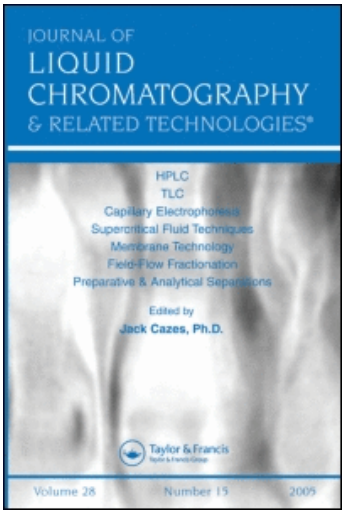

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The Determination of 3-Amino-5-ethyl-6-methyl-pyridin-2-one in Human Plasma by High Performance Liquid Chromatography with Fluorescence Detection

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To cite this Article Woolf, E. , Fu, I. and Matuszewski, B.(1993) 'The Determination of 3-Amino-5-ethyl-6-methyl-pyridin-2-one in Human Plasma by High Performance Liquid Chromatography with Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 16: 5, 1209 — 1222

To link to this Article: DOI: 10.1080/10826079308019582

URL: http://dx.doi.org/10.1080/10826079308019582

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(5), 1209-1222 (1993)

THE DETERMINATION OF 3-AMINO-5-ETHYL-6-METHYL-PYRIDIN-2-ONE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A method for the determination of 3-amino-5-ethyl-6-methyl-pyridin-2-one in human plasma is described. Buffered (pH 8) plasma samples are extracted with 7.5% 2-propanol in chloroform after the addition of an internal standard. The extracts are evaporated to dryness and reconstituted in mobile phase prior to analysis via HPLC with fluorescence detection (λ_{ex}=314 nm, λ_{em}=390 nm). The assay has been validated in the concentration range of 5-500 ng/ml when 1 ml samples are extracted. Application of the assay to the analysis of samples collected after oral dosing of a 2-pyridinone based specific HIV-1 reverse transcriptase inhibitor is demonstrated.

INTRODUCTION

Pyridinone derivatives such as 3-{[(4,7-dichlorobenzoxazolyl)methyl]amino}-5-ethyl-6-methyl-pyridin-2-one (Compound I, Figure 1) have been found to be potent *in vitro* inhibitors of human immunodeficiency

virus type 1 (HIV-1) reverse transcriptase (RT) [1-2]. Compounds such as **I** may prove to be clinically useful for the treatment of acquired immunodeficiency syndrome (AIDS). *In vitro* metabolism studies of **I** enabled the identification of 3-amino-5-ethyl-6-methyl-pyridin-2-one (Compound **II**, Figure 1) as a potential metabolite [3]. In order to determine whether **II** was present in plasma samples collected after dosing human subjects with **I** it was necessary to develop a sensitive and specific assay for **II**. The development of an HPLC method with fluorescence detection for the quantitation of **II** in human plasma and its application to the analysis of selected plasma samples is the subject of this paper.

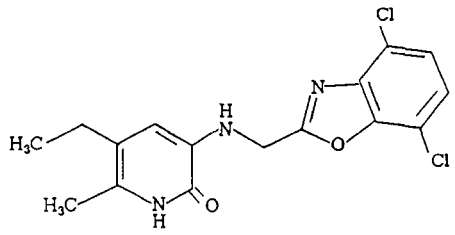
EXPERIMENTAL

Materials

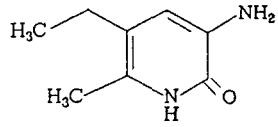
Compounds **I**, **II**, and the internal standard, 3-amino-5-propyl-6-methyl-pyridin-2-one (Compound **III**, Figure 1) were obtained from the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA). Acetonitrile, methanol, and 2-propanol (Omnisolve HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Chloroform (preserved with ethanol) was from Fisher Scientific (Springfield, NJ, USA, Catalog #C298-4). Drug-free heparinized human plasma was supplied by Sera-Tech Biologicals (New Brunswick, NJ, USA). 2-Aminopyridine (2-AP) was from Aldrich (Milwaukee, WI, USA). All other reagents were of ACS grade and were used as received.

Instrumentation

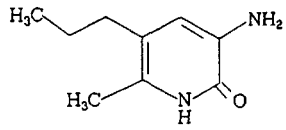
The HPLC system consisted of a Waters Associates (Milford, MA USA) model 6000 solvent delivery system, a WISP (Waters Associates) 712 autosampler, and a McPherson



I



II



III

FIGURE 1: Structures of compounds **I**, **II** and **III**

Instruments (Boston, MA USA) model FL-750 HPLC fluorescence detector. The detector was equipped with a Xe-Hg lamp and dual monochromators. The detector output was connected to a Hewlett Packard 3357 laboratory automation system via a Hewlett Packard 18652A analog to digital interface.

Absorption and fluorescence spectra were obtained using a diode array spectrophotometer (HP8452, Hewlett-

Packard, Palo Alto, CA, USA) and a Perkin-Elmer (Norwalk, CT, USA) model 650-10S spectrofluorometer.

The cyclic voltammograms were recorded with a Bioanalytical Systems (West Lafayette, IN, USA) model CV-1B potentiostat equipped with a model VC-2 cell with glassy carbon and Ag/AgCl electrodes.

Chromatographic Conditions

The mobile phase was composed of 15/85 v/v% acetonitrile:10 mM dibasic sodium phosphate. The pH of the mobile phase was adjusted to 7.0 with concentrated o-phosphoric acid and filtered through a nylon filter (0.20 μm) prior to use. The HPLC column (150 x 4.6 mm I.D.) was packed with BDS-Hypersil C_{18} 5 micron, 120 Å material (Keystone Scientific, Bellefonte, PA USA). The column was operated at ambient temperature. The flow rate of the mobile phase was 1.5 ml/min. Injection volumes were 125 μl. Fluorescence detection was used with an excitation wavelength of 314 nm (16 nm bandpass) and an emission wavelength of 390 nm (8 nm bandpass).

Preparation of Standards

A 20 μg/ml stock solution of **II** was prepared by weighing 1.0 mg of reference material into a 50 ml red volumetric flask, dissolving the compound in 25 ml of methanol and filling the flask to the mark with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20.0 μg/ml solution to 50 ml with 1:1 (v/v) methanol/water.

Working standards of 10, 8, 4, and 2 μg/ml **II** were prepared by dilution of the 20 μg/ml stock solution with 1:1 (v/v) methanol/water. Working standards of 1, 0.4, 0.2, and 0.1 μg/ml **II** were prepared by dilution of the 2.0 μg/ml stock solution with 1:1 (v/v) methanol/water.

A 10 μg/ml stock solution of internal standard was prepared by weighing 0.5 mg of reference material into a 50 ml red volumetric flask, dissolving the compound in 25 ml of methanol and filling the flask to the mark with water. A working internal standard solution of 4 μg/ml was prepared by dilution of the 10 μg/ml stock solution with 1:1 (v/v) methanol/water.

Plasma standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma. The resulting standards ranged in concentration from 5.0 to 500 ng/ml.

Plasma Extraction Procedure

A 1 ml aliquot of plasma (sample or standard) and 50 μl of working internal standard solution were pipetted into a 20 ml disposable borosilicate screw cap culture tube (150 x 16 mm). The tube was vortexed for 30 seconds after which 1 ml of 0.1 M pH 8 phosphate buffer was added. After vortexing for 30 seconds, 10 ml of extraction solvent (7.5% 2-propanol in chloroform) was pipetted into the tube. The tube was capped and mixed on a rotator (Glas Col, Terre Haute, IN, USA) set at 30 r.p.m. The resulting mixture was centrifuged at 2000 *g* for 10 minutes in a centrifuge cooled to 10°C. The upper aqueous layer of the sample was aspirated to waste. Seven ml of the remaining organic phase were pipetted into a 16 x 100 mm disposable borosilicate glass culture tube. The solvent was evaporated under a gentle stream of nitrogen in a 45°C water bath. The residue in the tube was reconstituted in 250 μl of HPLC mobile phase. Particulate matter was removed from the sample by centrifugation (0.5 ml polypropylene tube) at 10000 *g* for 4 minutes. The sample was then transferred to an amber autosampler vial containing a polymethylpentene low volume insert (Waters Assoc.) prior to analysis.

Fluorescence Quantum Efficiency

The fluorescence quantum efficiency (Φ_f) of **II** was determined relative to 2-AP as standard. The necessary corrections of the fluorescence intensities due to slightly different absorbances (adjusted to about 0.1) at the wavelength of excitation (300 nm) were performed using a similar procedure as described earlier.[4] The uncorrected spectrum of 2-AP was compared with that of **II**. The fluorescence intensities in various solvents were corrected by multiplying the intensities by η^2, where η is the refractive index of the solvent.

RESULTS

Assay Specificity

Figure 2 shows chromatograms of extracted drug-free plasma, a 50 ng/ml plasma standard, and a plasma sample obtained from a volunteer 12 hours after receiving a 500 mg oral dose of **I**. A comparison of Figure 2A with Figure 2B illustrates that no endogenous peaks elute in the region of **II**. The specificity of the method is further illustrated by the fact that all pre-dose plasma samples from subjects involved in clinical trials were free of interfering peaks. Under the conditions specified in this assay, compound **I** did not interfere with the determination of compound **II**.

Linearity

Weighted (weighting factor = 1/y where y = peak height ratio) least squares regression calibration curves, constructed by plotting the peak height ratio of **II** to the internal standard versus the concentration of **II**, yielded coefficients of regression typically greater than 0.999 over the concentration range of 5 to 500 ng **II**

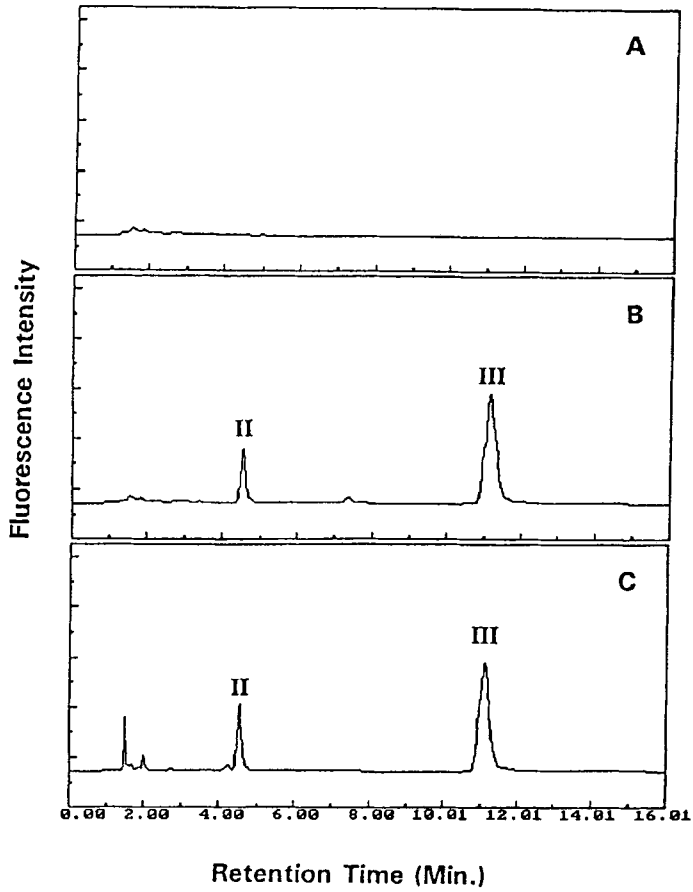


FIGURE 2: Chromatograms of A)drug-free plasma, B)a 50.0 ng/ml plasma standard and C)a plasma sample obtained 12 hours after oral administration of 500 mg I. The concentration of II is equivalent to 59.8 ng/ml.

per ml plasma. The use of the weighted least squares regression resulted in less than a 10% deviation between the nominal standard concentration and the experimentally determined standard concentration calculated from the regression equation.

Extraction Recovery

The fraction of analyte recovered during the extraction procedure was determined by comparing the responses (peak area) of standard solutions of **II** injected directly onto the HPLC column with those of extracted plasma standards. The results (Table 1) indicate that the mean analyte recovery over the concentration range of 5 - 500 ng **II**/ml plasma is 81.6%.

Assay Precision and Accuracy

Replicate standards (n=5) were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentration, as well as the coefficient of variation (%CV) of the plasma replicate standards are shown in Table 2.

Quality control samples containing concentrations of 15 and 300 ng **II**/ml plasma were prepared and frozen (-20°C) in 1.25 ml aliquots. Two pairs of quality control samples were analyzed with each of 7 standard curves over a period of 2 weeks. The results (Table 3) indicate that the interday variability of the method is less than 5% CV. Additionally, the fact that assayed values remained constant over the period indicates that plasma samples containing **II** are stable for at least 2 weeks when stored at -20°C.

Limit of Quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded an intraday

TABLE 1

Recovery of II from Human Plasma and Within-day Variability of the Assay

Nominal Concentration (ng/mL)	Mean Recovery (%, n=5)	Accuracy[a] (%)	Precision[b] (%)
5.00	91.4	91.5	8.1
10.00	80.2	99.5	2.9
20.00	81.0	97.5	4.5
50.00	78.6	102.8	2.0
100.00	78.7	105.3	1.4
200.00	82.6	103.6	2.3
400.00	84.5	100.3	1.9
500.00	75.8	99.9	3.7
	Mean	100.1 ± 4.3%	

[a]Calculated as (mean (n=5) observed concentration / nominal concentration) x 100

[b]Expressed as coefficient of variation.

TABLE 2

Inter-Day Variability of the Assay of II in Plasma as Assessed by Coefficients of Variation (%CV) of Low and High Quality Control Samples

Nominal Conc. (ng/mL)	Mean (n=14) Analyzed Conc.[a] (ng/mL)	%CV
15.0	15.8	4.1
300.0	303.9	5.0

[a]Results represent 7 standard curves over a 2 week period

TABLE 3

Representative Plasma Concentrations of **II** after a 500 mg Oral Dose of **I** to Human Subjects

	Concentration (ng/ml)					
Time (hour) Subject #	0	1	2	3	12	24
1	ND	5.2	16.6	66.9	59.8	7.5
2	ND	ND	7.1	23.4	39.1	12.5
3	ND	ND	9.3	77.5	54.4	13.7
4	ND	ND	5.5	28.0	43.7	10.3

ND = Not Detected

coefficient of variation of less than 10% and an intraday accuracy between 90 and 110% of nominal concentration, was 5 ng/ml.

Fluorescence Quantum Efficiency

The Φ_f values for **II** were determined in the mobile phase used in the assay and in methanol. 2-AP was utilized as a fluorescence standard with a known Φ_f of 0.71 in water.[5] The uncorrected maximum of emission of 2-AP was at 360 $\pm$ 5 nm. A relatively good overlap between the emission spectrum of **II** and 2-AP was observed. Based on comparison of uncorrected spectra of **II** and 2-AP, the Φ_f values for **II** were estimated at 0.029 and 0.055 in mobile phase and methanol respectively.

DISCUSSION

Compound **I** has been found to be active *in vitro* at low nanogram/ml concentrations [1-2], hence an assay to detect low nanogram levels of **II**, a possible metabolite of **I**, was needed. The spectral (UV absorbance and

fluorescence) and electrochemical (EC) properties of **II** were evaluated in order to establish the most sensitive method of detecting **II** after HPLC separation.

UV and fluorescence spectra of **II** were obtained under varying pH conditions in solutions of acetonitrile /methanol/0.01 M phosphate buffer (47:5:48 v/v/v, pH adjusted with 85% H_3PO_4 or 10 M NaOH). A strong absorbance band with a maximum at 318 and 324 nm was observed at pH 2 and 7, respectively. A band of similar intensity was noted for **I**. This absorbance is due to the aminopyridone moiety in the molecule. Additionally, **II** showed two bands of lower intensity with maxima at 252 and 214 nm at pH 7 and at 230 and <210 nm at pH 2. Only small changes in the absorption spectrum of **II** were observed in different solvents. For example, in methanol, three characteristic absorption bands with maxima at 214, 254 and 326 nm were present with corresponding molar absorption coefficients (ϵ) of 11800, 5800 and 10400 $M^{-1}cm^{-1}$, respectively.

The pH effect was especially well pronounced in the fluorescence spectrum of **II**. The compound was found to be fluorescent at pH 7, with an emission maximum (uncorrected) at 390 nm. This emission maximum was shifted to 375 nm when the pH of the solution was lowered to 2. A significant decrease in fluorescence was also observed at pH 2. Upon raising the pH back to 7, the intensity and the maximum of the emission band increased to the original values, indicating that protonation/deprotonation of the molecule is reversible and that this process is probably responsible for the observed pH dependent changes in the UV and fluorescence spectra. The Φ_f of **II** was also solvent dependent. In methanol, the Φ_f value of 0.055 (measured against 2-AP) was almost twice as high as that in mobile phase (Φ_f = 0.029). The fluorescence intensity of **II** was high enough

to achieve better detection sensitivity and selectivity than with UV absorption detection.

Cyclic voltammetry (CV) measurements, obtained in the solvent used for the spectral studies, indicated the presence of three oxidation peaks at +0.56, +0.80, and +1.05 V. In addition, several peaks in the reduction mode were observed. The electrochemical reaction was fully reversible as indicated by the presence of the same CV peaks after repeating the oxidation/reduction cycle. The low half-wave potential of +0.56 V created the possibility of development of a selective HPLC assay based on EC detection. However, due to the relative experimental simplicity of an assay based on fluorescence detection, and comparable to EC or better sensitivity and specificity, detection based on fluorescence was chosen for assay development.

An excitation wavelength of 314 nm was used for HPLC detection in order to take advantage of the high intensity of excitation present in the emission spectrum of the mercury lamp at this wavelength.

Compound **II** possesses a primary amino functional group. Basic analytes such as **II** are known to chromatograph with poor peak shape on conventional reverse phase columns under neutral pH conditions. Peak shape may be improved by lowering the pH of the mobile phase to 2.8, however, in the case of **II**, a low pH mobile phase would significantly decrease the fluorescence of the analyte. Use of a "base-deactivated" column with a mobile phase of pH 7.0 resulted in symmetrical peaks for both **II** and the internal standard while maintaining conditions of maximum detectability.

Initial attempts to use liquid-liquid extraction with hexane, ethyl acetate, methyl t-butyl ether, methylene chloride and chloroform to extract **II** from aqueous buffers over the pH range of 2-13 showed that the

best analyte recovery was obtained with chloroform from solutions buffered at pH 8. However, overall recovery of the chloroform extraction was only approximately 50%. In an effort to improve analyte recovery, small amounts of a polar modifier, i.e., 2-propanol, were added to the extraction solvent. Use of an extraction solvent composed of 7.5% 2-propanol in chloroform improved analyte recovery to approximately 80%. Chromatograms of extracts of plasma samples obtained using this solvent were free of interferences in the regions where **II** and the internal standard eluted.

Although chloroform is a hazardous substance to work with, no other solvent yielded a comparable recovery. Attempts to use solid phase extraction for the isolation of **II** also resulted in poor analyte recovery.

Compound **I** in clinical samples may potentially degrade during the extraction procedure and form compound **II**. In order to explore this possibility, a control plasma sample was spiked with **I** at a level of 1000 ng/ml. The sample was then extracted and analyzed according to the assay procedure for **II**. No **II** was detected in the resulting chromatogram.

The assay has been used to analyze clinical samples collected after the oral administration of **I**. Representative data is shown in Table 3. The concentrations of **II** ranged from 0 - 14% of the corresponding level of **I**, as determined via a separate HPLC assay of **I** [6] in the same plasma samples. The data obtained indicates that **II** is formed *in-vivo* after dosing human subjects with **I**.

ACKNOWLEDGEMENTS

The human plasma samples were obtained from a clinical study directed by Dr. O. Laskin, Department of Clinical Pharmacology, Merck Research Laboratories.

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Received: July 30, 1992
Accepted: August 11, 1992