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Sensitive and selective liquid chromatographic assay of memantine in plasma with fluorescence detection after pre-column derivatization

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Abstract

A procedure was developed for the determination of memantine in plasma using liquid chromatography with fluorescence detection. Following a liquid–liquid extraction from 1 ml of plasma containing the internal standard amantadine, the extract was derivatized at room temperature with dansyl chloride, and the highly fluorescent derivatives were chromatographed with a reversed-phase C_{18} column and a mobile phase of phosphate buffer and acetonitrile. Dansylated memantine and amantadine were eluted in less than 13 min with no interference from endogenous material. The calibration curve was linear over the concentration range of 3–400 ng/ml with inter- and intra-assay imprecision (C.V.) of less than 10%. The limit of quantitation was 3 ng/ml, and no major antidepressant, neuroleptic or their respective metabolites interfered with the quantitation of memantine. This method could also be applied to the quantitation of amantadine.

Keywords: Memantine

1. Introduction

Memantine HCl (1-amino-3,5-dimethyladamantane hydro-chloride) is a tricyclic amine chemically and pharmacologically related to the antiviral prototype amantadine (Fig. 1) and its α-methyl derivative rimantadine. While amantadine and rimantadine have been approved in the U.S. for the prophylaxis and treatment of influenza, and amantadine is also approved for the treatment of Parkinsonism, memantine is used in Parkinson's disease and movement disorders [1]. Recently, it has been demonstrated to be useful in dementia syndrome [2]. Memantine is a noncompetitive NMDA antagonist in clinical use for many years in Europe. It produces few side effects, even among the geriatric patients, who are typical candidates for this drug [3,4].

The NMDA receptor, a glutamate receptor subtype, may play a significant role in the development

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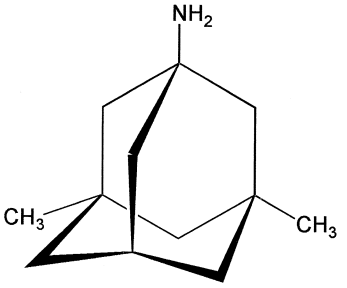
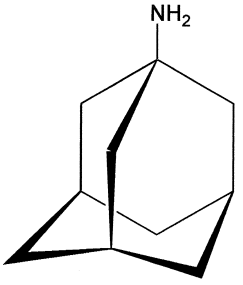


MEMANTINE



AMANTADINE

Fig. 1. Chemical structures of memantine and amantadine (internal standard).

and maintenance of dependence on opioids, nicotine, and cocaine [5,6]. In laboratory animals, low doses of NMDA antagonists inhibit the development of opioid tolerance and dependence [7], and attenuate established morphine (mμ) opioid tolerance [8]. It has been suggested that the development of tolerance, dependence and/or sensitization to virtually all psychoactive drugs can be attenuated or abolished by pretreatment with an NMDA antagonist [9]. As such, memantine is a promising agent for the treatment of substance use disorders. Unlike other noncompetitive NMDA antagonists, such as phencyclidine and ketamine, memantine has rarely been associated with the significant adverse side effects of agitation, confusion, and psychosis [10,11].

Studies requiring the quantitation of memantine in plasma, cerebrospinal fluid, urine, and other tissues have used a gas chromatographic–mass spectrometric (GC–MS) method [12]. No other published analytical procedures are available. However, analytical assays that measure amantadine or rimantadine could most likely be adapted to memantine. Amantadine was determined in plasma using GC with electron-capture detection following derivatization with pentafluorobenzoyl chloride (PFBC) [13], and trichloroacetyl chloride [14,15]. These methods reported low ng/ml sensitivity. Similarly, rimantadine and its metabolites have been assayed by GC–MS with negative chemical ionization following derivatization with PFBC resulting in a sensitivity of about 2.5 ng/ml of plasma [16,17]. Liquid chromatography (LC) has seldom been used for quantitation of this class of compounds because of the lack of a strong chromophoric, fluorophoric, or electrochemical group necessary for adequate detection. A complex on-line solid-phase derivatization procedure using 9-fluoreneacetate enabled amantadine to be determined in plasma using LC with fluorescence detection [18].

Dansyl chloride reacts readily with most primary and secondary amines in alkaline buffer, and it is regarded as the derivatizing reagent of choice in the preparation of highly fluorescent compounds [19]. Therefore, memantine, a primary amine, appeared to be a likely substrate for dansylation. The addition of the structurally similar internal standard amantadine achieved the goal of analyzing memantine in plasma in the low ng/ml range.

Little published data exists with regard to plasma levels of memantine in humans. In a limited study, serum levels of memantine were reported in the range of 4 to 94 ng/ml in dementia patients treated with 5 to 30 mg/day, p.o. [20]. Cerebrospinal fluid (CSF) in the same subjects was also assayed and found to be in a CSF/serum ratio of 0.52, and highly correlated with dose. These concentrations were adequate to interact with the PCP binding site of the NMDA receptor.

In this protocol, memantine was studied utilizing a laboratory model of cocaine self-administration by humans. The methods are described elsewhere [21]. Briefly, however, following a six-day slow dosage escalation using a double-blind design, individuals were maintained on memantine (0 and 20 mg) for approximately 12 to 15 days. Blood samples were assayed for memantine on days 9, 14–18, 23, 30, 35–39, and 44, some of which corresponds to

placebo dosing and others correspond to dosage titration up or down to placebo.

2. Experimental

2.1. Reagents and chemicals

Memantine hydrochloride, amantadine hydrochloride and isoamyl alcohol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium bicarbonate, monobasic potassium phosphate, sodium carbonate, *o*-phosphoric acid, sodium hydroxide, hydrochloric acid, *n*-heptane, and *n*-butylamine were all HPLC or reagent grade from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile was HPLC–UV grade from J.T. Baker (Phillipsburg, NJ, USA), and dansyl chloride was obtained as a 100 g/l solution in acetone from Pierce Chemical Co. (Rockland, IL, USA).

2.2. Preparation of stock and standard solutions

Stock solutions (1.0 mg/ml as the free base) of memantine hydrochloride and amantadine hydrochloride were prepared in 0.1*M* HCl. Working solutions (1.0 ng/μl) of each were prepared in 0.01*M* HCl, and stored at 4°C for six months. Water used for preparation of standards, as well as for buffers and the mobile phase, was produced using a Milli-RO 10-Plus and Milli-Q Plus water purification system (Millipore Corp., Bedford, MA, USA).

2.3. Sample collection and processing

Blood was drawn from each individual using one of two procedures. On outpatient days (9,23,30, and 44), approximately 7 ml of venous blood was collected from a peripheral vein using a 23-gauge butterfly needle connected to a Vacutainer® adapter. On inpatient days (14–18 and 35–39), approximately 7 ml of blood was collected from an indwelling intravenous catheter, which allowed for repeated blood collection during laboratory sessions. The indwelling catheter was maintained for up to 72 h with saline (but no anticoagulant) flushes every 8 h. All samples were collected in gray-top Vacutainer® tubes containing sodium fluoride and potassium oxalate. Following centrifugation, the plasma was transferred to cryogenic tubes and was stored frozen at −20°C prior to analysis.

To extract the drug from the samples, 1.0 ml of plasma was transferred to a 16×100 mm disposable screw-capped borosilicate tube to which 50 μl (50 ng) of the internal standard amantadine, 1.0 ml 1*M* NaOH, and 4.0 ml of 1.5% (v/v) isoamyl alcohol in *n*-heptane were added. The contents were mixed for 10 min on a rocking platform at low speed, followed by centrifugation at 1500 *g* for 10 min. The supernatant (organic) phase was transferred to a 5 ml screw-thread tapered disposable borosilicate centrifuge tube containing 150 μl of 0.1*M* HCl, mixed for 10 min at moderate speed, and centrifuged at 1500 *g* for 10 min. The top (organic) layer was aspirated and discarded. The aqueous acid phase was evaporated to dryness under reduced pressure and moderate heat (45°C) in a vacuum centrifuge for approximately 60 min. To the residue, 50 μl of 1*M* carbonate buffer (pH 10.3) and 25 μl of 1% dansyl chloride in acetonitrile were added, vortexed, and allowed to react at room temperature for 45 min. The mixture was then evaporated to dryness in a vacuum centrifuge for 30 min at 45°C, and reconstituted with 125 μl of 25:75 (v/v) mixture of water–acetonitrile. Following a brief vortex and centrifugation (3–5 min), the supernatant was transferred to small-volume glass vials for automated sampling.

A seven-point calibration standard curve in the concentration range of 3–400 ng/ml was prepared with the working solution added to drug-free plasma, and processed similarly with each batch of samples. Three levels of quality control samples (run in duplicate) were included with each days' analyses.

2.4. Instrumentation

Chromatographic separation was achieved using a Model 590 programmable solvent-delivery pump and a Model 717+ autosampler (Waters Corp, Milford, MA, USA). A Model 980 Spectroflow programmable fluorescence detector equipped with a 5 μl flow cell and a deuterium source lamp (Applied Biosystems, Foster City, CA, USA) was used to detect the analytes. Chromatographic signals were acquired with a PC-based data system using ChromPerfect for Windows v.3.5 (Justice Laboratory Software, Palo

Alto, CA, USA) for data collection, construction of the calibration curves, sample analyses and storage of quality control data. Chromatographic tracings were recorded on a Recordall strip chart recorder (Fisher Scientific, Springfield, NJ, USA).

A Speed-Vac Concentrator (Savant Instruments, Holbrook, NY, USA) with a built-in heater and a Model VP190 oil-vacuum pump were used to evaporate sample extracts during sample preparation and derivatization.

2.5. Chromatographic conditions

The mobile phase consisted of 270 ml of 0.025*M* mono basic potassium phosphate and 730 ml of acetonitrile with 500 μl of *o*-phosphoric acid and 600 μl of *n*-butylamine added. Following filtration and degassing, the mobile phase was pumped at 1.8 ml/min through a 25×0.46 cm column containing 5 μm particles of octadecyl bonded silica (Supelcosil LC-18, Supelco, Bellafonte, PA, USA) resulting in a back pressure of about 8.28 MPa. The excitation wavelength of the fluorescence detector was set at 235 nm, and a 470 nm cutoff filter (emission wavelength) was used to monitor the fluorescence. The detector signal response time was set at 1 s and the attenuation of 0.08 μA full scale adequately capture the peak signals on the recorder. The detector signal to the data system was set at 1 V full scale.

Memantine was quantified by calculating the peak-height ratios to the internal standard amantadine. The data system performed the least-square linear regression analysis for each calibration curve with equal weighting for each point.

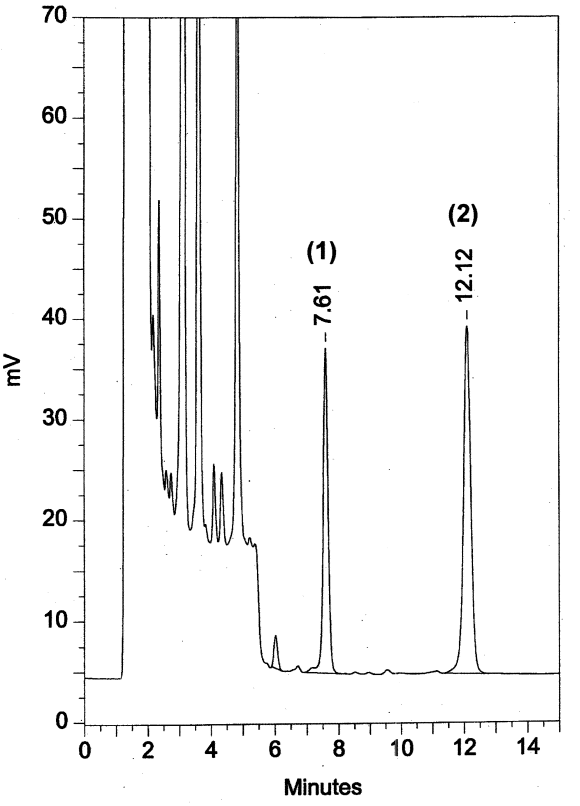


Fig. 2. Chromatogram of a 20 μl injection of a derivatized 1 ml supplemented plasma extract containing 100 ng/ml of memantine (2), and 50 ng/ml of the internal standard amantadine (1).

3. Results and discussion

3.1. Chromatography

Memantine in plasma was quantitated using LC coupled with fluorescence detection following pre-column derivatization with a fluorescent tagging agent. The addition of a structurally related compound, amantadine, for the internal standard reacted similarly to form the dansyl derivative. A chromatogram (Fig. 2) shows the separation of the dansyl derivatives of memantine and amantadine. Total analysis time did not exceed 15 min. A liquid–liquid extraction from plasma produced a relatively clean extract free from interfering endogenous peaks as demonstrated in the drug-free plasma blank (Fig. 3). A large solvent front is typical for dansyl extracts [22], but can be substantially reduced, if necessary, by the addition of L-proline during the derivatization step [23]. Dansyl derivatives are usually stable so that re-injection of the extract the following day is possible using the same calibration curve. Re-injection of the memantine calibration standards 24 h following derivatization did not indicate any deterioration in peak height ratios or overall sensitivity.

The excitation and emission wavelengths for the detection of dansylated memantine and amantadine were optimized to afford the greatest sensitivity with the analytical conditions described. Using a different

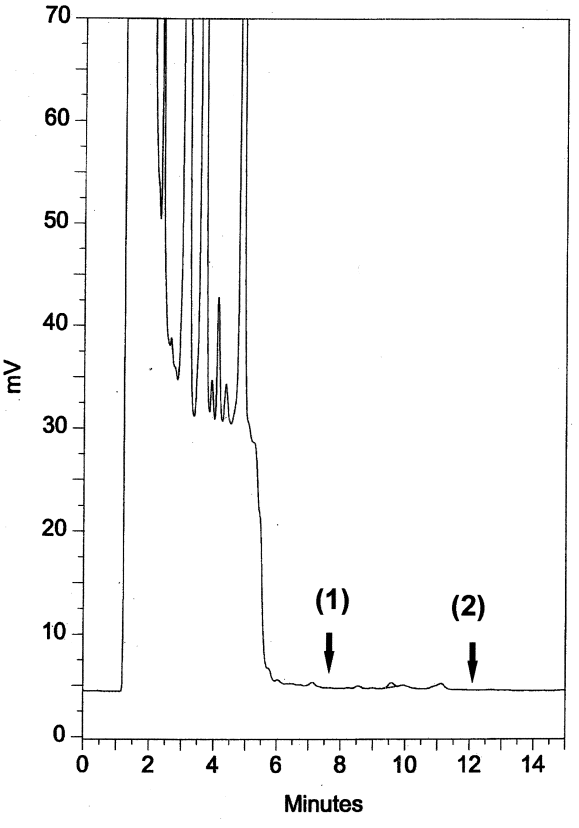


Fig. 3. Chromatogram of a 40 μl injection of a derivatized 1-ml drug-free plasma extract.

energy source (e.g. xenon) would probably require re-optimizing these wavelengths.

3.2. Precision, accuracy and recovery

The intra-day precision of the method was determined by supplementing 12 1 ml aliquots of drug-free plasma with various amounts of memantine. After addition of the internal standard, the samples were processed as described, together with a standard calibration curve. The average result for each added concentration and the respective C.V.s are listed in Table 1. Inter-day variability was assessed by analyzing quality-control samples with each batch of samples. The quality-control samples were prepared at 10, 75, and 250 ng/ml, and stored frozen at −20°C. The respective day-to-day variations (C.V.s) are listed in Table 1. The accuracy of the method, measured as the intra- or inter-day percentage difference between the mean concentration found and the amount added, did not exceed 4%. Inspection of the daily calibration curve parameters, i.e. slope, intercept and correlation coefficient, was another measure of day-to-day consistency.

The absolute recovery of memantine from plasma was determined by preparing an aqueous solution of known concentrations of memantine. The internal standard was added to each solution, evaporated to dryness, and derivatized with dansyl chloride. An aliquot of the extract was injected into the chromatograph. The same concentrations were added to drug-free plasma and processed routinely, but quantitatively. The internal standard was added to the final acid extract, and then dansylated as previously described. An aliquot of this extract was injected as above. The difference between the ratio of standards to internal standard in the processed samples vs the direct-injection samples indicated the overall extraction recovery. The percent recovery of memantine from plasma at 400, 100, and 10 ng/ml was found to be 95.6 (C.V.=1.9%), 90.3 (C.V.=2.7%), and 93.6 (C.V.=3.8%), respectively.

3.3. Selectivity, sensitivity and stability

No endogenous peaks from plasma appeared in the areas of interest (Fig. 3). Table 2 lists a number of drugs and their respective metabolites, with their retention times, that were processed by this method. Only propranolol at high levels would interfere with the internal standard amantadine. Because dansyl chloride reacts readily with primary and secondary amines, drugs with a tertiary amine moiety (e.g., imipramine, amitriptyline, etc.) will not be detected, thereby increasing specificity of the method. As this list is essentially psychopharmacological, there are, of course, other amine-containing compounds that must be tested for interference.

The limit of quantitation (LOQ) was determined to be 3 ng/ml (inter-assay variation=7.6%, n=12, signal-to-noise approximately 20). Fig. 4 represents a chromatogram of 3 ng/ml of memantine plasma standard. A LOQ of less than 1 ng/ml might be achieved with an acceptable C.V. using a higher detector response time greater than 1 s.

The stability of memantine in plasma was assessed

Table 1
Plasma memantine intra-assay and inter-assay variation

Memantine added (ng/ml)	Memantine recovered (ng/ml)	Coefficient of variation (%)
Intra-assay variation		
400	394	1.4
200	197	2.3
100	99.5	3.9
50.0	50.8	4.9
25.0	24.5	2.1
10.0	10.0	4.3
3.0	3.0	7.6

$N=12$ at each concentration.

Memantine quality controls (ng/ml)	Memantine recovered (ng/ml)	Coefficient of variation (%)
Inter-assay variation		
250	252	2.1
75.0	76.6	4.3
10.0	10.4	2.4

$N=12$ consecutive days; each concentration was run in duplicate on each day.

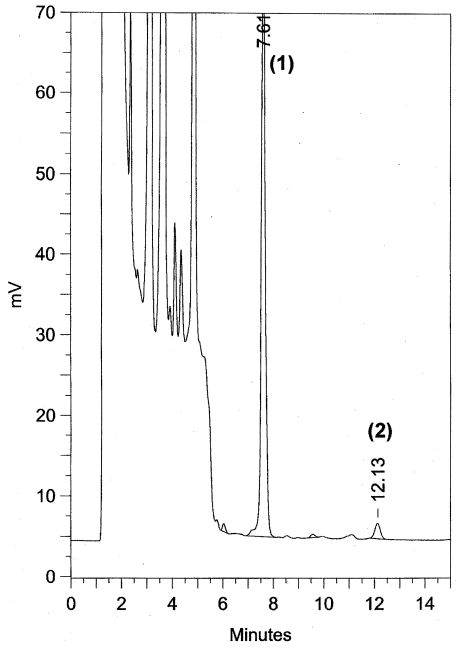


Fig. 4. Chromatogram of a 40 μl injection of a derivatized 1 ml supplemented plasma extract containing 3 ng/ml of memantine (2) and 50 ng/ml of the internal standard amantadine (1). This illustrates the LOQ for the assay.

by the 'freeze–thaw' procedure. A set of eight tubes each of three levels of quality controls (10, 75, and 250 ng/ml) were allowed to thaw at room temperature (in darkness) for 2 h three times during a 1 week period. Each set was then processed with eight tubes that were kept frozen during the same period, along with the standard calibration curve. There was no significant difference between the thawed set and the frozen set. (Student's *t*-test, $P>0.05$). Furthermore, the quality controls that were stored at −20°C and included with each run did not indicate any degradation of memantine over a 5 month period. Human plasma samples stored at −20°C for 1 year from the original analysis were reanalyzed to check for long-term stability. Memantine plasma concentrations decreased an average of about 6.6% from the original values (individual differences ranging from +4 to −13%; sample range 37 to 89 ng/ml; $n=10$). This trend, however, was not corrected for the assay variability since each set of samples was analyzed 1 year apart. Stability of memantine plasma samples at room temperature was determined by preparing 20 samples, each containing 50 ng/ml. Ten samples were stored frozen at −20°C and 10 were left at room temperature (23°C) in darkness for 24 h. Both

Table 2
Retention times of some commonly used drugs and their metabolites

Drug/Metabolite	Retention times (min)
Drugs/metabolites extracted and derivatized	
Norfenfluramine	5.32
Trans-10-hydroxynortriptyline	5.48
Cis-10-hydroxynortriptyline	6.18
Desmethylcitalopram	6.68
Propranolol	7.11
Amantadine	**7.61**
m-chlorophenylpiperazine	8.21
Norclozapine	8.35
Fluvoxamine	8.36
Clovoxamine	8.45
Norfluoxetine	8.50
Desmethylmaprotiline	9.56
Fenfluramine	10.02
Paroxetine	10.15
Desmethyldoxepin	10.55
Memantine	**12.16**
Desmethylmianserin	12.87
Fluoxetine	12.94
Rimantadine	13.48
Amoxapine	13.72
Desipramine	14.54
Protriptyline	14.59
Desmethylsertraline	15.41
Maprotiline	15.67
Nortriptyline	17.45
Desmethyltrimipramine	19.06
Desmethylclomipramine	20.05
Sertraline	31.70
Drugs/Metabolites not extracted and/or not derivatized, or elutes at solvent front	
2-hydroxyimipramine	Citalopram
2-hydroxydesipramine	Moclobemide
Clozapine	Loxapine
Clomipramine	Bupropion and metabolites
Amitriptyline	Haloperidol
Imipramine	Olanzapine
Doxepin	Risperidone

sets of samples were processed identically the following day. There was no significant difference in memantine plasma concentrations between the two groups (Student's *t*-test, $P>0.05$. Finally, dansylated memantine and amantadine were determined to be stabile for at least 24 h. This permitted the processing of an extended number of samples for unattended automated analyses.

3.4. Application to biological samples

This method was developed for the purpose of monitoring individuals taking memantine 20 mg orally once daily. Fig. 5 illustrates an actual plasma sample containing 31 ng/ml from a subject who was being tapered off memantine following a 15 day memantine maintenance (20 mg/day) phase, three days of memantine 10 mg, and one day of memantine 5 mg (on the day prior to the drug level assay). Blood levels appeared to increase (number of samples too small to test significance) during the course of each inpatient week (despite a period of outpatient maintenance on memantine 20 mg daily), which is consistent with the likelihood that medication com-

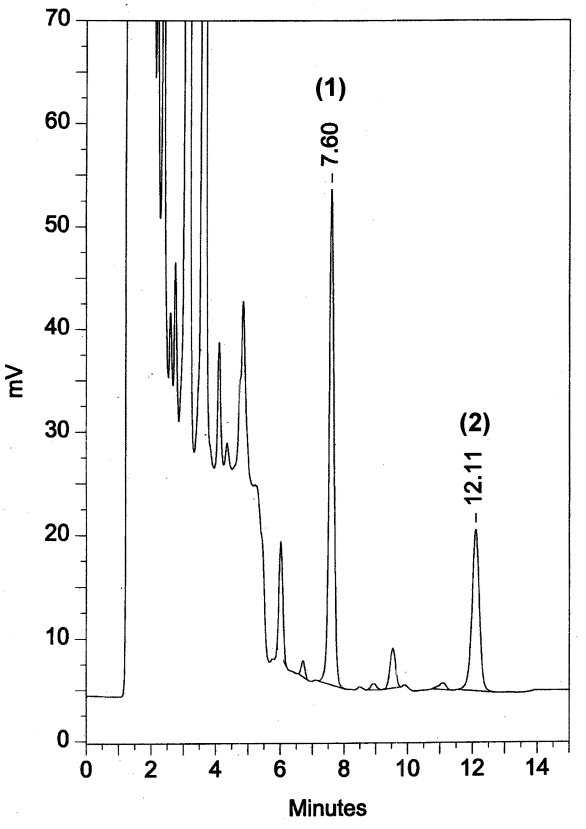


Fig. 5. Chromatogram of a 35 μl injection of a derivatized 1-ml plasma extract from a subject receiving memantine 5 mg p.o. prior to the day of the drug level assay: the concentration of memantine (2) was determined to be 31 ng/ml. (The injection volume for all sample extracts was set at 35 μl.)

pliance was better during inpatient hospital stays. Maintenance on 20 mg memantine daily produced plasma memantine levels ranging from 37 to 121 ng/ml [21]. Several hundred plasma samples have already been assayed over a 5 month period demonstrating the robustness of the procedure.

4. Conclusion

A highly sensitive and selective assayed was developed for the purpose of determining memantine in human plasma. Memantine, as well as other structurally related compounds such as amantadine and rimantadine, are not easily detected by LC using UV detection because of the absence of a chromophoric group. Derivatization with dansyl chloride is a simple and very effective means of enhancing the chromatographic detection of these compounds.

Acknowledgements

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