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Review

Separation methods for tricyclic antiviral drugs

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Abstract

A review of the published analytical methodology for the tricyclic antiviral (TAV) drugs is presented. While amantadine and rimantadine are the only two approved drugs for the prophylaxis and treatment of the influenza A virus, amantadine has also been approved for the treatment of Parkinson's disease. In addition, a few structurally related compounds are finding important clinical applications in other central nervous system-related disorders. To effectively evaluate the pharmacokinetics, biotransformations, stability, and other critical parameters that are necessary for pre-clinical and clinical studies, analytical methodology that conforms to the rigors of regulatory requirements must be developed and made available. This review discusses the analytical methods used in the determination of amantadine, rimantadine, tromantadine and memantine and the pre-clinical and clinical application of these techniques.

Keywords: Reviews; Amantadine; Rimantadine; Tromantadine; Memantine

Contents

1. Introduction

The tricyclic antiviral (TAV) drugs constitute a small but unique part of a diverse class of non-nucleoside therapeutic agents that have been established as effective in the prophylaxis and treatment of influenza A virus infections. The prototype amantadine (1-adamantanamine) was first in this class to be clinically approved in 1966, followed by the α-methyl analog rimantadine in 1993. While immunization remains the preferred mode of prevention

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of many viral diseases, chemoprophylaxis and chemotherapy with these TAVs are complementary to the immunizations for influenza A. The TAVs offer protection during the interim period prior to the production of optimal antibody concentrations following a vaccination, and may have an additive effect of the immunization. These agents often hasten the clinical recovery from influenza A, and generally decrease the subsequent transmission of the virus [1]. While considered equally effective in the prophylaxis and treatment of the influenza A virus, rimantadine has become the TAV of choice, especially in the elderly, due to significantly less central nervous system (CNS) side effects than with amantadine [1,2]. This could be attributed to lower clinically effective plasma concentrations of rimantadine [3]. Thus far, despite a number of reports evaluating numerous adamantane derivatives for their antiviral activity [4–7], amantadine and rimantadine remain as the only approved TAVs. Tromantadine, an N-substituted derivative of amantadine was found to have limited use in the treatment of herpesvirus, but is inactive against influenza A [7].

It is quite common for drugs to have more than one approved clinical application, and the aminoadamantane group of compounds is no exception. While early indications for this class of compounds was limited to antiviral activity only, it was discovered fortuitously that amantadine had reduced the symptoms of Parkinson's disease (PD). It was subsequently investigated and approved for this indication at the same dose [8]. While rimantadine was not considered to be useful in PD, a recent report has suggested that it could have some benefits, and warrants further studies [9]. Memantine, a dimethyl ring-substituted aminoadamantane, is another compound in this class that was originally synthesized for possible use as an antidiabetic agent but was shown to be ineffective. Also ineffective as an antiviral agent, memantine, not surprisingly, was discovered to have potential for the treatment of PD [7,10]. Extensive studies on the pharmacology of memantine have been carried out and are presented in a recent comprehensive review [11]. Most noteworthy are the promising results in the efficacy of memantine in Alzheimer's disease shown in recent clinical trials [12].

The clinically useful aminoadamantane class of compounds has a characteristic 10-carbon rigid tricyclic saturated ring structure (Fig. 1). Because of the primary amino group, they are basic compounds (pK_a=8–10). Substitutions at various locations on the ring will afford differences in therapeutic activity and affect the pharmacokinetics and biotransformations, which could contribute to the overall activity, including side effects [7]. Amantadine and its structural analog rimantadine are extraordinarily stable compounds. Full antiviral activity was retained by both compounds following extended storage (25 years) at room temperature, at 4°C (20 years), and in solutions subjected to various elevated temperatures including boiling [13].

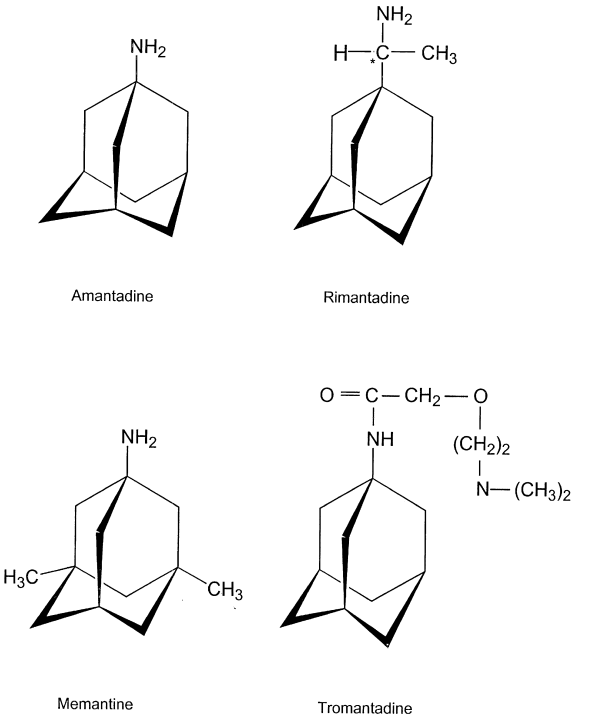

Fig. 1. The chemical structures of the TAVs and related compounds. The asterisk denotes a chiral center.

2. Metabolism and pharmacokinetics

One of the purposes for developing analytical assays for any drug is to determine its fate in living organisms. This includes the absorption, distribution, transformation to one or more metabolites, and finally the excretion both parent drug and metabo-

lite(s). Early pharmacokinetic studies indicated that amantadine is totally absorbed from the gut in all species including man [14]. Over 90% of the amantadine dose was recovered in urine unchanged, while other species excreted lesser amounts. No identifiable metabolites were reported in man, but *N*-methylamantadine was found in blood and urine of the dog. Wesemann et al. [15] identified one hydroxylated metabolite of amantadine, 1-amino-3-hydroxyadamantane, in rat urine. A more definitive study using gas chromatography–mass spectrometry (GC–MS) discovered metabolic transformations in humans following standard therapeutic dosing [16]. While most of the dose (65–85%) was found to be excreted unchanged, *N*-acetylamantadine (5–15%), *N*-methylamantadine and *N*,*N*-dimethylamantadine were also detected in urine. Other minor metabolites all involving the 1-amino group were characterized, but no ring-hydroxylated products were formed in humans. The plasma elimination half-life is reported to be 16.7±7.7 h [17]. Another application of bioanalysis is the attempt to establish a therapeutic plasma concentration range with respect to its antiviral use. Using three different doses in healthy adults (50, 200 and 300 mg/day), steady state levels were achieved after 3 days. Steady state plasma concentrations were determined to be 110±39, 302±80, and 572±207 ng/ml, respectively [18].

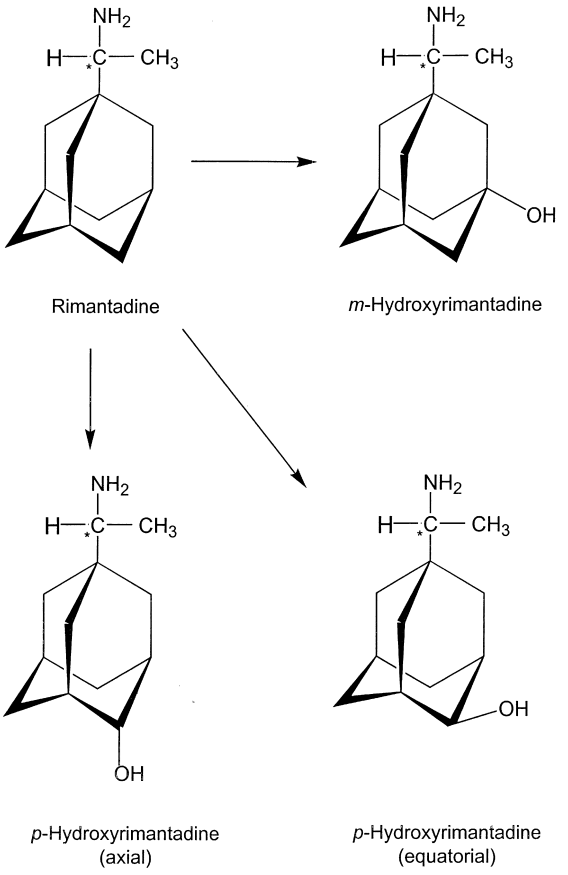

Fig. 2. The primary metabolic pathways of rimantadine in humans.

The metabolic fate of rimantadine differs from amantadine. Only about 22% is excreted in the urine unchanged, but several conjugated and unconjugated ring hydroxylated metabolites were identified in humans [19]. These constituted about 42% of the dose [20]. Hydroxylation occurred on C-3 (*meta*) and C-4 (*para*), with axial and equatorial epimers at the C-4 position (Fig. 2). Because rimantadine is chiral and the subsequent hydroxy metabolites retain the chiral center, each metabolite can and does exist as an enantiomeric pair. Choma et al. [21] demonstrated the existence of all six hydroxy enantiomers in human plasma with significant differences in the formation of the epimers of *p*-hydroxyrimantadine. The antiviral activity of these metabolites are considerably less than rimantadine in vitro, and it is unlikely that they contribute to the overall efficacy of rimantadine. However, their significance in vivo may depend upon their plasma concentrations in rimantadine-treated patients [22]. The plasma elimination half-life of rimantadine in humans is about 36±15 h [17].

The metabolism of memantine, a 3,5-dimethyl substituted amantadine has been studied in rats and man [15,23]. Following a single oral 10 mg dose of memantine in a volunteer, 1-amino-3-hydroxymethyl-5-methyladamantane was identified along with unchanged memantine in urine. It is unlikely that this metabolite contributes to the therapeutic effect of memantine since it was not present in brain tissue of rats, and it corresponded to less than 1% of the parent compound [24]. Memantine has a plasma elimination half-life of about 60 to 100 h [10]. Steady state plasma concentrations in humans following a 20 mg/day dose are reported to be in the range of 37–121 ng/ml [25]. Serum levels of memantine were reported to be in the range of 4–94 ng/ml in dementia patients treated with 5 to 30 mg/day [26]. Cerebrospinal fluid (CSF) in the same

subjects were also assayed and found to be in a CSF/serum ratio of 0.52 and highly correlated to dose. These concentrations are sufficient to interact at certain binding sites of the NMDA receptor.

Tromantadine is clinically used as a topical antiviral, but there is little published data of the pharmacokinetics and metabolism of this drug. In a report that described the metabolic fate of tromantadine, an oral dose of 120 mg was administered to three healthy volunteers and urine was collected over 48 h. More than 50% of the tromantadine dose was eliminated unchanged. Cleavage of the amide bond or the ether bond led the to formation of two principle metabolites, amantadine and 1-adamantyl-(2-hydroxy)acetamide, respectively [27]. Except for amantadine, all other metabolites were conjugated. Allergic contact dermatitis from topical use appears to be a relatively frequent occurrence among patients, and cross-reactivity to other structurally related aminoadamantanes is a possibility [28,29].

3. Analytical methods

A review of the analytical methods for the TAVs was presented in 1990 as part of a comprehensive evaluation of all the antivirals [30]. Since then, a number of new analytical procedures have been published. The following review includes the new procedures as well as the earlier seminal methods. The adamantadine class of compounds are suitable candidates for analysis by GC, GC–MS and, more recently, liquid chromatography (LC) following derivatization to permit adequate detection in biofluids. While the methods discussed claim to be specific for each of the drugs listed, it is very likely that many of the procedures could be easily adapted to their structurally similar analogs. Table 1 summarizes the published GC and LC procedures for the quantitation of amantadine, rimantadine and memantine in biological samples.

3.1. Amantadine

Since amantadine is a primary amine, a number of analytical approaches are available. A GC procedure was introduced in 1965 as part of study to determine the absorption, distribution and excretion of amantadine in various species and tissues [14]. Using various combinations of programmed and isothermal conditions, amantadine was separated on a Carbowax 20M column and detected by flame ionization detection (FID). This followed an extraction procedure using sodium hydroxide and benzene for the initial extraction from blood, urine and tissue samples, then back-extracted into dilute hydrochloric acid, and re-extracted into a small volume of benzene after alkalization. An aliquot of the benzene extract was injected into the gas chromatograph. While this method provided valuable initial data on the disposition of amantadine animals and man, the major disadvantages were the lack of sensitivity (0.1 μg), the long retention time (~20 min) and the lack of a suitable internal standard. Human urine was analyzed for amantadine by Stumph et al. [31], again, by FID but altering the column conditions as well as adding an internal standard β-phenethylamine. This was a poor choice of internal standard because of its natural presence in urine. A single extraction from 5 ml of urine made basic with sodium hydroxide into 0.3 ml of chloroform and injecting 1–3 μl into the gas chromatograph produced a relatively clean chromatogram. The limit of detection was reported to be 500 ng/ml. Belanger and Grech-Belanger [32] also employed FID but used a different column stationary phase and temperature condition to effect a more rapid elution of amantadine (<5 min). Further advantages over the previous two methods include a non-endogenous internal standard (chlorphentermine), and less toxic extraction solvents. Application to plasma and urine samples from a number of human subjects demonstrated its usefulness in the pharmacokinetics of amantadine. However, the detection limits remained high, which would preclude this mode of detection in more rigorous pharmacokinetic studies.

The need for a more sensitive assay for amantadine was satisfied by the use of ^{63}Ni electron-capture detection (ECD) following conversions to electron-rich derivatives. Biandrate et al. [33] derivatized amantadine and the internal standard amphetamine with 2% trichloroacetyl chloride (TCA) in toluene at 70°C for 30 min following a triple extraction from 1 ml of plasma sample. Both derivatives were separated on 100–200 mesh Chromosorb Q coated with 3% OV-17 isothermally at 220°C. The

Table 1
Summary of chromatographic methods for the determination of the clinically useful aminoadamantane drugs in biological samples

Drug	Tissue (volume)	Extraction	Derivatization	Separation stationary phases	Detection	Recovery (%)	Range (ng/ml)	LOQ (ng/ml)	I.S.	Ref.
Gas chromatography										
Amantadine	Blood (5 ml), urine, tissues	LLE	No	20% Carbowax 20M on Chromosorb W	FID	NS	NS	100	No	[14]
Amantadine	Urine (5 ml)	LLE	No	10% Apiezon L 2% KOH on 80–100 mesh Chromosorb W	FID	97	500–1000 (U)	500	Yes	[31]
Amantadine	Plasma (1–3 ml), urine (1 ml)	LLE	No	5% Apiezon L on 100–120 mesh Gas Chrom Q	FID	100 (P) 100 (U)	100–5000 (P) 4–160 μg/ml (U)	NS	Yes	[32]
Amantadine	Plasma (1 ml)	LLE	Yes	3% OV-17 on 100–120 mesh Chromosorb Q	ECD	99	25–1000 (P)	25	Yes	[33]
Amantadine	Plasma (1 ml), urine (1 ml)	LLE	Yes	5% SE on 80–100 mesh Chromosorb W-HP	ECD	100	10–1000 (P, U)	10 (P, U)	Yes	[34]
Amantadine	Plasma (2 ml)	LLE	Yes	10% OV-1 on 80-100 mesh Chromosorb W	ECD	NS	10–500 (P)	10	No	[3]
Amantadine	Plasma (1 ml)	LLE	Yes	HP-1 Megabore capillary	ECD	90–105	2.3–402.9	2.3	Yes	[35]
Rimantadine	Plasma (1 ml)	SPE	Yes	10% OV-1 on 80-100 mesh Chromosorb W	ECD	NS	NS	10	No	[3]
Rimantadine	Plasma (1 ml)	SPE	Yes	10% OV-1 on 80-100 mesh Chromosorb W	ECD	54–72	10–10 000	10	Yes	[47]
Rimantadine	Plasma (1 ml), urine (0.2 ml)	SPE	Yes	Chrompak CP Sil 8 CB capillary	MS-NCI	38 (P) 61 (U)	4.2–416 (P), 21–2077 (U)	4.2 (P), 21 (U)	Yes	[48]
Rimantadine	Plasma (1 ml)	SPE	Yes	DB-1 capillary	MS-EI	>80	0–1500	10	Yes	[49]
Rimantadine & metabolites	Plasma (1 ml), urine (0.2 ml)	LLE	Yes	DB-5 capillary	MS-NCI	87 (P) 90 (U)	5–250 (P), 25–1250 (U)	5 (P) 25 (U)	Yes	[50]
Rimantadine enantiomers	Plasma (1 ml), urine (0.2 ml)	SPE	Yes	DB-1 capillary	MS-NCI	NS	2.5–250 (P) 12.5–1250 (U)	2.5 (P) 12.5 (U)	Yes	[51]
Rimantadine, *m*-hydroxy-, *p*-hydroxy (*a*, and *e*) epimers metabolites enantiomers	Plasma (1 ml)	LLE	Yes	DB-5 capillary	MS-NCI	NS	2.5–250 2.5–50 1.25–62.5 1.25–62.5	2.5 2.5 1.25 1.25	Yes	[21]
Memantine	Various tissues	LLE	No	2% Dexsil 300 on 80-100 mesh Gaschrom P	MS	NS	15–300	NS	No	[24]
Memantine	Blood (5 g) Brain (1.5 g) Liver (5 g)	LLE	No	2% EmulphorOU on 80–100 mesh Gaschrom P	FID	70–80	NS	60	Yes	[23]
Memantine	Plasma (0.5 ml), urine (0.5 ml)	LLE	Yes	HP1 methyl silicone capillary	MS	NS	8.4–267 (P), 80–16 000 (U)	NS	Yes	[53]
Liquid chromatography										
Amantadine	Urine (5 ml)	LLE	Yes	Hypersil ODS 5 μm	UV	NS	100–20 000	75	No	[38]
Amantadine	Plasma, urine (50 μl)	SPE	Yes	NS	FL	97 at room temperature (water)	200–4000 (P, U)	15	No	[39]
Amantadine	Urine (0.1 ml)	No	Yes	TSK gel ODS-80TM, 5 μm	FL	NS	NS	2000	Yes	[40]
Amantadine	Plasma (0.1 ml)	LLE	Yes	Phenomenex ODS	CL	NS	NS	NS	No	[42]
Amantadine	Plasma (50 μl)	LLE	Yes	Cosmosil ODS-MS, 5 μm	CL	74	NS	NS	Yes	[44]
Memantine	Plasma (1 ml)	LLE	Yes	Supelcosil ODS, 5 μm	FL	>90	3–400	3	Yes	[41]

LLE=Liquid–liquid extraction; NS=not stated; P=plasma; U=urine; SPE=solid-phase extraction; FL=fluorescence; UV=ultraviolet absorbance.

method showed linearity between 25 and 1000 ng/ml, and the identity of the GC derivatized peak was confirmed using GC–MS. No retention time data were stated. Using the same internal standard, Sioufi and Pommier [34] employed a 5% SE-30 on Chromosorb W HP 80–100 mesh at 200°C to separate the same TCA derivatives also using ECD. A slight difference in the final extraction procedure removed the excess underivatized TCA. A decrease in the limit of quantitation (10 ng/ml) was a result of the modifications made in this method. As with the previous procedure, chromatographic run-time was not stated, and both methods required extensive column conditioning of greater than 24 h. In attempting to compare the differences in the pharmacokinetics of amantadine and rimantadine, Hayden et al. [3] used GC–ECD following derivatization with pentafluorobenzyl chloride (PFB-Cl) in toluene. No internal standard was used, and the extraction procedure was based upon a previous method [14]. The lower limit of detectability of 10 ng/ml was no improvement over the previous method. Finally, Rakestraw [35], using ECD, improved upon nearly all aspects of the previous methods. From 1 ml of plasma sample made alkaline with sodium hydroxide, amantadine together with the internal standard rimantadine was extracted with toluene. To an aliquot of the toluene extract, a 0.04% solution of pentafluorobenzoyl chloride (PFBO-Cl) in toluene was added and allowed to react for 30 min at 60°C.

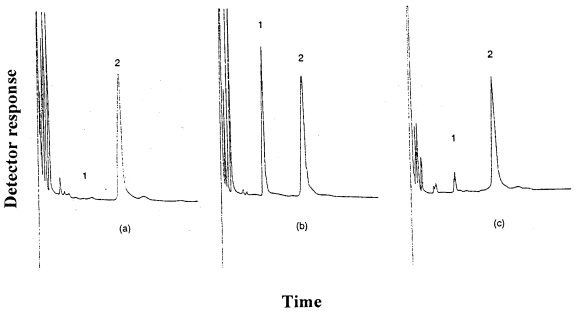

Fig. 3. Chromatographic separation of amantadine (1) and the internal standard rimantadine (2) in human plasma extracts derivatized with PFBO-Cl: (a) 1 ml of control plasma with 100 ng/ml of (2) added; (b) 1 ml control plasma sample with 40.3 ng of (1) added; and (c) 1 ml plasma sample 72 h after receiving a single 100 mg oral dose of (1). (Reproduced from Rakestraw [35] with permission from Elsevier Science).

Unreacted PFBO-Cl was hydrolyzed by sodium hydroxide, and the PFBO derivative of amantadine and internal standard injected on a HP-1 fused-silica megabore capillary column at 180°C. Chromatographic run-time was less than 15 min, with excellent precision at all levels (400 to 2.3 ng/ml) (Fig. 3). This procedure has several advantages over previous methods; increased sensitivity, a less labor-intensive extraction procedure, and an internal standard that is structurally similar to amantadine. The method was used to determine the bioequivalance between to amantadine formulations after a single 100 mg oral dose, with levels being detected up to 5 days.

Although never quantitated using GC with specific nitrogen–phosphorus detection (NPD), amantadine was used as an internal standard (500 ng/ml) in a method developed for the determination of plasma phentermine [36]. Since a derivatization step is not necessary for NPD, this method could be developed into a useful alternative to ECD provided the same sensitivity and specificity as in ECD are retained.

The use of GC–MS for routine quantitation of amantadine has not been reported. However, GC–MS was used in the investigation of the metabolism of amantadine as well as some structurally similar compounds in animals [15]. GC–MS has been extensively used in the bioanalysis of rimantadine, and will be discussed below. The use of NMR was also described in the identification of metabolites of amantadine. Quantitation of amantadine hydrochloride in capsules and syrups by NMR has also been reported [37].

LC with various modes of detection has not been widely reported as a method for routine analysis of amantadine and its structurally similar analogues. This might be explained in part due to the inability to directly detect amantadine by the most common modes of LC detection unless derivatized. Furthermore, GC methodology was probably considered adequate for most analytical applications. As with most of the GC procedures, derivatization is also a necessary step for detection of amantadine and its analogues in LC. Most often, a fluorescent tag is added to the primary amine group following sample extraction. Pre-column derivatizations have been reported using either 1-fluoro-2,4-dinitrobenzene (DNFB) [38], 9-fluoreneacetic acid (9-FA) [39], 3-

(7′-methoxycoumarin-3′-carbonyl)-benzoxazoline-2-thione (MCBT) [40] or dansyl chloride [41]. Van Der Horst et al. [38] used micelle mediated pre-column derivatization with DNFB permitting amantadine to be determined in urine. Using a reversed-phase ODS packing with a mobile phase of acetonitrile–citrate buffer (75:25), the absorption of amantadyl-DNP was monitored at 350 nm using an absorbance detector. The detection limit of amantadine in urine was 75 ng/ml with a retention time of about 5 min. An automated but complex sample preparation was described by Zhou and Krull [39] who reported a direct determination of amantadine in plasma and urine using an automated solid-phase derivatization with 9-FA. A fluorescence detector monitored the resulting amantadine derivative with excitation at 254 nm and emission from 305 to 395 nm. Simultaneous extraction and derivatization of the amine were performed by an automated on-line pre-column reaction that excluded the protein components of the plasma and urine samples. In spite of a detailed description of the preparation of the solid-phase reagent material, the type of analytical column used for the final separation is not mentioned. The practical application of this novel method appears limited to high concentrations of amantadine found in urine since the calibration curves were in the range of 0.2 to 4 μg/ml. No internal standard was used. Another method involving derivatization converts amantadine to its methoxy coumarin derivative [40]. This fluorescent tag permits separation from the internal standard *n*-decylamine using a reversed-phase ODS column with methanol–water (10:1) as the eluent, and monitored at excitation and emission wavelengths of 355 and 405 nm, respectively, at a flow-rate of 0.8 ml/min. Again, this method would be limited to urine samples as the limit of detection is 2.0 μg/ml. Another derivatizing reagent CY5, a dicarbocyanine dye was found to be an efficient far red chemiluminophore tag. Ellingson and Karnes [42] investigated the applicability of this reagent with amantadine in human plasma. Adequate separation of the CY5-amantadine derivative from the unreacted CY5 was achieved using a reversed-phase C_{18} column with a mobile phase of acetonitrile in water adjusted to pH 7 with 0.1% imidazole. The limit of detection was found to be 200 fmol on-column. While a theoretical limit of quantitation was stated at 0.05 ng/ml using 1 ml of plasma sample, this was not actually demonstrated nor validated. Nagaraj et al. [43] applied the same CY5-amantadine derivative to a laboratory constructed capillary electrophoresis system coupled with visible diode laser-induced fluorescence (VDLIF) detection. Validation of the method in plasma provided an LOQ of 1.8 ng/ml with acceptable precision and accuracy, but was not applied to the quantitation of actual plasma samples. Yoshida et al. [44] used amantadine to demonstrate a new chemiluminescence (CL) derivatizing reagent 4-(6,7-dihydro-5,8-dioxoxthiazolo-[4,5-*g*]phthalazin-2-yl)benzoic acid. A commercially available CL detector coupled with LC separated amantadine from the internal standard rimantadine in a run time of less than 30 min. A limited validation study was performed for amantadine, but the LOQ was not specifically determined. Schmermund and Locke [45] described the development of a photoionization detector for reversed-phase liquid chromatography. The somewhat complex procedure involving sample cleanup and trace enrichment through automated valve switching techniques, used a liquid-to-vapor interface, and was capable of detecting 25 ng of underivatized amantadine injected. The application to actual samples was not reported.

Micellar electrokinetic chromatography (MEKC) is a separation technique using conventional capillary electrophoresis instrumentation that selectively analyzes neutral compounds directly in biological fluids. Szulc and Krull [46] reported the application of such a procedure to the analysis of amantadine in urine and plasma with acceptable precision in the concentration of 5–50 ppm in plasma and 25–100 ppm in urine.

3.2. Rimantadine

Not surprisingly, the analytical methods for rimantadine are quite similar to those reported for amantadine, i.e., GC–ECD and GC–MS. Because rimantadine is a chiral compound, several methods report the separation and quantitation of both enantiomers. Additionally, rimantadine appears to undergo extensive metabolism compared to amantadine, and is thus reflected in several analytical methods.

Hayden et al. [3] first reported the analysis of

rimantadine in plasma during a comparative pharmacokinetic study with amantadine. While the method was described previously for amantadine, analysis of rimantadine differed by using a solid-phase extraction technique using cyano-bonded extraction columns. The derivatization (PFB-Cl) and chromatographic conditions (ECD) were the same as for amantadine. Details concerning limit of quantitation and precision for rimantadine were not stated. However, this method was subsequently modified by Hoffman et al. [47] by using amantadine as the internal standard and applied to the pharmacokinetics of rimantadine in various tissues and biofluids of mice and dogs. A limit of detection was reported to be 5 ng/ml with good reproducibility. The quantitative determination of rimantadine in plasma and urine was reported by Fukuda et al. [48] using a GC–MS procedure. Using a solid-phase extraction from cyano-bonded columns, a 1-ml plasma sample or 0.2 ml urine sample containing rimantadine and tetradeuterated rimantadine (internal standard) was eluted from the columns with methanol, evaporated to dryness, and derivatized with PFBO-Cl. Using a CP Sil 8 CB capillary column at 265°C, the retention time was 2.8 min. The mass spectrometer was set for negative chemical ionization (NCI) using methane as the reagent gas. Selected ion monitoring (SIM) was used for quantitation using the ion ratio at (m/z 353)/(m/z 356). The LOQs for plasma and urine were 4.2 and 20.7 ng/ml, respectively. Application of the method to a single dose pharmacokinetic curve was demonstrated. Another GC–MS procedure was reported by Herold et al. [49] that claimed to be a simplified procedure of the previously described method by Fukuda et al. This method introduced a procedure for synthesizing the deuterated internal standard (d_3-rimantadine), and used a *tert*.-butyldimethylsilyl derivative. Using a programmed temperature gradient from 50 to 300°C (at 15°C/min), rimantadine and the internal standard eluted at about 11 min. The peak was monitored at m/z 236 and m/z 239 which are major fragments resulting from the loss of $C_4H_9^{\cdot}$ from both compounds. The limit of detection was 10 ng/ml with good precision across the calibration range. Application of this assay was demonstrated in a group of elderly volunteers receiving 200 mg of rimantadine. Following a report describing the formation of free and conjugated hydroxy metabolites of rimantadine [18], Rubio et al. [50] described a procedure to simultaneously quantitate rimantadine and several hydroxy metabolites in human plasma and urine. Using a liquid–liquid extraction from alkalinized plasma or urine containing the $^2H_4$-labeled reference standards, the extractants were derivatized within the solvent by 2% PFBO-Cl. After evaporating to dryness, the residue was re-dissolved in toluene and injected on a DB-5 capillary column. Rimantadine and its three hydroxylated metabolites were eluted in less than 10 min using a temperature-programmed gradient. As in the previous methods, NCI with methane as the reactant gas was used for detection. The base peak for PFBO-rimantadine is m/z 353 which is the $[M-HF]^-$ ion. The derivatized hydroxy metabolites similarly lose HF under these conditions affording the base peak at m/z 369. The limit of detection for rimantadine and the *m*-hydroxy metabolites was 5 ng/ml, and 2.5 ng/ml for the two epimers of the *p*-hydroxy metabolite in plasma. The values in urine were 25, 25, and 12.5 ng/ml, respectively. Short- and long-term stability of these compounds in plasma and urine were demonstrated.

Unlike amantadine, rimantadine has a chiral center and is administered as a racemate. It is of importance to monitor the disposition of chiral compounds as profound differences could occur in the pharmacokinetics of the individual enantiomers. Miwa et al. [51] reported a GC–MS method for the quantitation of the enantiomers of rimantadine in human plasma, again, using methane NCI mode of detection. Following a solid-phase extraction procedure previously described in an earlier paper [48], rimantadine and tetradeuterated rimantadine were derivatized using the optically active reagent (+)-α-methyl-α-methoxy(pentafluorophenyl) acetic acid (MMPA) in the presence of catalysts to impart NCI sensitivity and to prevent racemization. Removal of excess reagent was effected by a base extraction into dichloromethane, evaporated and redissolved in ethyl acetate. Separation of the formed diastereomers was achieved using a DB-1 capillary column with temperature program gradient, and monitoring the ions at m/z 379 ($[M-HF-CH_3OH]^-$) for the unlabeled enantiomers, and m/z 383 for the tetradeuterated rimantadine. The LOQs in plasma and urine were 2.5 and 12.5 ng/ml, respectively. Several phar-

macokinetic examples in humans illustrate the utility of the method, and indicates that there is no appreciable difference in the unconjugated individual enantiomers in plasma or urine, but one enantiomer, not both, was released following enzymatic hydrolysis in urine. A logical outcome of the enantiomeric method for rimantadine would be the assay of its chiral hydroxy metabolites. Choma et al. [21] presented the method using a similar technique employing MMPA as the derivatizing reagent. Using GC–MS (NCI mode), the diastereomers of the derivatized rimantadine and their diastereomeric derivatized hydroxy metabolites were separated in less than 6 min on a DB-5 capillary column temperature programmed gradient from 150 to 300°C (20°C/min). The actual ions monitored were *m/z* 379 for rimantadine and *m/z* 395 for the hydroxy metabolites (Fig. 4). The LOQ for each enantiomer of rimantadine and *m*-hydroxyrimantadine is 2.5 ng/ml and the enantiomeric epimers of *p*-hydroxyrimantadine is 1.25 ng/ml. Absolute configuration of each enantiomer was assigned based upon model aromatic amine MMPA derivatives. Actual pharmacokinetic data is presented indicating stereospecific differences in the metabolism of rimantadine (Fig. 5).

Rimantadine has also been assayed by capillary zone electrophoresis following derivatization with either 4-methylbenzylamine or 1,2-naphthoquinone-4-sulfonic acid resulting in detection limits of 0.1 or 2 ppm, respectively [52]. The method was applied to rimantadine in pharmaceutical products and for dissolution testing.

3.3. Tromantadine

Tromantadine is a topical antiviral derived from amantadine having a dimethylaminoethoxy acetamide group. A report [27], which discusses the metabolism of tromantadine, briefly described an analytical method using GC–MS with EI or CI mode of detection. Following an extraction from urine with diethyl ether at pH 2 and 9, the solvent was evaporated to dryness and the residue reconstituted with methanol. A 1 μl aliquot was injected on a BD-5 capillary column temperature programmed from 75 to 300°C. This procedure was used for metabolite identification and not for quantitation.

3.4. Memantine

Although devoid of any antiviral activity, the analytical procedures for memantine is included in the review because of the structural similarity to the other previously mentioned tricyclic antivirals. The first reported assay was a GC–MS procedure that featured both amantadine and memantine as the analytes in urine [15]. It was primarily a study in the metabolism of these two compounds, and neither one

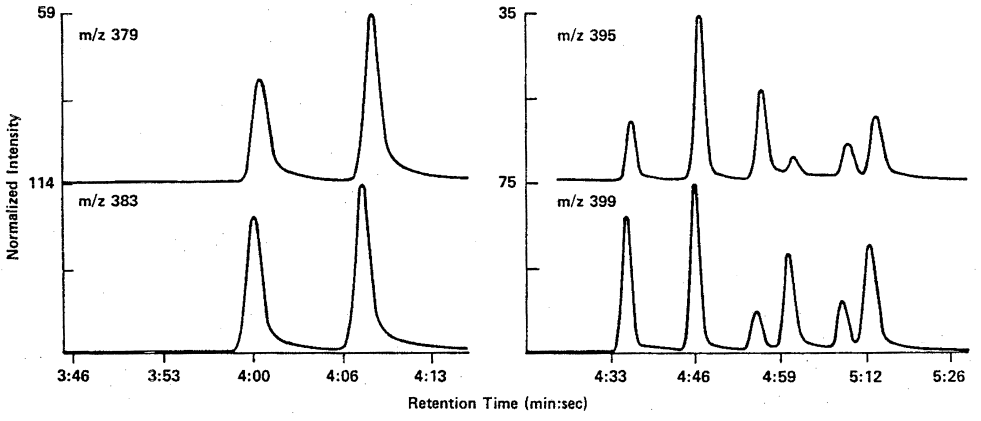

Fig. 4. Selected ion current profiles from a MMPA derivatized extract of a 48 h post-dose plasma sample following a 200 mg oral dose of rimantadine given to a healthy human volunteer. The lower tracings are that of the [$^2H_4$]reference standards. The first diastereomeric pair of ions at *m/z* 379 is rimantadine followed by *m*-hydroxyrimantadine, *p*-hydroxyrimantadine (equatorial) and *p*-hydroxyrimantadine (axial) at *m/z* 395. (Reproduced from Choma et al. [21] with permission from Wiley).

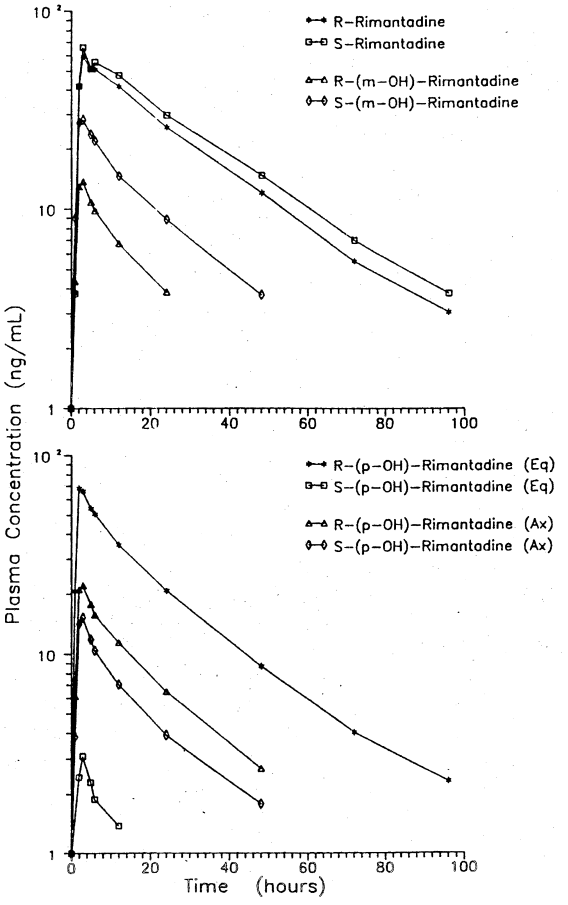

Fig. 5. Plasma concentration–time curves of the enantiomers of rimantadine and its hydroxy metabolites from a human subject following a single 200 mg oral dose. (Reproduced from Choma et al. [21] with permission from Wiley).

was quantitated. Useful mass spectra of amantadine, memantine and some of their respective metabolites found in rats were presented. In a distribution study of memantine in brain, liver and blood of rats, Wesemann et al. [23] reported a method for quantifying memantine with the internal standard *N*-ethyladamantanamine using GC–FID. Using a liquid–liquid extraction procedure from basified sample, both compounds were extracted into *n*-heptane, back-extracted into dilute HCl, then re-extracted into a small volume of *n*-heptane. A 5 μl injection onto a Gaschrom P 80–100 mesh column at 180°C separated both memantine and the internal standard in less than 4 min. Sensitivity was reported to be 1–2 ng/5 μl injection corresponding to about 60 ng/g of tissue. In another study of the distribution and metabolism of memantine in humans, Wesemann et al. [24] applied his previously described GC–MS procedure for the quantitation of memantine. No internal standard was used, and the calibration curve was linear from 15 to 300 pg/ml. A study showing the influence of urinary pH on the excretion of memantine in humans used a validated GC–MS procedure [53]. Using 0.5 ml of alkalinized plasma or urine, memantine (and the internal standard amantadine) was extracted with *n*-hexane. The extract was then derivatized with *N*-methyl-bis-trifluoroacetamide (TFA) and injected on a HP1 capillary column and eluted using a three-step programmed temperature gradient from 50 to 250°C.

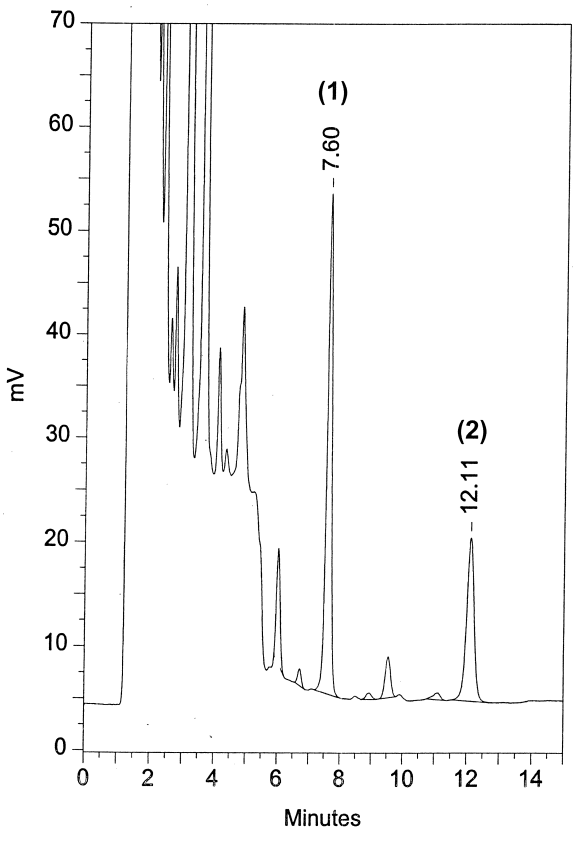

Fig. 6. A chromatogram of a dansyl derivative of memantine (2) and internal standard amantadine (1) of a 1 ml plasma extract from a subject receiving 5 mg orally. Under the same chromatographic conditions, rimantadine elutes at ~13.5 min. (Reproduced from Suckow et al. [41] with permission from Elsevier Science).

The TFA derivatives of memantine and amantadine were monitored at m/z 275 and m/z 247, respectively. Calibration curves for plasma and urine ranged from 8.4 to 267 ng/ml and 0.08–16 μg/ml, respectively, with good inter-assay precision.

Memantine was monitored in plasma as part of a study to determine the effectiveness of this NMDA receptor antagonist in substance abuse patients. A liquid chromatographic method was developed using dansyl chloride as a fluorescence tag for memantine and the internal standard amantadine [41]. Both compounds were extracted from basified plasma with 1.5% isoamyl alcohol in *n*-heptane, then back extracted into dilute acid, evaporated to dryness and derivatized with dansyl chloride at room temperature. The derivatized memantine and amantadine were injected on a reversed-phase C_{18} column, eluted with a mixture of phosphate buffer–acetonitrile (27:73) and monitored at excitation and emission wavelengths of 235 and 470 nm, respectively. The LOQ was 3 ng/ml in plasma with acceptable inter- and intra-day precision, and the run time did not exceed 15 min (Fig. 6). Clinical and stability data for memantine were presented. This procedure could be a suitable alternative to the GC and GC–MS methods for amantadine and rimantadine since both compounds were similarly extracted and derivatized.

4. Conclusions and perspectives

Analytical methodology is an essential component not only in the developmental phase of a drug substance but also in the continuing evaluation of clinical efficacy. It is not usually necessary to routinely monitor patients receiving amantadine or rimantadine since the suggested dose of these agents most often result in steady-state plasma levels that correspond to effective antiviral activity. In cases where compliance is in question, or in the development of toxicity, or when other pathological conditions co-exist that could alter the pharmacokinetics and metabolism, plasma concentration data could be informative and corrective action could be taken. Although memantine is approved in some European countries for several neurological disorders, little data exist relating clinical effects and plasma concentration.

In summary, GC–ECD and GC–MS have proven to be well suited for the separation and quantitation of the aminoadamantane class of drugs. The fact that these compounds lack a suitable chromophore may account for the paucity of LC methods for amantadine and rimantadine. But more than likely, existing GC methodology was more than adequate for separation and quantitation of the aminoadamantanes. Among the published methods reviewed here, the procedure by Rakestraw [35] appears to be a good choice for the quantitation of amantadine for reasons previously discussed. For rimantadine, the choice of methods becomes dependent upon intent. For the analysis of rimantadine only, the method by Fukuda et al. [48] would be a good choice. If the hydroxy metabolites are to be determined, the method by Rubio et al. [50] offers a sensitive, selective and rapid separation. Finally, to simultaneously determine the enantiomers of rimantadine and the enantiomers of its three hydroxy metabolites, the method by Choma et al. [21] is an excellent choice. All of these validated methods for rimantadine involve GC–MS. A validated method for memantine in plasma was published by Suckow et al. [41] using LC with fluorescence detection following derivatization with dansyl chloride. It is very likely that this method could also be adapted to rimantadine or amantadine. Other reports illustrate the potential usefulness of LC and CE as suitable alternatives for assaying these compounds. However, some these new procedures would be difficult to adapt to routine assays of the TAVs since some of the instrumentation and reagents are not commercially available. Unless new TAVs are discovered, it is likely that the existing methodology will suffice. However, new uses for the aminoadamantanes (e.g., memantine) will undoubtedly foster the continuation of new and sensitive procedures that might eliminate the time-consuming derivatization step necessary for detection in both GC and LC procedures. LC–MS and LC–MS–MS, with its various interfaces, have the potential of achieving this goal, but currently have not been reported in the literature.

5. Nomenclature

9-FA	9-Fluoreneacetic acid
CI	Chemical ionization

CL	Chemiluminescence
CNS	Central nervous system
DNFB	1-Fluoro-2,4-dinitrobenzene
ECD	Electron-capture detection
EI	Electron impact
FID	Flame ionization detection
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
LC	Liquid chromatography
LOQ	Limit of quantitation
MCBT	3-(7′-Methoxycoumarin-3′-carbonyl)benzoxazoline-2-thione
MEKC	Micellar electrokinetic chromatography
MMPA	(+)-α-Methyl-α-methoxy(pentafluorophenyl) acetic acid
NCI	Negative chemical ionization
NMR	Nuclear magnetic resonance
NPD	Nitrogen–phosphorus detection
PD	Parkinson's disease
PFB-Cl	Pentafluorobenzyl chloride
PFBO-CL	Pentafluorobenzoyl chloride
SIM	Selected ion monitoring
TAV	Tricyclic antiviral
TCA	Trichloroacetyl chloride
TFA	*N*-Methyl-bis-trifluoroacetamide

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