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Fluorimetric liquid chromatographic analysis of amantadine in urine and pharmaceutical formulation☆

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Abstract

A simple and sensitive liquid chromatographic method is described for the analysis of amantadine and memantine. The method is based on the derivatization of amantadine and memantine extracted from alkalified samples with (2-naphthoxy)acetyl chloride at mild conditions. The resulting derivatives were analyzed by isocratic HPLC with a fluorimetric detector (λ_{ex}, 227 nm; λ_{em}, 348 nm). The linear range for the determination of amantadine or memantine spiked in urine (1.0 ml) was 1.0–10. 0 nmol with a detection limit of about 0.2 nmol ($S/N = 3$; injected sample 20 μl). Only amantadine preparations are available on our local market, and application of the method to the analysis of amantadine in formulation and in the urine of a dosed subject was demonstrated and proved feasible. Quantitation of AT in tablets or capsules is capable in the linear range of 2.0–50.0 μM. Toluene was used as the solvent for extracting amantadine or memantine in samples and the resulting toluene extract was directly subjected to subsequent derivatization without solvent replacement leading to a simpler analytical procedure.

Keywords: Derivatization; LC; Fluorimetry; Amantadine; Memantine

1. Introduction

Amantadine (AT) is used for the treatments of influenza A virus infections and parkinsonism [1], and memantine (MT) is used for parkinsonism, spasmodic and Alzheimer's diseases [2]. AT and MT (Fig. 1) comprise no practical chromophore for being analyzed by common absorption spectrophotometry. Consequently, Detection-oriented derivatization techniques [3,4] coupled with chromatography are frequently used for enhancing a sensitivity. Analytical derivatization of AT or MT in biological samples has been performed, including derivatizing the analyte as trichloroacetamide or pentafluorobenzamide derivative for GC with radioactive electron-capture detector [5,6], as dinitrobenzene derivative for LC-UV [7], as substituted fluorene, coumarin, quinoxaline or dansyl derivative for LC-fluorimetry [8–11], and as Cy5.29 derivative for CE-LIF [12], all leading to improve the sensitivity for analyzing AT or MT. Several methods are reported for the analysis of AT or MT in pharmaceutical formulations, including acid-dye spectrophotometry [13], oscillopolarography [14], ion-selective electrode [15] and capillary isotachophoresis [16] methods, usually giving less sensitivity and specificity as compared to that based on fluorimetry. Additional step for a solvent replacement is needed in the derivatization or sample preparation [7,10–12]; direct analysis of AT in biosample with special solid-phase reagent has been performed [8] and the derivatization of AT in benzene (an eco-unfriendly solvent) at high temperature is also reported [9].

In this work, (2-naphthoxy)acetyl chloride (NAC) [17] was used as a fluorescent reagent for the derivatization of AT and MT in toluene without further solvent replacement for subsequent analysis. NAC is one of the most simple fluorescent reagents for labeling nucleophilic analytes; in structure,

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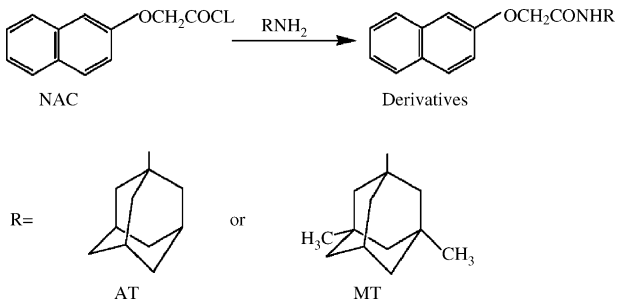

Fig. 1. Reaction scheme for the derivatization of amantadine (AT) and memantine (MT) with (2-naphthoxy)acetyl chloride (NAC). RNH_2 stands for AT or MT.

NAC (Fig. 1) has an alkoxy substitute (auxochrome) attached to the chromophoric/fluorophoric naphthalene system that favors in the UV and fluorescence detection of the resulting derivatives. In addition, NAC contains no amino (basic) or phenol (acidic) function linking to its aromatic system. This may make the resulting derivatives least susceptible to a pH change (such as in a mobile phase) in UV/fluorescence detection.

Application of the method to the analysis of AT in pharmaceutical product and in the urine of a dosed subject proved simple and feasible (MT product is not available for study on our local market). AT is primarily excreted unchanged in urine; it accumulates in plasma and other tissues when renal function declines [18]. Therefore, monitoring AT levels in urine or plasma is useful for therapeutic and toxicological purposes.

2. Experimental

2.1. Chemicals and solutions

Amantadine (AT), memantine·hydrochloride (MT), benserazide, carbidopa and levodopa (Sigma, St Louis, MO, USA), NAC (prepared in our laboratory) [17], 2-isobutoxynaphthalene (internal standard, IS-1, for the analysis of AT in formulation) and 9,10-diphenylanthracene (IS-2, for the analysis of AT and MT in urine) (TCI, Tokyo, Japan), toluene, methanol, tetrahydrofuran and triethylamine (Tedia, Fairfied, OH, USA) were used without further treatment. Other chemicals were of analytical-reagent grade. Solutions of AT and MT were prepared in 0.1 M HCl. Solution of NAC at various concentrations was prepared in toluene. Solutions of IS-1 and IS-2 were prepared by dissolving each compound in toluene containing triethylamine (15 mM).

2.2. LC conditions

A Waters LC system with a model 1515 pump, a Model 717 plus autosampler and a Model 474 fluorimetric detector and a Breeze data system were used. A Merck Purospher Star RP-18e (125 mm × 4 mm I.D.; 5 μm) and a mixed solvent of methanol–water (91:9, v/v) at a flow rate of 0.8 ml/min were used for the analysis of pharmaceutical sample. A Supelco Discovery HS C18 (150 mm × 4.6 mm I.D.; 5 μm) and a mixed solvent of methanol–tetrahydrofuran–water–triethylamine (75:10:15:0.02, v/v) at a flow rate of 1.0 ml/min were used for the analysis of urine sample. The column separated derivatives were detected fluorimetrically (λ_{ex} 227 nm; λ_{em} 348 nm).

2.3. Sample preparation

2.3.1. For pharmaceutical sample

Sample solutions of commercial amantadine capsules (labeled amount of 100 mg amantadine per capsule) and amantadine tablets (labeled amount of 100 mg amantadine per tablet) were prepared as follows: 20 capsules of amantadine content or 20 tablets of amantadine were weighed and finely powdered in an agate mortar. An aliquot of 10.0 mg of the powder was placed in a screw-capped test tube (8 ml) and added with 0.5 ml of water. The mixture was sonicated for 15 min and then alkalified with NaOH (5 M, 100 μl). The alkalified mixture was extracted twice with toluene (1.0 ml each) by vortex mixing (2 min). The combined toluene layers were filtered to a volumetric flask (10.0 ml) and diluted with toluene to the volume (10.0 ml). A 100 μl aliquot of the toluene solution from AT capsule or 200 μl of the toluene solution from AT tablet was further diluted with toluene to a final volume 10.0 ml. Aliquots (300 μl) of the diluted solutions of the samples were subjected to the derivatization (Section 2.4).

2.3.2. For urine sample

For AT and MT spiked urine, aliquots (1.0 ml) of normal urine were added with AT and MT (10 nmol each in 100 μl of 0.1 M HCl); in parallel, for AT dosed urine, a 1.0 ml aliquot was used (diluted with 0.1 M HCl if necessary). The urine sample (spiked or dosed, 1.0 ml each) was alkalified with NaOH (4 M, 100 μl) and extracted with toluene (1.0 ml) by vortex mixing (1 min). The mixture was then centrifuged at 1800 × *g* for 10 min. Aliquots (300 μl) of the supernatant toluene layer were subjected to the derivatization (Section 2.4).

2.4. Derivatization of AT and MT

Aliquots (300 μl) of the toluene solutions for the analysis of AT in pharmaceutical samples or aliquots (300 μl) of the toluene extracts for the analysis of AT and MT in urine were taken to series of screw-capped test tubes (25 ml). IS-1 (12 μM, 100 μl) was added to the formulation samples and IS-2 (300 μM, 100 μl)) was added to the urine sample, and then 100 μl of NAC (1 mM) was added to each tube. The reactants were shaken at 30 °C for 6 min in a thermostated shaker. After derivatization, 250 μl of methanol was added and the solution was further reacted for 8 min to inactivate excess

NAC (methanol also makes the resulting solution compatible with the mobile phase for LC analysis). The methanol-treated solution was subjected to LC analysis.

3. Results and discussion

For optimizing the conditions for extracting and derivatizing AT (15 nmol) from pharmaceutical products or AT and MT from spiked urine (10 nmol each of AT and MT), several key parameters were studied, including the volume of toluene for extraction and the amount of the reagent (NAC) for derivatization. The optimization was evaluated basing on the resulting peak-area ratios of the derivatives to the IS-1 or IS-2. As an internal standard, IS-1 was used for the analysis of AT in pharmaceutical preparations leading to a short run time (<8 min); but IS-1 is coeluted with unknown urine matrix peaks and can not be used as an internal standard for the analysis of AT in urine. Therefore IS-2 with higher retention (than IS-1) was selected for the analysis of AT in urine (see latter section). Both IS-1 and IS-2 are fluorescent compounds that were directly used as internal standards in the study without derivatization.

3.1. Extracting and derivatizing AT from pharmaceutical formulations

An amount (10.0 mg) of the pulverized powder from AT tablet or capsule was macerated with water (0.5 ml) in an 8 ml test tube. The mixture was alkalified with NaOH (5 M, 100 μl) and extracted with toluene (in the range of 0.3–2.0 ml) by sonication for 15 min. The plateau formation of the AT derivative from various toluene extracts is attainable with the toluene volumes ≥0.5 ml. The effects of various concentrations of NAC (0.1–10 mM) on the derivatization (Section 2.4) of the extracted AT indicate that plateau formation of the derivative is obtainable using NAC ≥1 mM. The optimum derivatizing time (at 30 °C) is rapid in about 6 min, reflecting the high reactivity of the acyl reagent.

3.2. Extracting and derivatizing AT and MT from urine

Aliquots (1.0 ml) of urine samples were alkalinized (4 M NaOH, 100 μl) and extracted with various volumes of toluene (0.5–3.0 ml). The results (data not shown) indicated that equilibrium extraction of AT or MT is attainable using toluene with volumes ≥1.0 ml.

The effects of various concentrations of NAC (0.1–10 mM with 100 μl each) on the derivatization (at 30 °C, 6 min) of AT and MT extracted from urine shows that (Fig. 2) the plateau formation of the derivatives of AT or MT can be attained using NAC at concentrations ≥1 m*M*. The optimized conditions obtained were formulated in the procedures for the preparation and derivatization of AT and MT in the previous sections. Obviously the procedures are simple and rapid for the analysis of the analytes in urine.

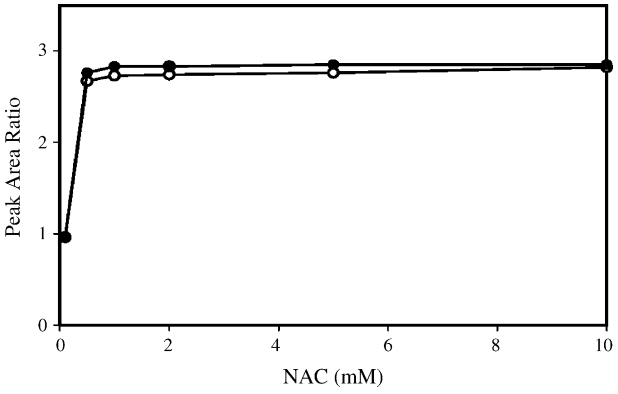

Fig. 2. Effects of various concentrations of the derivatizing reagent (NAC) on the formation of the derivatives. Amantadine (◯) and memantine (●) derivatized with NAC at 30 °C for 6 min. See Section 3.2 for the details.

3.3. Stability of the derivatives

The stability of AT or MT derivative at room temperature after the derivatization was studied up to 48 h. No significant changes in the peak-area ratios of the analytes to the IS-1 or IS-2 were observed. This indicates that the derivatives are sufficiently stable for the time required for the LC analysis.

3.4. Analytical calibration for AT in pharmaceutical formulation

The quantitative applicability of the method for the determination of AT was evaluated at five different concentrations of AT over the range 2–50 μM. Calibration graph was established with *y* for the peak-area ratios of AT derivative to the IS-1 and *x* for the concentration (μM) of AT. The linear regression equation was obtained as follows: $y = (0.0452 \pm 0.0008)x - (0.0015 \pm 0.0001)$ with an $r = 0.999$ ($n = 5$). The results indicate that a good linearity between *y* and *x* is attainable over the range studied (2–50 μ*M*). The relative standard deviation (RSD) of the method based on the peak-area ratios for replicate determination of AT at 3, 20 and 40 μ*M* was studied. The results in Table 1 indicate that the percentage

Table 1
Precision and accuracy for the determination of AT

Concentration known[a] (μM)	Concentration found (μM)	RSD[b] (%)	R.E.[b] (%)
Intra-day ($n = 5$)			
3.0	2.9 ± 0.0	0.0	−3.3
20.0	20.4 ± 0.2	1.0	2.0
40.0	40.3 ± 0.3	0.7	0.8
Inter-day ($n = 7$)			
3.0	2.9 ± 0.1	3.4	−3.3
20.0	20.3 ± 0.5	2.5	1.5
40.0	40.1 ± 0.6	1.5	0.3

[a] Intra- and Inter-day analyses, respectively for five replicate analysis and seven consecutive days.

[b] RSD and R.E., respectively for relative standard derivation and relative error [(value found − value known)/value known].

RSD and relative errors (R.E.) for the intra-day ($n = 5$) and inter-day ($n = 7$) are numerically all below 3.5%.

3.5. Analytical calibration for AT and MT spiked in urine

The quantitative applicability of the method was studied for AT and MT. spiked in urine (1.0 ml) at five different amounts of AT and MT each over the range of 1–10 nmol. The calibration graphs were established with the peak-area ratios of AT or MT derivative to the IS-2 as the ordinate (y) versus the amount of AT or MT (nmol) spiked in urine as the abscissa (x). The linear regression equations obtained are $y = (0.272 \pm 0.002)\ x + (0.028 \pm 0.001)$ with an $r = 0.999$ ($n = 5$) for AT, and $y = (0.277 \pm 0.008)\ x - (0.025 \pm 0.001)$ with an $r = 0.999$ ($n = 5$) for MT. The results indicate that the applicable quantitation range for AT or MT spiked in urine is 1–10 nmol. The upper limit of the quantitation for AT or MT was not extensively studied, because AT or MT at higher levels can be diluted before this analysis. The detection limit ($S/N = 3$) of AT or MT was 0.2 nmol spiked in urine, equivalent to about 1.6 pmol of AT or MT injected (20 μl).

The intra- and inter-day precision (RSD) and accuracy (R.E.) were studied on the analysis of AT and MT each at three levels. The results (data not shown) indicate that the RSD and R.E. for intra- and inter-day analyses are numerically below 2.5%.

3.6. Selectivity of the method

The selectivity of the method was briefly studied on the separation of a standard mixture of some antiparkinsonian related drugs with amino function that could be derivatized with the fluorescent reagent (NAC), including benserazide, carbidopa and levodopa (in addition to AT and MT). The analytes (20 μM each) were derivatized by the derivatization procedure (Section 2.4). The results indicate that no interference peaks overlap with that of amantadine, memantine and IS-2. In structure, benserazide, carbidopa and levodopa each has an additional polar group (carboxyl function) that can make the resulting derivatives more polar than that of amantadine or memantine leading to their faster elution (than AT, MT or IS-2) at the present LC conditions.

3.7. Application of the method to the analysis of AT in pharmaceutical product

The method was applied to the analysis of AT in capsules and tablets that were available on the local market. The results in Table 2 indicate that all the analytical values fell within 95–105% of the labeled range usually required by a pharmacopoeia [19]. Fig. 3 shows the typical chromatograms for the analysis of AT in pharmaceutical products.

The recoveries of the method were briefly studied by spiking and mixing known amounts of AT to suitable amount of finely triturated capsule or tablet contents. After extraction and dilution as indicated in Section 2.3.1, the resulting sample solutions were prepared to contain known levels of added AT at 6.6, 13.2 and 19.8 μM in addition to the unknown level of AT existed in the formulated product. The analytical results (Table 3) indicate that the average recoveries for the analysis of the spiked drug analytes are all above 98.0%.

3.8. Application of the method to the analysis of AT in urine

Drug products of MT are not available on the local market for study. Therefore, application of the method was demonstrated to the analysis of AT in biosample. A normal subject (female, 38 years, 50 kg) was orally dosed with AT capsule (100 mg of AT·HCl per capsule). After dosing, the urines were successively collected at suitable times up to 72.5 h. Fig. 4 shows the typical chromatograms for the analysis of AT in urine, indicating that no significant peaks from blank urine interfered with that of the analytes and the IS-2. But IS-1 can not be used as an internal standard for the analysis of AT in urine, because it elutes faster than AT derivative (peak 1 in Fig. 4A) and overlaps with those complicate peaks from

Table 2
Assay results for amantadine in pharmaceutical products

Sample[a]		Amount found[b] (mg)		RSD (%)		Claimed content (%)	
A	B	A	B	A	B	A	B
A1	B1	101.6 ± 0.9	100.9 ± 0.5	0.8	0.5	101.6	100.9
A2	B2	99.5 ± 1.8	100.3 ± 0.9	1.8	0.9	99.5	100.3
A3	B3	97.7 ± 1.0	102.1 ± 0.7	1.0	0.7	97.7	102.1
A4	B4	101.3 ± 1.3	100.2 ± 1.2	1.3	1.2	101.3	100.2
A5	B5	99.7 ± 1.4	100.7 ± 1.4	1.4	1.4	99.7	100.7
A6	B6	100.7 ± 0.7	99.6 ± 1.1	0.7	1.1	100.7	99.6
A7	B7	101.5 ± 1.8	101.8 ± 0.6	1.8	0.6	101.5	101.8
A8	B8	99.8 ± 1.4	100.9 ± 1.4	1.4	1.4	99.8	100.9
Mean						100.2	100.8
SD						1.3	0.8

[a] AT capsules (A; 100 mg of AT hydrochloride per capsule) and AT tablets (B; 100 mg of AT sulfate per tablet) obtained on Taiwan market.
[b] Mean ± SD ($n = 3$).

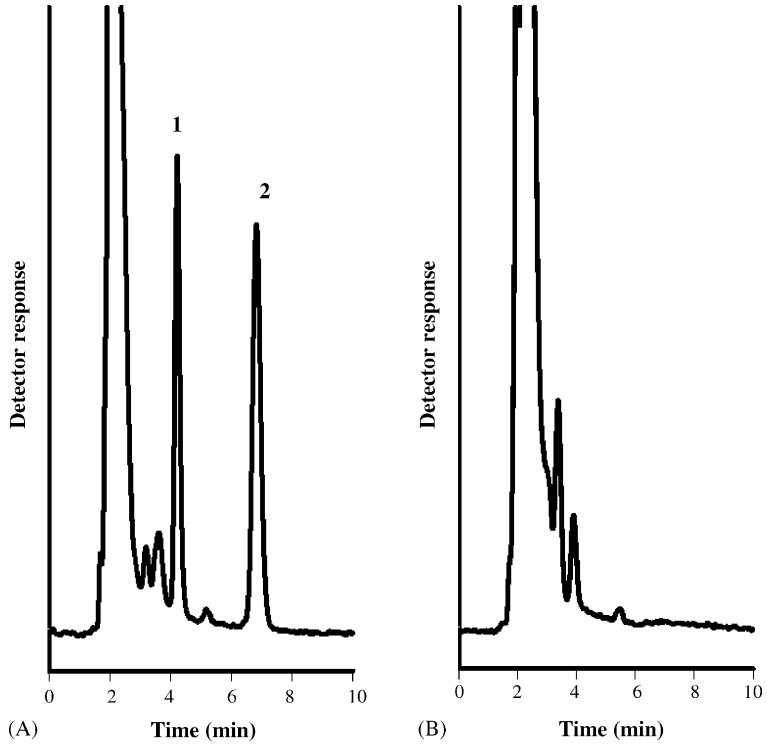

Fig. 3. Chromatograms for the analysis of amantadine (AT) in tablets (A) and reagent blank (B). Peaks: 1 for the internal standard (IS-1) and 2 for AT derivative. LC conditions: C_{18} column (125 mm × 4 mm I.D.; 5 μm); mobile phase (methanol–water, 91:9, v/v) with flow-rate (0.8 ml/min); fluorimetric detection (λ_{ex} 227 nm, λ_{em} 348 nm).

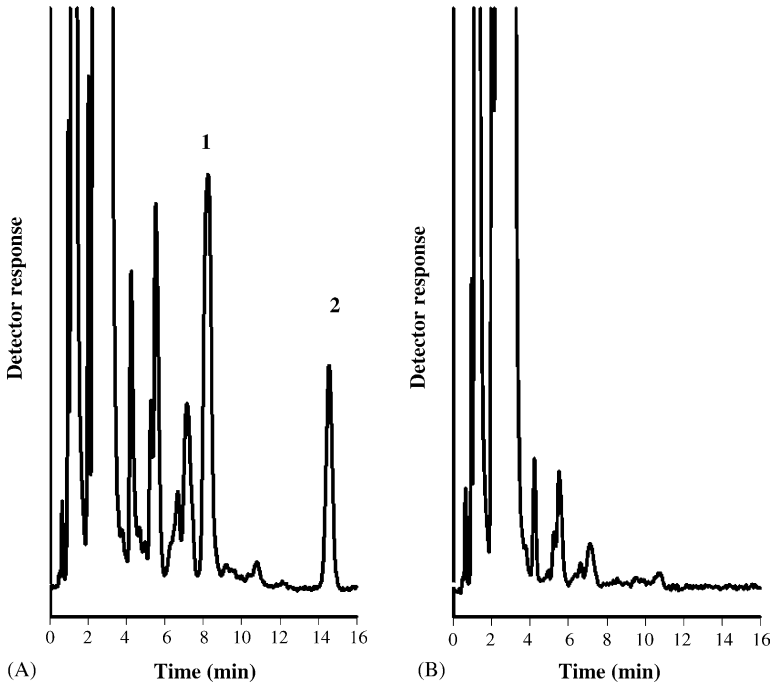

Fig. 4. Liquid chromatograms for amantadine (AT) extracted from urine and derivatized with NAC (A) and blank urine (B). Peaks: 1 for AT derivative and 2 for the internal standard (IS-2). LC conditions: C_{18} column (150 mm × 4.6 mm I.D.; 5 μm); mobile phase (methanol–tetrahydrofuran–water–triethylamine, 75:10:15:0.02, v/v) with flow-rate (1.0 ml/min); fluorimetric detection (λ_{ex} 227 nm, λ_{em} 348 nm).

Table 3
Analytical results for the recovery of amantadine in pharmaceutical products

Sample[a]	Concentration spiked (μM)	Concentration found (μM)	Recovery[b] (%)
AT capsules			
A	0.0	26.5 ± 0.3	–
B	6.6	33.0 ± 0.6	98.5
C	13.2	39.7 ± 0.8	100.0
D	19.8	46.7 ± 0.2	102.0
AT tablets			
E	0.0	11.7 ± 0.0	–
F	6.6	18.2 ± 0.4	98.5
G	13.2	25.0 ± 0.5	100.8
H	19.8	31.9 ± 0.2	102.0

[a] AT capsules and AT tablets were from Taiwan market; sample A in AT capsules and sample E in AT tablets respectively prepared from finely powdered AT capsules and AT tablets products without addition of the known levels of the drugs expressed as 0 μM spiked.

[b] Recovery defined as the term of (amount found − amount spiked) divided by the amount found in the zero spiked sample; mean ± SD (n = 3).

urine matrix. Fig. 5 shows the cumulative excretion of AT in urine, indicating that AT is primarily excreted unchanged in urine as reported [18,20]. The LC conditions (see Fig. 3) of the column and the mobile phase for the analysis of AT in formulation can not be used for the analysis of AT and MT in urine with complicated matrix. Therefore, a longer separation column of C18 with a mixture of mobile phase was used (see Fig. 4) for the separation of AT and MT resulting in a good resolution of the analytes and IS (Fig. 6).

In conclusion, a highly sensitive liquid chromatographic method was established for the analysis of AT and MT. Application of the method to the analysis of AT in urine and formulations proved feasible. The method is simple and efficient, using the same solvent for the extraction and derivatization of AT and MT prepared from tested or spiked sample. Further application of the method to the analysis of other lipophilic amines with simple and sensitive detection can be expected.

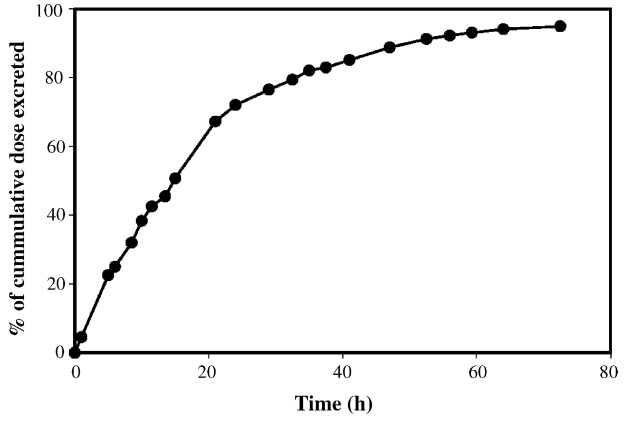

Fig. 5. Cumulative urinary excretion of amantadine for a volunteer dosed orally with 100 mg of amantadine·HCl capsule.

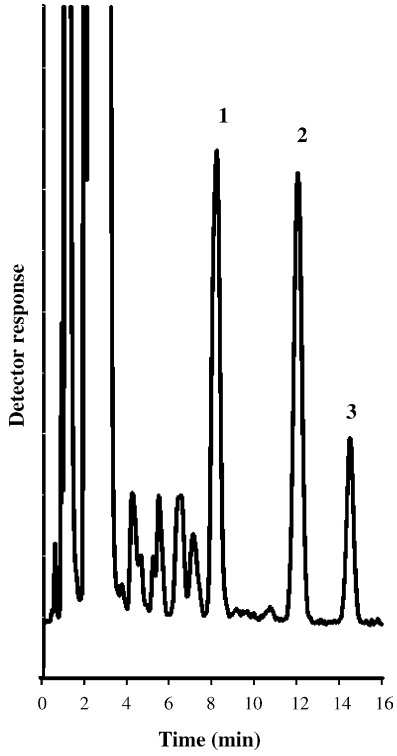

Fig. 6. Liquid chromatogram for amantadine (AT) and memantine (MT) (10 nmol each) spiked in urine and derivatized with NAC. Peaks: 1 for AT derivative, 2 for MT derivative and 3 for the internal standard (IS-2). LC conditions as indicated in Fig. 4.

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