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Development of a liquid chromatography–mass spectrometric method for measuring the binding of memantine to different melanins

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Abstract

A sensitive and selective liquid chromatography–mass spectrometric method was validated for the determination of free memantine in melanin binding studies. The sources of melanin studied were sepia, synthetic and bovine melanin. Memantine was chromatographed on a reversed-phase column (Prodigy 5 μm, ODS(3), 100 Å, 100×4.6 mm) using gradient elution with mobile phases of 0.1% formic acid in deionised water and 0.1% formic acid in methanol at a flow-rate of 0.8 ml/min. The mode of ionisation was atmospheric pressure–electrospray and detection by single ion monitoring of the memantine ion *m*/*z* 180. Validation of the method showed that the assay was linear from 0.1 to 1200 n*M* and 0.5 to 1200 n*M* memantine in deionised water and phosphate-buffered saline (PBS), respectively. Accuracy for sample preparations in deionised water was between 80 and 108% and between 80 and 123% for PBS. For both media, intra- and inter-day precision was below 1% for retention time and below 5% for analyte peak area. At the LLOQ, the variation of peak area was less than 17%. Binding of memantine to melanin was measured indirectly by determining the unbound fraction of memantine. After incubation of melanin with memantine, the sample was centrifuged and filtered to separate the memantine–melanin complex effectively from suspension. The filtrate was then assayed for free memantine from which the extent of binding was then calculated.

Keywords: Drug binding; Memantine; Melanins

1. Introduction

Memantine (1-amino-3,5-dimethyladamantane hydrochloride, Fig. 1) has putative neuroprotective properties by blocking the calcium channels activated by *N*-methyl-D-aspartate (NMDA) receptor

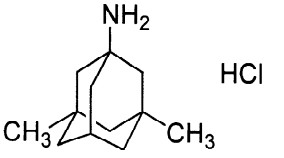

Fig. 1. Structure of memantine hydrochloride. Molecular formula: $C_{12}H_{21}N \cdot HCl$; molecular mass: 215.76 (free base: 179.20); partition coefficient, log(*P*): 3.28; basicity, pK_a: 10.42; solubility of the hydrochloride salt: 3.5% in a pH 6.5 aqueous solution at 25 °C.

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stimulation [1,2]. Excessive activation of NMDA receptors is thought to mediate the calcium-dependent neurotoxicity associated with neurodegenerative diseases. Currently, memantine is used for the treatment of Alzheimer's and Parkinson's disease. However, its neuroprotective properties have suggested that memantine may be of benefit for the treatment of glaucoma. The mode of action is thought to be due to the prevention of damage to retinal ganglion cells as a result of increased intraocular pressure [3–6].

The accumulation of a drug in melanin-rich tissues may have serious physiological consequences as it could lead to potentially toxic effects [7–11]. Despite several investigations into the nature of drug–melanin binding, the exact mechanism of the interaction remains unknown [12–16]. Drug–melanin binding is a phenomenon that has been observed with structurally and pharmacologically unrelated drugs following administration by ocular and other routes. Of the drugs with known melanin affinity, many are positively charged at physiological pH and it is generally accepted that ionic interactions are a major contributor. Other factors involved in the reversible binding are the drug's lipophilicity, van der Waals forces and the ability to form charge–transfer complexes.

Whereas in vitro studies may reveal the underlying mechanism of interaction and affinity between melanin and drug, in vivo studies demonstrate which physiological processes predominate. In particular, cellular barriers restrict access to the melanin binding sites and cellular location also influences accessibility. Isolated melanin has a smaller particle size and is suspended in solution while intracellular melanin is surrounded by membranes which hinder accessibility. Drugs with a higher lipophilicity diffuse through these membranes more easily than less lipophilic ones. However, this may be of minor importance with respect to the binding mechanism of the drug to melanin. Moreover, proteins bound to melanin do not affect the binding characteristics as shown by comparison of the findings of native and hydrolysed (protein free) melanin [17].

Memantine free base, which is both highly basic (pK_a 10.42) and lipophilic (log P 3.28), suggests that it may show binding to ocular melanin due to ionic interactions of its basic primary amine group. The high partition coefficient implies that there would be a good permeability of memantine through biological tissues. A mechanistic understanding of the melanin binding of memantine is critical to analysing its ocular disposition.

Since memantine lacks a useful chromophore, it cannot be readily assayed by HPLC–UV techniques [18]. Consequently, memantine has to be either derivatised for HPLC–fluorescence measurement [19–21], determined by capillary zone electrophoresis with indirect UV detection [22], measured by GC without derivatisation [23–26] or for enhanced sensitivity derivatised and analysed by GC [27–30]. Liquid chromatography–mass spectrometry provides high sensitivity with high selectivity while avoiding a tedious derivatisation step.

The purpose of this study was to validate a LC–MS method for the determination of memantine in memantine–melanin binding studies. Three sources of isolated melanin were used (bovine, sepia and synthetic) and the incubation media were deionised water and phosphate-buffered saline. As melanin is virtually insoluble in aqueous media, the unbound fraction of memantine was separated from the melanin–memantine complex by centrifugation and subsequent filtration. The unbound fraction was then assayed and quantified and from these data, the binding kinetics of memantine to melanin was determined.

2. Experimental

2.1. Chemicals and reagents

Memantine hydrochloride was obtained from Merz & Co (Frankfurt, Germany). Melanin sepia officinalis, synthetic melanin (by oxidation of tyrosine with hydrogen peroxide) and formic acid were purchased from Sigma–Aldrich (St Louis, MO, USA). Bovine melanin was isolated according to an in-house procedure [32]. Potassium phosphate monobasic was purchased from EM Science (Gibbstown, NJ, USA). Sodium chloride and sodium phosphate dibasic heptahydrate were provided by Allergan (Irvine, CA, USA). Phosphoric acid (85%) and sodium hydroxide (50% w/w) were purchased from Mallinckrodt–Baker (Phillipsburg, NJ, USA). Methanol was of HPLC grade and obtained from Burdick & Jackson (Muskegon, MI, USA). Deion-

ised water was obtained from a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Sample preparation

Silanised glassware was used to avoid memantine adsorption. Solutions were stored at room temperature for a maximum of 3 days.

2.2.1. Phosphate-buffered saline (PBS)

Phosphate-buffered saline prepared in deionised water contained sodium chloride (154 m*M*), potassium phosphate monobasic (1.5 m*M*) and sodium phosphate dibasic (8.5 m*M*). The pH of the buffer was adjusted to 7.4 using either sodium hydroxide or phosphoric acid.

2.2.2. Determination of linearity, accuracy and precision

Separate standard solutions of memantine HCl were prepared in deionised water and PBS. Stock solutions of memantine HCl (10 μ*M*) were prepared from 1000 μ*M* solutions. Appropriate volumes of the stock solution of memantine HCl (10 μ*M*) were used to prepare solutions in the concentration range 0.1–1200 n*M*. A series of solutions in deionised water ($N=9$) and PBS ($N=8$) were prepared to determine linearity between concentration and detector response. Only four standard solutions were used for daily calibration of the instrument.

Samples for the determination of accuracy were prepared in deionised water and PBS. Weights (20 mg) of sepia, synthetic and bovine melanin were separately suspended in deionised water (10 ml) or PBS (10 ml) and sonicated for 15 min. Each suspension was split into several aliquots and transferred into centrifuge tubes (Flex-tube, polypropylene, Eppendorf, Hamburg, Germany) and centrifuged at 14 900 *g* for 15 min. The supernatants were withdrawn into a syringe and filtered through individual syringe filters (Nylon Acrodisc, 0.2-μm pore size, 25-mm diameter, Gelman Sciences, Ann Arbour, MI, USA). The first 0.5–0.6 ml of each filtrate were discarded to avoid interferences by leachable contaminants. The filtrates from the split samples were then combined for every melanin type and suspension medium.

For the assessment of accuracy, equal volumes of the six filtrate pools were mixed with an equal volume of the memantine HCl solution of the respective solvent. The concentrations of the memantine HCl solutions were 2400, 600 and 20 n*M*. A blank for every melanin type and suspension media was prepared by mixing equal volumes of filtrate and respective solvent. Controls were prepared by mixing equal volumes of memantine HCl solution and respective solvent.

2.2.3. Preparation of binding samples

Suspensions (2.0 mg/ml) of sepia, synthetic and bovine melanin were prepared in deionised water and PBS, sonicated for 15 min and warmed up to 37 °C prior to incubation with memantine HCl. While being stirred, a volume (1.0 ml) of the melanin suspension was transferred into an incubation container (5 ml, polypropylene, VWR, San Diego, CA, USA) and mixed with a memantine HCl solution (1.0 ml, 1500 n*M*) of the appropriate solvent (deionised water or PBS).

To improve sample homogeneity, the containers were placed horizontally in the temperature-controlled shaker, set to 37 °C and 100 rpm. Binding samples were prepared in triplicate for each time point. Controls were prepared by incubating memantine HCl solution separately with deionised water and PBS. In a similar fashion, blanks were prepared by incubating melanin suspension separately with deionised water and PBS.

At 10, 20, 30, 45, 60 and 90 min, the samples were transferred into a centrifuge tube and centrifuged at 14 900 *g* for 15 min. The supernatant was then drawn into a syringe and filtered through a Nylon Acrodisc syringe filter. The first 0.5–0.6 ml of the filtrate were discarded and the remaining solution was filtered directly into a HPLC vial (0.75 ml, polypropylene, VWR, San Diego, CA, USA), which was vortexed and analysed.

2.3. Instrumentation

LC–MS analyses were carried out using a HP1100 Series HPLC system connected to a HP 1100 MSD mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and integration was controlled by Agilent Technologies ChemStation software.

Buffer solution pH values were measured using a Beckman–Coulter Φ10 pH Meter (Fullerton, CA,

USA), calibrated prior to use with Beckman–Coulter pH standard buffer solutions of pH 4 and 7.

Melanin binding samples were incubated using an Environ-shaker from Lab Line Instruments (Melrose Park, IL, USA). Separation of the melanin–memantine complex from the unbound memantine fraction was accomplished using a Baxter Biofuge A (Deerfield, IL, USA).

2.4. Analytical conditions

HPLC separations were carried out on a Prodigy ODS(3) column (100×4.6 mm, 5-μm particle size, 100 Å pore size) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were 0.1% (v/v) of formic acid in deionised water and 0.1% (v/v) of formic acid in methanol, respectively. The mobile phases were filtered through a 0.45-μm Nylaflo nylon membrane filter from Gelman Sciences (Ann Arbour, MI, USA) prior to use.

The ratio of mobile phase A and B at the start of the analysis was 50% A and 50% B. A linear gradient over 3 min was then used to increase the fraction of mobile phase B to 70% and was followed by an isocratic period for 2 min. Over the following 0.5 min, a linear gradient was used to restore the mobile phase ratio to initial conditions and retained for 3.5 min. In all experiments, the flow-rate was 0.8 ml/min with an injection volume of 100 μl. The autosampler and column were not temperature controlled.

Splitless positive electrospray ionisation was accomplished at atmospheric pressure. The flow of the drying gas was set to 13.0 l/min at a temperature of 300 °C. The nebuliser pressure was set to 206.84 kPa (30 p.s.i.). The capillary entrance was set to a voltage of 3500 V and the exit to 70 V. Detection was carried out in single ion mode monitoring an ion of m/z 180. The electron multiplier was set to 10 000 V, gain to 1.0, dwell time to 580 ms and ion width was set to 0.05 m/z.

2.5. Quantification

Minitab 13 (State College, PA, USA) was used to assess calibration curves by linear regression of peak area to memantine concentration with the use of a weighting factor of $1/x^2$ (where x is the concentration of memantine HCl). Samples were bracketed by sets of four standard solutions.

2.6. Validation

Selectivity was determined by LC-MS analysis of blank and spiked samples of melanin. Sepia, synthetic and bovine melanin were suspended in both deionised water and PBS and prepared in the same manner as the binding samples. Selectivity was demonstrated when no interfering peaks co-eluted with memantine.

Precision for the retention time and peak area of memantine HCl was determined from the replicate measurement of three standard solutions between 2 and 1200 nM on one day (intra-day) and again on a different day (inter-day). Acceptance criteria for intra-day and inter-day precision (RSD) were less than 5% for retention time and less than 10% for peak area.

The acceptance criterion for accuracy for each melanin type in deionised water and PBS was set to be within 80 and 120% of the nominal concentration, for the three concentrations employed.

The acceptance criteria for linearity were a correlation factor (r^2) above 0.97 and an intercept not significantly different from zero. The regression equations obtained were tested for zero intercept using a published method [31].

The lower limit of quantification was set to the concentration that had an intra-day precision for peak area just below 20%.

The syringe filters that were used in the process of sample preparation were tested for contamination of the sample and adsorption of memantine to the filter. A solution of memantine HCl (5.66 nM) in deionised water was filtered through syringe filters at a filtration rate of about two drops per second. Two fractions of 0.5 ml and one of 1.0 ml were collected for analysis.

3. Results and discussion

3.1. Selectivity

Representative LC–MS chromatograms of blank and spiked preparations of sepia, synthetic and

bovine melanin are shown in Fig. 2. The chromatograms demonstrate that the assay was selective for memantine with a single peak for memantine (t_R = 3.6 min) which was not present in the melanin controls.

3.2. Precision

Precision (N = 6) determined for three solutions of memantine HCl ranging between 2 and 1200 nM prepared in deionised water and PBS showed a

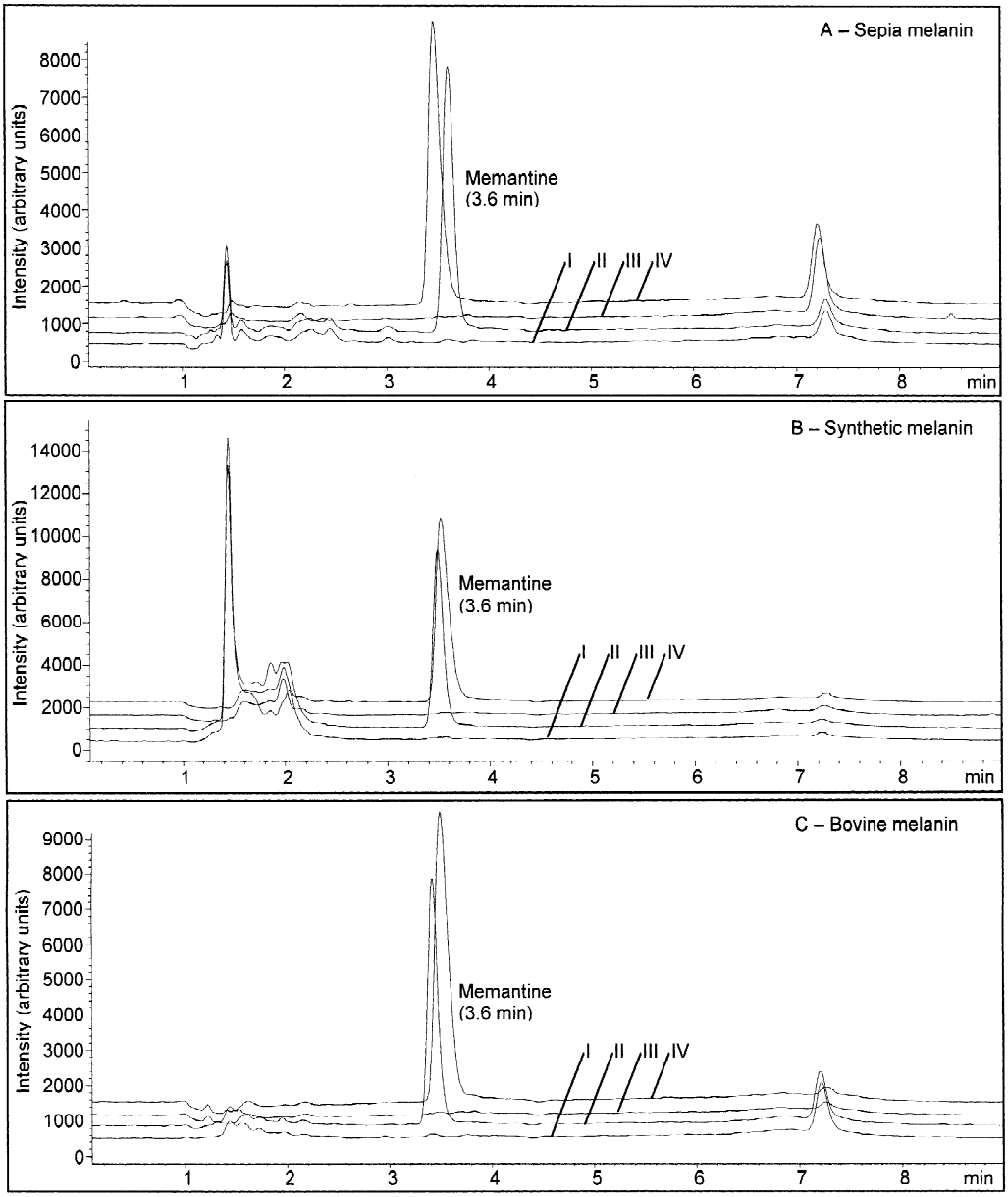

Fig. 2. Single ion LC–MS chromatograms of sepia melanin (A), synthetic melanin (B) and bovine melanin (C). I: blank, melanin suspended in deionised water; II: melanin suspended in deionised water and spiked with memantine (10.2 nM); III: blank, melanin suspended in PBS and IV: melanin suspended in PBS and spiked with memantine (10.2 nM).

variability of less than 5% for peak area and less than 1% for retention time. Determined on a different day, the precision values for peak area and retention time were within the same limits.

The intra-day precision ($N=6$) determined for suspensions of sepia, synthetic and bovine melanin prepared in deionised water and PBS containing memantine HCl was less than 2% and 1% for peak area and retention time, respectively (Table 1).

The set acceptance criteria for peak area (10%) and retention time (5%) were met at all memantine concentrations and for both sample solvents. Chromatographic and mass spectrometric reproducibility of the assay was thus demonstrated for memantine HCl solutions prepared in deionised water and PBS and melanin as sample matrix.

3.3. Accuracy

The accuracy data for melanin preparations in deionised water and PBS and spiked at three concentrations are summarised in Table 2.

For both media, matrices and all concentrations sample accuracy was within the given range of 80–120% of the nominal concentration with one exception. For the preparation of synthetic melanin in PBS at the lowest memantine concentration, accuracy exceeded the set range (122.4%). However, this was accepted as it was only slightly above the limit and at the lower concentration level where a greater variance can be tolerated. This will not affect the performance of this assay for its intended purpose.

3.4. Linearity

Results from weighted linear regression and test for zero intercept of standard solutions prepared in deionised water and PBS are summarised in Table 3. Variations of slope and intercept were within acceptable limits and the correlation coefficients were more significant than the lower limit ($r^2=0.97$). For all regression equations, the calculated *t*-value was well below the critical *t*-value, demonstrating that the intercept was not significantly different from zero. A zero intercept also demonstrated that there was no effect by factors not investigated (e.g. solvent, adsorption) on method accuracy.

3.5. Lower limit of quantification

Standard solutions of 0.10 n*M* memantine HCl in deionised water and of 0.50 n*M* memantine HCl in PBS showed a precision ($N=6$) of 14.3 and 16.7%, respectively. The limit for intra-day precision for the LLOQ was set to be just below 20% as this would result in adequate accuracy and precision for the intended use of this assay. These concentrations were set as the LLOQ for preparations in deionised water and PBS, respectively, as the precision values were close to the acceptance criteria.

3.6. Sample contamination with filter material

The analysis of the filtrate fractions obtained after filtration of memantine solution through a nylon syringe filter showed that substances leached from the filter into the filtrate. Although these substances

Table 1
Intra-day precision ($N=6$) of melanin suspensions in deionised water (H_2O) and PBS containing memantine (305.56 n*M*)

		Sepia		Synthetic		Bovine	
		t_R (min)	Peak area	t_R (min)	Peak area	t_R (min)	Peak area
H_2O	Mean	3.620	1 776 067	3.551	1 870 625	3.430	1 710 232
	SD	0.010	17 213	0.022	26 849	0.002	8481
	RSD	0.3	1.0	0.6	1.4	0.1	0.5
PBS	Mean	3.466	1 484 503	3.519	1 498 322	3.504	1 496 462
	SD	0.003	10 449	0.002	9986	0.004	7339
	RSD	0.1	0.7	0.1	0.7	0.1	0.5

Table 2
Accuracy of spiked samples prepared in deionised water (H_2O) and PBS

Sample	Solvent	N[a]	Nominal conc. (nM)	Calculated conc. (nM)	Accuracy (%)	RSD
Sepia	H_2O	4	1222.24	1168.8	95.6	0.2
	PBS	4	1222.24	981.3	80.3	2.4
	H_2O	8	305.56	315.8	103.3	0.9
	PBS	8	305.56	267.2	87.5	0.6
	H_2O	4	10.19	9.2	90.3	0.3
	PBS	4	10.19	10.6	104.1	0.6
Synthetic	H_2O	4	1222.24	1199.2	98.1	0.5
	PBS	4	1222.24	1022.3	83.6	2.2
	H_2O	8	305.56	328.3	107.4	2.4
	PBS	8	305.56	284.8	93.2	1.0
	H_2O	4	10.19	10.2	99.7	1.0
	PBS	4	10.19	12.5	122.4	1.1
Bovine	H_2O	4	1222.24	1124.3	92.0	1.9
	PBS	4	1222.24	994.6	81.4	0.2
	H_2O	8	305.56	308.4	100.9	0.7
	PBS	8	305.56	285.0	93.3	0.6
	H_2O	4	10.19	8.2	80.1	3.4
	PBS	4	10.19	12.0	118.2	1.3
Control	H_2O	2	1222.24	1241.0	101.5	0.4
	PBS	2	1222.24	998.3	81.7	0.2
	H_2O	2	305.56	314.4	102.9	0.2
	PBS	2	305.56	295.3	96.6	0.2
	H_2O	2	10.19	9.2	90.6	2.6
	PBS	2	10.19	11.9	116.5	1.0

[a] For every concentration, two samples and one control were prepared and assayed twice, but of the medium concentration one sample was assayed six times for the determination of precision.

Table 3
Linearity and test for zero intercept of standard solutions prepared in deionised water (H_2O) and PBS

Solvent	Set	N	Linearity			Zero intercept	
			Slope (nM^{-1})	Intercept	Correlation coefficient (r^2)	t-value ($\alpha = 0.05$)	Calculated t-value
H_2O	A	9	5571	614	0.985	2.306	0.0003
	B	9	5820	269	0.993	2.306	0.0001
	C	9	5801	−98	0.998	2.306	<0.0001
	Mean		5731	262	0.992		
	SD		139	256	0.005		
	RSD		2.4	136.1	0.5		
PBS	A	8	6185	3908	0.989	2.365	0.0014
	B	8	6232	2620	0.989	2.365	0.0010
	C	8	5929	2697	0.975	2.365	0.0013
	Mean		6115	3075	0.984		
	SD		163	722	0.008		
	RSD		2.7	23.5	0.8		

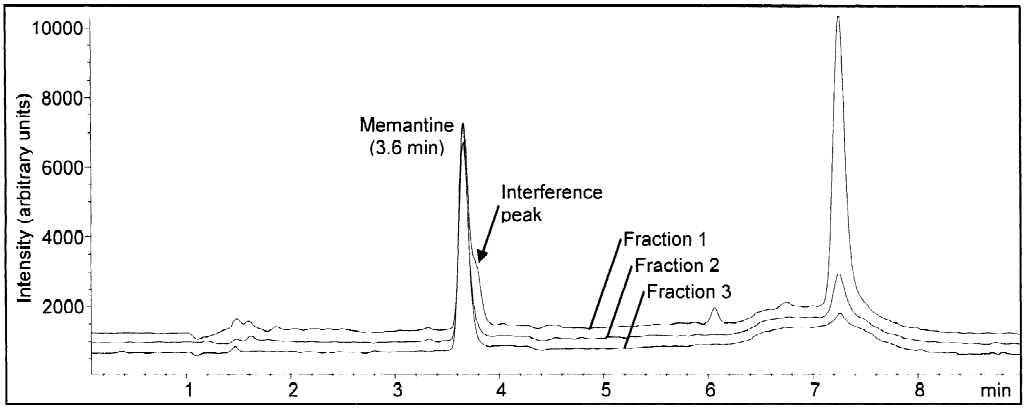

Fig. 3. Analysis of fractions of memantine solution (5.66 n*M*) in deionised water filtered through syringe filter. Fraction 1: 0–0.5 ml; Fraction 2: 0.5–1 ml; Fraction 3: 1–2 ml.

have the same molecular mass as memantine, only one was of concern as it eluted only slightly later than memantine (Fig. 3, fraction 1). However, the analysis of the memantine filtrate fractions showed that this interferent was present only in the first 0.5 ml of filtrate (Fig. 3).

From these results, it was concluded that in the process of sample preparation the first 0.5 ml of filtrate had to be discarded. With this procedure, any interference from the co-eluting compound and adsorption of memantine to the filter material was avoided.

To avoid the presence of compounds which might interfere, filters of the same pore size and diameter but different membrane materials (polysulfone, hydrophilic polyethersulfone and hydrophilic polyvinylidene) were tested. Although these filters did not leach any substances, they were not used because they adsorbed memantine (approximately 10% of concentration tested), which would have necessitated a larger volume of elution (>2 ml) to saturate the filter.

3.7. Melanin binding

The data for the binding of memantine to sepia, synthetic and bovine melanin in deionised water and PBS for various times are shown in Fig. 4. For all types of melanin and incubation media the melanin–memantine binding process appeared to occur virtually instantly and the binding remained constant after the initial 10-min incubation time. Experimental variation, as indicated by the error bars, was small for each melanin type and incubation media.

Interestingly, the differences in the extent of melanin–memantine binding depend on the melanin type and the nature of incubation medium (Fig. 4). In deionised water, sepia and bovine melanin bound about 97% of the available memantine and synthetic melanin bound about 70%. However, when incubated in PBS, the binding characteristics were significantly different. Of the available memantine sepia melanin bound about 59%, synthetic about 41% and bovine melanin bound about 30%.

4. Conclusion

A simple, sensitive and selective LC–MS method for the analysis of memantine in melanin–binding samples was developed and validated. It was demonstrated that the method was linear in the range from 0.1 to 1200 n*M* with deionised water as sample matrix and from 0.5 to 1200 n*M* with PBS as matrix. Appropriate precision and accuracy were shown for both sample media.

Centrifugation and subsequent filtration of the binding sample proved to be a simple and effective means of separating the melanin–memantine complex from unbound memantine that required no further sample preparation.

The results clearly illustrate that the binding of

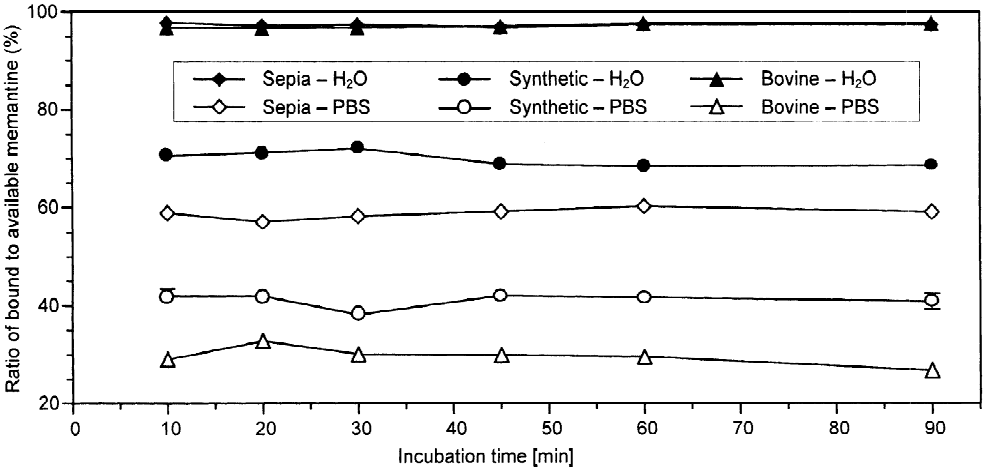

Fig. 4. Binding of memantine to different melanins in deionised water (H_2O) and PBS. Error bars indicate the standard deviation of three measurements and are generally encompassed by the symbol. All samples contained 750 n*M* memantine HCl and 1 mg/ml melanin.

memantine to melanin occurs rapidly as has been previously reported [32,33] and is virtually complete within an incubation time of 10 min. Bovine and sepia melanin suspended in deionised water showed the highest binding capacity for memantine. However, when melanin was prepared as a suspension in PBS, the overall binding was lower and bovine melanin, in contrast to synthetic melanin in deionised water, bound the least amount of memantine. This indicates that the binding to melanin is strongly influenced by the presence of electrolytes. This observation is in agreement with previously reported studies with different ligands where the extent of melanin binding was also altered [7,34]. Moreover in this study, the magnitude of binding of memantine to melanin (synthetic, sepia and bovine) was affected differently by the presence of electrolytes. Hence, for studies of binding of drugs in the eye, the use of the appropriate melanin source appears to be important [35] and further studies are in progress to investigate the influence of melanin binding when memantine is administered by various ocular routes.

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