has been validated in the concentration range of 1 to 25 ng/mL. The performance of the HPLC-FD assay was evaluated by comparing the results of the analysis of selected clinical samples using an independent method based on HPLC with tandem mass spectrometric detection (LC-MS/MS).

A good correlation between the data obtained using the two methods was found confirming the selectivity and reliability of the HPLC-FD method.

INTRODUCTION

Compound **I**, (L-368,899) (Figure 1), is an orally-active non-peptide oxytocin antagonist (OA) drug candidate.[1] The hormone oxytocin is involved in the initiation and maintenance of uterine contractions associated with labor.[2-4] The objective of developing an OA drug, therefore, is the inhibition of uterine contractions to manage or prevent cases of premature births.[5-8]

In order to support human pharmacokinetic studies of **I**, a HPLC assay in plasma with the limit of quantification (LOQ) of 1 ng/mL was required and has been developed. Due to the lack of native fluorescence for the molecule and the relatively poor absorption in the accessible UV region, efforts began to develop an assay based on chemical derivatization.

The presence of a primary amino group in **I** provided a desirable functional group for chemical derivatization using the well known dialdehyde/nucleophile (NU)[9-14] chemistry. Because of our earlier experience[12-14] with the naphthalene-2,3-dicarboxaldehyde (NDA) derivatization reaction, the NDA/NU system was utilized for assay development.

Reaction of **I** with NDA in the presence of a nucleophile led to the formation of a highly fluorescent benzo[*f*]isoindole derivative, but the selection of a suitable nucleophile for the NDA reaction was critical in the case of **I** where the primary amino group was attached to a secondary carbon atom. The nucleophile utilized may effect the reaction kinetics, derivative stability, solubility, and fluorescence efficiency. In addition, the nucleophile effects the chromatographic retention of the derivative.

Initially, cyanide ion was used extensively as the nucleophile both by us and others.[9-12] We later explored a number of structurally different thiols as nucleophiles: initially to avoid the use of cyanide in the laboratory,[14] and later to increase the rate and yield of the reaction with analytes that were sterically hindered or relatively non polar.

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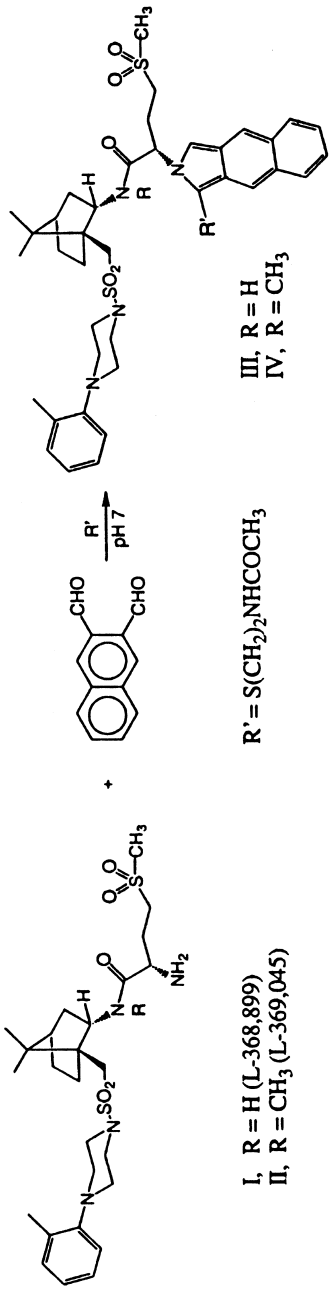

Figure 1. Chemical structures of **I** and **II**, and their products **III** and **IV** of derivatization with naphthalene-2,3-dicarboxaldehyde and N-acetylcysteamine.

Four different nucleophiles were explored in the reaction of **I** with NDA: cyanide ion, N-acetyl-D-penicillamine (NAP), N-acetyl-cysteine (NAC), and N-acetyl-cysteamine (CEA). The NDA/CEA reaction was finally selected for the assay of **I**. It was found that the NDA/CEA reaction with **I** occurred at pH 7 which allowed for the use of conventional reversed-phase analytical columns, and was efficient at low ng/mL concentrations of **I.**

The HPLC-FD plasma assay based on the reaction of **I** and a structurally similar internal standard L-369,045 (**II**) with NDA/CEA, producing derivatives **III** and **IV** (Figure 1), has been validated in the concentration range of 1 - 25 ng/mL.

In addition, the performance of the assay was further evaluated by comparing the results of the analysis of post-dose human plasma samples using an independent method based on HPLC with tandem mass spectrometric (LC-MS/MS) detection.[15] The details of the assay development, validation, and comparison with LC-MS/MS results are presented in this paper.

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EXPERIMENTAL

Materials

All solvents and reagents were of HPLC or analytical grade (Aldrich, Milwaukee, WI, USA; EM Science, Gibbstown, NJ, USA; Fisher, Fairlawn, NJ, USA). Compounds **I** and **II** were obtained from Merck (Rahway, NJ, USA). The NDA was purchased from Molecular Probes (Eugene, OR, USA). The NAC was purchased from Aldrich, Milwaukee, WI, USA. The NAP was obtained from Fluka, Ronkonkoma, New York, USA. The CEA was obtained from Schweizerhall (South Plainfield, NJ, USA).

B & J Brand high purity water was used (Burdick and Jackson, Muskegon, MI, USA).

Instrumentation

A Varian HPLC system (Sugarland, TX, USA) interfaced to Access*Chrom Data System, version 1.7 (Perkin-Elmer Nelson Systems, Inc., Cupertino, CA, USA) was used. A McPherson fluorescence detector (Model FL-750B) equipped with a xenon-mercury lamp, high-sensitivity attachment, and auto zero was utilized (McPherson, Acton, MA, USA). Column switching was achieved using a 10-port switching valve (Valco Instruments, Houston, TX, USA). The columns used were (1) Zorbax SB-CN (4.0 x 80 mm) and (2) Waters Symmetry™ column (3.9 x 150 mm).

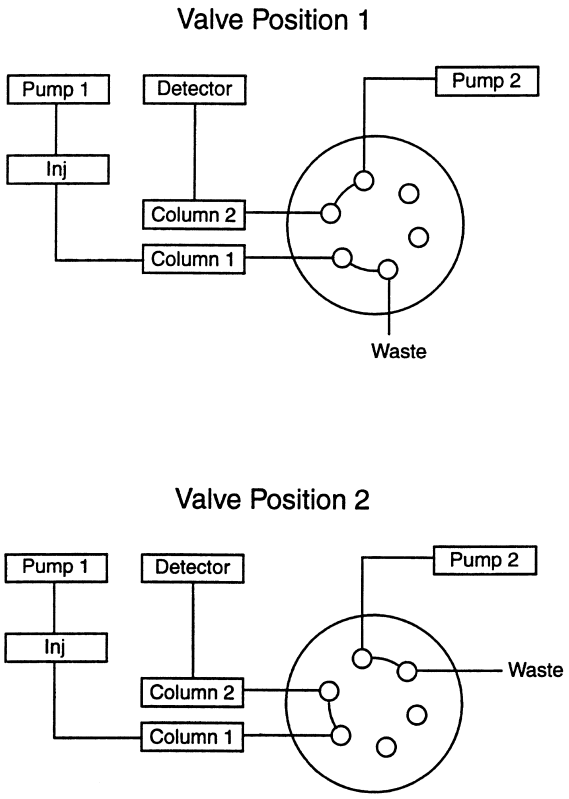

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Figure 2. HPLC system configuration with column switching used for separation and quantification of the derivatives **III** and **IV**.

The absorption and fluorescence sprectra were taken with a diode-array spectrophotometer (HP 8452) and Hitachi San Jose, CA, USA) F-4500 fluorescence spectrophotometer, respectively. LC-MS/MS analysis of the product of derivatization of I with CEA were performed using a SCIEX API III system (Perkin-Elmer/Sciex, Concord, ON, Canada).

Chromatographic Conditions

The HPLC system (Figure 2) was composed of two pumps delivering two mobile phases to two HPLC columns. The NDA/CEA derivatives **III** and **IV** were initially injected onto column 1 (Zorbax SB-CN) using a mobile phase of acetonitrile:water (57.5:42.5, v/v) and containing 0.01 mM ethylene-diaminetetraacetic acid (EDTA).

The system was programmed so that initially the column-switching valve was in position #1 until just prior to the elution of the derivatives when it was switched to position #2. The derivatives, eluted from column #1 between 3.2 and 5.2 minutes, were "heart cut" and loaded onto column 2 (Waters Symmetry™). After both derivatives were transferred onto column 2, the column switching valve was returned to position #1.

The derivatives were then eluted from column #2 with a mobile phase composed of acetonitrile:water (69:31, v/v) containing 0.01 mM EDTA and were monitored by fluorescence detection. The retention times for **III** and **IV** were approximately 10 and 12 minutes, respectively. Total analysis time was 15 minutes. The fluorescence detector was set to the excitation wavelength (Σ) of 436 nm and a cutoff filter with Σ > 440 nm was placed in the emission path.

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Standard Solutions

A stock standard solution of **I** (100 μg/mL) was prepared in methanol:water (20:80, v/v). This solution was further diluted to give a series of working standards with concentrations of 0.02, 0.05, 0.10, 0.20, and 0.5 μg/mL. A stock solution of **II** (1 μg/mL) was also prepared in methanol:water (20:80, v/v).

Stock solutions were stored at -5°C and kept for a period of up to two weeks. A series of quality control (QC) samples in plasma were prepared by spiking 1-mL aliquots of plasma with a separately prepared working stock standard of I. The QC samples were stored at -20°C until assayed.

HPLC-FD Assay in Plasma Using NDA-CEA Derivative

The isolation of **I** and **II** was accomplished by buffering 1 mL of plasma with 1 mL of 0.2M borate buffer, pH 9. This was followed by liquid-liquid extraction with 10 mL of a 95/5 methylene chloride/methanol. The aqueous layer was then aspirated to waste. A 50 μL aliquot of 0.1% HCl in MeOH was added to the organic layer prior to evaporation to dryness in a turbovap at 42°C under nitrogen.

The sample was reconstituted in 400 μL of 40/60 acetonitrile:water (v:v) and transferred to an amber autosampler vial. Pre-column derivatization was performed automatically on the autosampler just prior to injection by adding 30 μL of 0.01M carbonate buffer (pH 10.7), 40 μL CEA (50 μL of neat CEA in 1 mL acetonitrile), and 40 μL NDA (15 mg NDA dissolved in 1 mL acetonitrile).

Following a 6.5 minute reaction time, a 50-μL aliquot of the solution was injected onto the column. The next sample placed on the autosampler tray was dervivatized in the same fashion while the previous sample was chromatographed.

Precision, Accuracy, Linearity, and Specificity

The precision of the method was determined by the replicate analyses (n=5) of human plasma containing **I** and **II** at all concentrations utilized for constructing calibration curves. Quality control (QC) samples were prepared to monitor the stability of **I** in plasma stored at -20°C. These QC samples were assayed in replicates (n=5), and the concentration of **I** was found to be within 10% of their spiked concentrations. The samples were assayed daily with unknown samples using daily constructed standard lines. The accuracy of the assay was expressed by (mean observed concentration)/(expected concentration) x 100.

The linearity of each standard curve was confirmed by plotting the ratio of the peak heights of **III** to **IV** *versus* the spiked concentration of **I**. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted (1/y) linear regression of the standard line. Specificity of the assay was confirmed by analyzing control plasma from five different sources. Endogenous interferences were not encountered.

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RESULTS

Assay Validation

The reaction between **I** and NDA/CEA was found to be highly effective, leading to the formation of a fluorescent derivative **III** (Figure 1). In order to preliminarily confirm the formation of **III**, the product was analyzed, without isolation, using ultraviolet (UV) absorption spectroscopy, spectrofluorometry, and by mass spectral analysis. A characteristic absorption band with maxima at 440 and 450 nm, and a fluorescence band with the emission maximum at 500 ± 5 nm (uncorrected) were observed that were indicative of the formation of a characteristic benzo[f]isoindole chromophore of **III**. A mass spectrum of the mixture after derivatization (17 ng equivalent of the derivatized **I** was injected under flow injection conditions) indicated the presence of an ion at m/z = 823 $(M+H)^+$ which was absent in a similar reaction mixture not containing **III**. The MS/MS product mass spectrum of this ion indicated the presence of fragments at m/z = 346 and 239, which may be indicative of the formation of **III** (Figure 3).

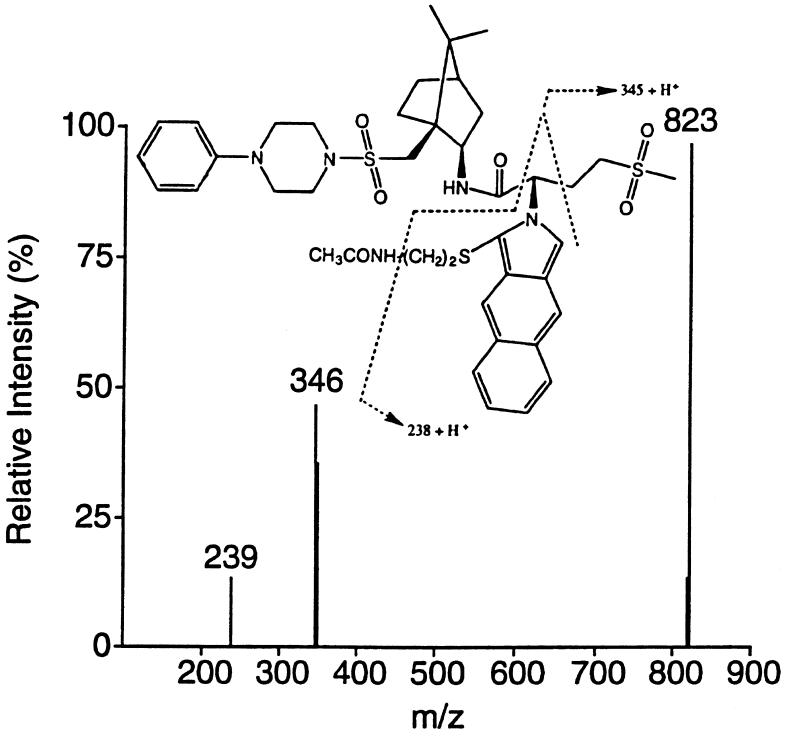

Figure 3. Production mass spectrum of the protonated molecule $(M+H)^+$ at *m/z* = 823 of the product formed after derivatization of **I** with NDA/CEA.

Following the procedure described in Experimental, the assay for **I** in plasma was validated in the concentration range of 1 to 25 ng/mL, and the limit of quantification (LOQ) was 1 ng/mL. The intra- and inter-day precisson and accuracy data are presented in Table 1.

The representative equation of a typical standard line was y = 0.0286x - 0.011 with a correlation coefficient of 0.9994. Representative chromatograms are presented in Figure 4.

Analysis of Samples From Clinical Studies

The HPLC-FD assay was used for the analysis of plasma samples from a human pharmacokinetic study. The results of the analysis of more than 30 post-

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Table 1

Precision and Accuracy Data for the Determination of I in Human Plasma

Concentration ng/mL	Intra-Day		Inter-Day	
	Precision[b] (%)	Accuracy (%)	Precision[b]	Accuracy (%)
1.0	1.3	97.0	---	---
2.0	---	---	6.9	110.0
2.5	3.9	105.6	---	---
5.0	2.3	98.0	---	---
10.0	7.1	103.3	---	---
20.0	---	---	7.7	107.5
25.0	8.6	99.5	---	---

[a] Based on a one week period, n = 7.
[b] Expressed as coefficient of variation (C.V., %), n = 5.
[c] Calculated as (mean observed conc.)/(nominal conc.) x 100.

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dose plasma samples using HPLC-FD methodolgy were compared with results obtained for the same samples using tandem mass spectrometric (LC-MS/MS) detection.[15] A correlation plot (Figure 5) demonstrates the good agreement obtained between these two assays.

DISCUSSION

The development of an assay for **I** based on chemical derivatization with NDA/NU required a careful selection of an appropriate nucleophile to achieve the desired efficiency of derivatization. Initially, NAP and NAC were used as nucleophiles but the yield of reaction was poor and the efficiency at room temperature was low.

One reason for poor reaction yield was probably steric hindrance due to derivatization of a primary amino group attached to a secondary carbon atom.

Our previous experience with the NDA/NU system included the analysis of an amino bisphosponate[12,14] and a cyclic heptapeptide,[13] both containing a readily accessible primary amino group attached to a primary carbon atom.

The poor efficiency of derivatization with **I** using bulky nucleophiles (NAP, NAC) made the finding of an appropriate NU for the reaction with NDA/I the most challenging aspect of the method development.

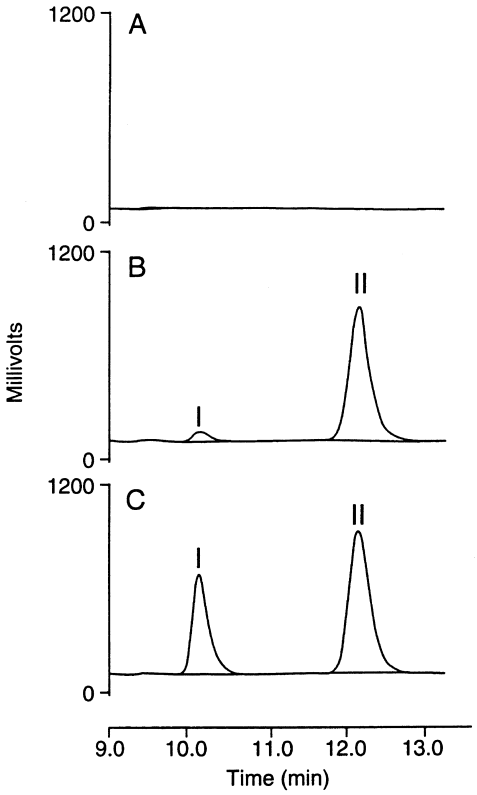

Figure 4. HPLC chromatograms of human plasma spiked with **I** and **II**, derivatized with NDA/CEA to **III** and **IV**, respectively, and analyzed using HPLC-FD. (A) Blank control plasma; (B) control plasma spiked with 2.5 ng/mL **I** and 50 ng/mL **II**, (C) control plasma spiked with 25 ng/mL **I** and 50 ng/mL **II**.

Four nucleophiles investigated in this work included CN^-, NAP, NAC, and CEA. The cyanide ion reacted readily but the resulting derivative was very non polar making timely HPLC analysis difficult (retention times of 40-50 minutes). The formation of a derivative with NAC only occurred when the concentration of **I** was above 1 μg/mL. In addition, the application of NAP, which we had previously utilized extensively,[12,14] did not produce any detectable derivative. The CEA was the only nucleophile found which produced a useful derivative of **I** in the desirable low ng/mL concentration range. The optimized reaction time of NDA/CEA and **I** at room temperature was about 6.5 minutes, which is relatively long when compared to the reaction times (1-2 minutes) of compounds containing a primary amine attached to a primary carbon atom studied previously.[12,14]

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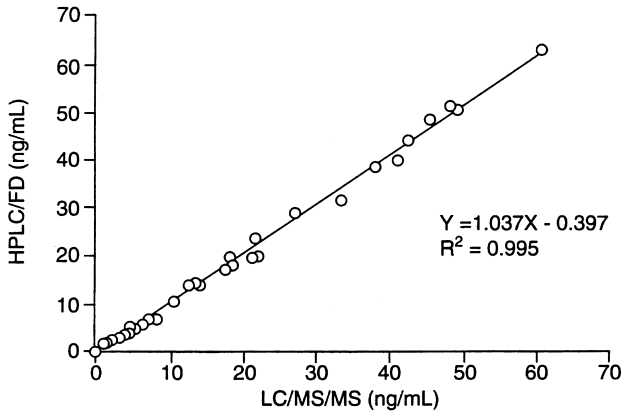

Figure 5. Correlation plot between the assayed concentrations of **I** in selected post-dose human samples obtained using HPLC-FD versus LC-MS/MS.

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A unique advantage of the reaction of NDA/CEA and **I** was that the reaction occurred near neutral pH. The pK_a of the amino group in **I** (pK_a = 6.67 in a solution of 23% ethanol in water) is much lower than for other compounds containing a primary amine attached to an aliphatic chain. The ability to form a derivative at pH 7 was an important aspect of the method development. In all previously studied cases very basic reaction conditions (pH >9) were required to achieve the desired reaction efficiency.

The need for this elevated pH for derivatization reaction limited chromatography to polymeric HPLC columns, which are not as efficient as the conventional HPLC analytical columns. The column switching system which was ultimately developed in this work required injection of the reaction mixture at neutral pH conditions, which was directly compatible with the reaction mixture after derivatization.

The liquid-liquid extraction utilized in this work did not provide alone the necessary clean-up to ensure that the derivatized sample would be free of endogenous interferences. Consequently, an additional on-line sample clean-up procedure using column switching was developed. The column switching system linked two unique chromatographic phases: Zorbax SB-CN and the Waters Symmetry™ C8. The Zorbax SB-CN was ideally suited as the initial column since this phase is generally less retentive than aliphatic phases and provided an adequate separation from the impurities in a short period of time while utilizing a relatively weak (low organic modifier content) mobile phase. The weak mobile phase allowed the derivatives to be well compressed on the second, more retentive Waters Symmetry™ C8 analytical column.

The selectivity of the HPLC-FD method for **I** was confirmed by the analyses of representative clinical samples which were previously analyzed using the concurrently developed LC-MS/MS method.[15] The excellent correlation of data obtained using the two methods confirmed the adequacy of both methods for analyses of post-dose clinical samples. The sensitivity of both methods (LOQ of 1 ng/mL for HPLC-FD and 0.5 ng/mL for LC-MS/MS) were comparable. Also, the required chromatographic run times of 14 and 15 minutes were practically the same for LC-MS/MS and HPLC-FD methods, respectively. In addition, both methods required derivatization to achieve the low ng/mL sensitivity. Therefore, the method based on HPLC-FD constitutes an attractive alternative method for assaying **I** in plasma when LC-MS/MS equipment is not available.

In conclusion, a sensitive assay in human plasma for the determination of **I** based on liquid-liquid extraction, automated pre-column derivatization with NDA/CEA and HPLC with column switching has been developed. The lowest limit of quantification for assaying **I** was 1 ng/mL. The efficiency of the method in terms of the analysis times and the time required for sample preparation was comparable to the analogous method based on LC-MS/MS.

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