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High-performance liquid chromatographic determination of amantadine in urine after micelle-mediated pre-column derivatization with 1-fluoro-2,4-dinitrobenzene*

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Abstract: Cationic micelles have been used for the derivatization of the anti-Parkinson drug amantadine with the chromophore 1-fluoro-2,4-dinitrobenzene in urine. In the presence of 90 mM cetyltrimethylammonium bromide (CTAB), the conversion of amantadine into its derivative is complete within 4 min at 60°C and pH 11. Such a short reaction time allows a fully automated pre-column derivatization of amantadine in an on-line combination with reversed-phase high-performance liquid chromatography. This cannot be attained when using purely aqueous derivatization mixtures because then the reaction takes some 20 min at the same temperature. Without the use of an internal standard, the repeatability of the automated determination at the 0.5 $\mu g\ ml^{-1}$ level is ca. 6%, whilst the detection limit is 75 $ng\ ml^{-1}$ (S/N = 3). The present study clearly demonstrates that micellar systems can be beneficially used for the on-line pre-column derivatization of amines in urine.

Keywords: *Amantadine; micelle-mediated pre-column derivatization; 1-fluoro-2,4-dinitrobenzene; urine; HPLC.*

Introduction

The derivatization of a drug with a label containing a chromophore or a fluorophore prior to high-performance liquid chromatography (HPLC) is often necessary to attain the required detection sensitivity and/or selectivity. Unfortunately, because of the solvation of (polar) analytes by water molecules, the reaction rate in aqueous physiological matrices may be reduced in the case of, e.g. nucleophilic substitutions such as those involving the reaction of amines with the UV label 1-fluoro-2,4-dinitrobenzene [1]. Traditionally, the derivatization rate is enhanced by increasing the reaction temperature or by extracting the drug from the aqueous matrix into a suitable organic solvent prior to derivatization. The latter approach is also often necessary in the case of carboxylic acids [2].

Surprisingly, only little attention has been paid to the application of micellar systems for the pre-column derivatization of drugs in bioanalysis [1, 3–5], despite the fact that the rate-enhancing properties of micelles have been amply demonstrated [6, 7]: reaction rates can be accelerated up to 100-fold compared with those in aqueous solutions. This means that very fast reaction rates can be obtained in aqueous (physiological) matrices solely by the addition of micelles. Generally speaking, the "catalytic" properties of micelles can be attributed to two factors [8]. Firstly, the reaction probability increases if the analyte and the reagent become concentrated in the much smaller volume of the micellar phase. Secondly, the aprotic and hydrocarbonaceous micellar core may cause an increase of the reaction rate such as is demonstrated for aromatic nucleophilic substitutions [1, 8].

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Another significant advantage of micellar derivatization systems is that they are compatible with subsequent reversed-phase high-performance liquid chromatography (RP–HPLC); that is, the injection of aqueous micellar derivatization mixtures will not adversely affect the performance of RP–HPLC systems [1, 4].

Recently, first evidence has been presented of the utility of micelles in the derivatization of carboxylic acids in aqueous matrices [4, 5, 9–11]. For example, it has been demonstrated that by using an on-line micelle-mediated pre-column derivatization procedure, plasma free fatty acids can be determined with RP–HPLC in a fully automated system [11]. The issue of the present study is to investigate the potential of micelle-mediated derivatization of amines in physiological matrices with the UV label 1-fluoro-2,4-dinitrobenzene (DNFB), prior to RP–HPLC analysis. As an example, the automated RP–HPLC determination of amantadine [1-aminoadamantane, Symmetrel^{r} (Fig. 1)] in urine is presented, which is carried out in the presence of cationic cetyltrimethylammonium bromide (CTAB) micelles. The anti-viral and anti-Parkinson drug amantadine was selected as a model compound, because it cannot easily be determined by common HPLC detection procedures; in practice, amantadine is only determined by gas chromatographic techniques [12–16].

The development of a simple HPLC determination for amantadine and related compounds may be useful for routine purposes as well as pharmacokinetic studies.

Experimental

Chemicals and solutions

Amantadine was purchased from Janssen Chimica (Beerse, Belgium) and disodium tetraborate decahydrate from Baker (Deventer, The Netherlands). DNFB, CTAB, tetramethylammonium bromide, trisodium citrate, acetonitrile, methanol and dioxane were from Merck (Darmstadt, FRG). All chemicals were of analytical reagent grade. Urine was collected from healthy volunteers.

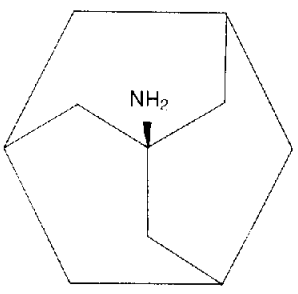

Figure 1
Structure of amantadine

DNFB was added to acetone to yield a concentration of 0.8 M and stored at 4°C. A 400 mM sodium borate buffer was prepared in demineralized water, and was adjusted to pH 11 by the addition of sodium hydroxide. CTAB was dissolved in methanol at a concentration of 300 mM.

Batchwise derivatization procedure

In order to investigate the kinetics of the derivatization reaction, batchwise experiments were carried out, as described previously for *n*-alkylamines [1]. The derivatization vials were prepared by pipetting a known volume of the methanolic CTAB solution into a type 3813 vial (Eppendorf, Hamburg, FRG). Next, methanol was evaporated at room temperature. These vials can be stored for at least several weeks at ambient temperature. A volume of 900 µl of spiked urine or water and 100 µl of borate buffer were pipetted into the derivatization vial which was, subsequently, vortexed for 15 s.

After addition of 100 µl reagent solution derivatizations were carried out in a laboratory-made thermostatted swerve-waterbath. After selected times the derivatization reaction was terminated by the addition of an equal volume of dioxane–concentrated hydrochloric acid (99:1, v/v). In this manner, precipitates which may form during the derivatization step were completely dissolved, and did not cause problems in subsequent HPLC processing of the samples.

Automated derivatization procedure

The samples were prepared as follows: 5.0 ml of spiked urine were adjusted to about pH 11 with 10 M sodium hydroxide and subsequently centrifuged at 3000 *g* for 10 min. The volume of the added sodium hydroxide solution was always <2% of the total volume. Next, 450 µl of urine and 50 µl of borate buffer were pipetted into the derivatization vials which were, subsequently, vortexed for 15 s and placed in a Gilson (Villiers-le-Bel, France) 232/401 autosampler.

These samples were processed fully auto-

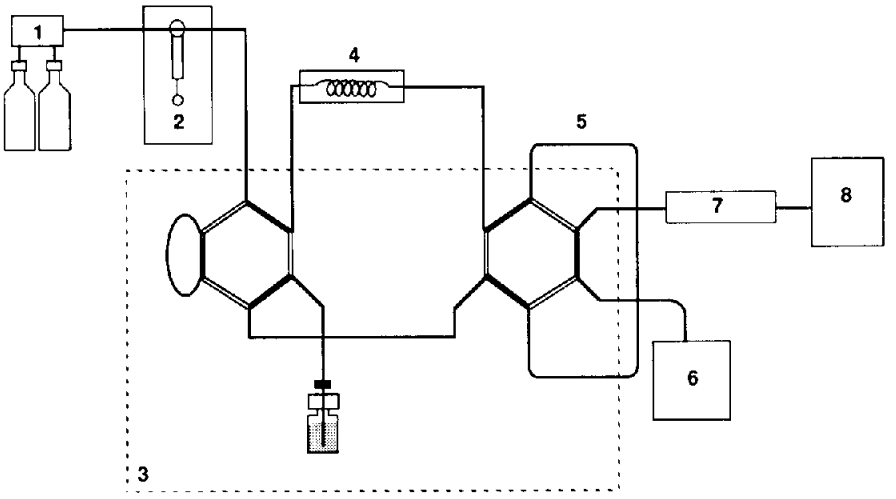

Figure 2
Schematic of the automated derivatization procedure of amantadine: (1) Low-pressure solvent selector; (2) Gilson 401 low-pressure syringe pump; (3) Gilson 232 autosampler; (4) 100 × 0.5 mm i.d. PTFE reaction coil (60°C); (5) 25-µl sample loop; (6) Spectroflow 400 pump; (7) analytical column; and (8) Spectroflow 757 UV absorbance detector (350 nm).

mated by the Gilson 232/401 autosampler (Fig. 2). After 25 µl of the DNFB reagent have been added to the sample, and mixing has been carried out, 100 µl of the sample are transferred to a laboratory-made reaction coil (PTFE tubing, 0.5 mm i.d.; Omnifit, Cambridge, UK) which is thermostatted at 60°C. After a 4-min residence time — with repetitive forward and backward moving — the sample plug is transferred to a 25-µl sample loop, and a heart-cut valve-switching procedure is used into transport the contents of the loop to the RP–HPLC system. The low-pressure PTFE tubing (0.5 mm i.d.; Omnifit) is subsequently rinsed with 1 ml of acetonitrile and 1 ml of water in a backflush mode. For this, a multipurpose stream-switch unit (MUST; Spark, Emmen, The Netherlands) that is connected to the inlet of the Gilson 401 pump serves as a solvent selector.

The sample processing and HPLC separation are carried out concurrently to ensure an optimal sample throughput.

Chromatography

The chromatographic system consisted of a Spectroflow 400 pump (Kratos, Ramsey, MA, USA) and a 150 × 3 mm i.d. analytical column packed with 5 µm Hypersil ODS (Shandon, Runcorn, Cheshire, UK). The mobile phase was acetonitrile–10 mM citrate buffer (pH 2.5) (75:25, w/w) which contained 20 mM tetramethylammonium bromide. The HPLC effluent was monitored at 350 nm using a Spectroflow 757 (Kratos) absorbance detector, while retention times and peak areas were measured with an SP 4270 integrator (Spectra-Physics, Santa Clara, CA, USA).

Results and Discussion

Figure 3 shows the schematic of the derivatization of amines with DNFB in a cationic micellar system [1]. Generally speaking, in the micellar system both the protonated and the deprotonated species of the amine are present. The ratio between the species is determined by

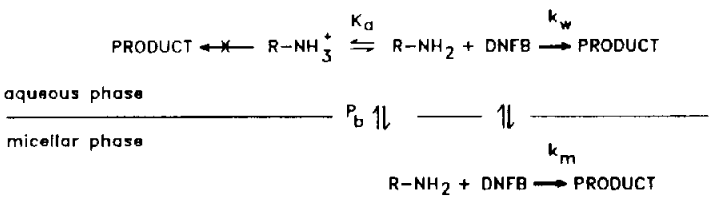

Figure 3
Schematic of the derivatization reaction in the micellar system. The partition coefficient, P_B, determines the partitioning of the deprotonated amine ($R—NH_2$) between the aqueous bulk and the micellar phase. In both the aqueous bulk and the micellar phase, the amine reacts with the reagent, DNFB; the rate constants are k_w and k_m, respectively. K_a is the acidity constant of the amine in the aqueous bulk solution.

the acidity constant (K_a) of the amine and by the pH of the micellar solution. Because only the deprotonated species exhibits significant reactivity, the pH of the derivatization medium should be chosen at least 1–2 units above the pK_a of the amine in the micellar solution to ensure a high reaction rate. Next to the concentration of the deprotonated species, the environment of the reaction complex also has an important effect on the derivatization rate. In micellar solutions two reaction constants can be discerned [8], viz. that for the aqueous bulk solution, termed k_w, and that in the micelle, termed k_m. The contribution of these reaction constants to the overall derivatization rate is, of course, significantly affected by the partition coefficient of the deprotonated amine, P_B, and of the reagent DNFB between the micellar and the bulk phase [1].

Optimization of the derivatization system

The initial experimental set-up for the micelle-mediated derivatization of amantadine in urine was based on a previous study [1]. Here a derivatization system was used which contained 35 mM CTAB, 8 mM DNFB and 33 mM borate buffer (pH 11). However, in the batchwise as well as the automated procedure, this system did not perform well in the case of urine samples.

Firstly, it was observed that both the reaction rate and the yield were less than those in buffer solutions. This could not be attributed to an insufficient amount of the reagent, but appeared to be due to a fast decrease of the apparent pH in the derivatization medium. This decrease is caused by the release of a large amount of hydrogen ions in the reaction between DNFB and significant quantities of compounds apparently present in urine, which can be derivatized. Obviously, the buffer capacity of the system was insufficient to maintain a pH above the pK_a of 9.1 (determined titrimetrically [1]) for amantadine in the micellar solution, which is required to attain a high reaction rate. The borate concentration could not be substantially increased because of the limited borate solubility in the aqueous stock solution. The pH problem was solved, however, by making the urine alkaline (ca. pH 11) prior to derivatization.

A second problem was that during the derivatization in urine, a flocculent precipitate formed in the micellar solution. This interfered with the automated processing of the samples and markedly affected the repeatability of the determination. The formation of a precipitate was attributed to the presence of compounds in urine which readily react with DNFB, because in buffer samples no such precipitation was observed. To improve the solubilization power of the micellar system, the final CTAB concentration was increased from the earlier 35 mM to 90 mM, at a slight expense of the reaction rate [1]. With derivatization times up to ca. 10 min this modification prevented precipitation and, simultaneously, the repeatability of the determination improved. The increase of the CTAB concentration give problems when preparing the micellar stock solution, because CTAB was insufficiently soluble in the concentrated borate buffer. To avoid undesired dilution of the urine sample, CTAB was separately added to the reaction vial, i.e. by prior precipitation from a methanolic solution or, simply, as the solid salt.

Influence of the temperature

At room temperature, the conversion of amantadine in the optimized micellar solution was complete after ca. 30 min. Obviously, this reaction time is too long for an efficient on-line coupling of the micelle-mediated derivatization system and the HPLC procedure. Figure 4 demonstrates that when increasing the temperature to about 60°C, amantadine is completely converted into its DNFB derivative within a mere 4 min. This temperature was selected for all further experiments, because the requirements for the on-line coupling with RP–HPLC are now fully met. It should be noted that under the same conditions, but in the absence of the CTAB micelles, the conversion of amantadine in urine is complete only after ca. 20 min.

The amantadyl–DNP derivative was stable for at least 30 min in the micellar solution at 60°C (Fig. 4). However, it was preferred to keep the derivatization time as short as possible, because (1) a precipitate gradually forms after somewhat longer reaction times, and (2) during the derivatization 2,4-dinitrophenol is formed as a result of the hydrolysis of the reagent. The presence of large amounts of 2,4-dinitrophenol in the derivatization mixture yields high reagent blanks during the chromatographic separation.

Analytical performance

Calibration curves. The calibration graph for

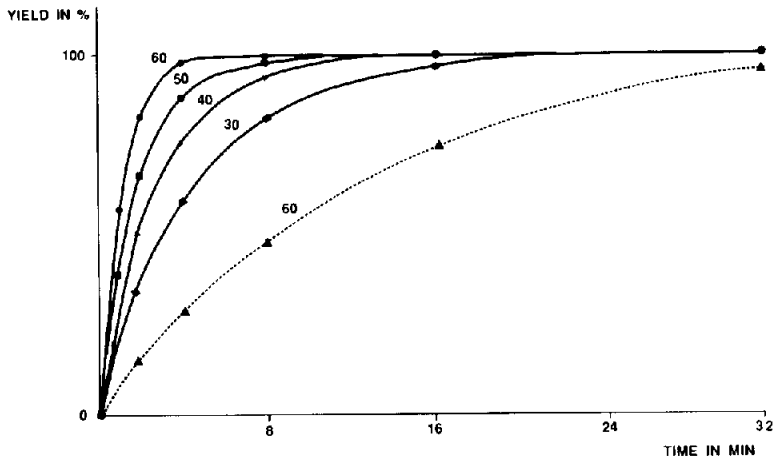

Figure 4
The influence of the temperature on the derivatization rate of amantadine with DNFB in an aqueous 40 mM borate buffer (pH 11) in the absence (dotted line) or the presence (solid lines) of 90 mM CTAB. The derivatization temperature are indicated for each curve.

the determination of amantadine in urine using the automated procedure showed a good linearity (r = 0.998, n = 10) in the range 0.1–20 μg ml^{-1}. The repeatability of the determination at the 10 μg ml^{-1} level was 3.8% (n = 6), whilst at the 0.5 μg ml^{-1} level it was 5.8% (n = 6). The inter-assay repeatability of the automated procedure was found to be 7.3%, when analysing six different blank urine samples, each separately spiked with 10 μg ml^{-1} of amantadine. Because of these satisfactory results, the use of an internal standard, e.g. n-nonylamine, was omitted.

The detection limit of amantadine in urine (at an S/N ratio of 3) was 75 ng ml^{-1}, which is below the concentration range commonly found in urine [14]. The present procedure is, therefore, suited for routine analysis of amantadine in urine. If a lower detection limit is required for, e.g. pharmacokinetic studies, it may be further reduced by using the column-switching technique, which has recently been described [17, 18]. However, this was not the main issue of the present study.

Chromatography. Figure 5A shows a chromatogram of urine spiked with 150 ng ml^{-1} amantadine and Fig. 5B that of blank urine. The chromatograms demonstrate that the amantadine derivative is well separated from the injection peak. In agreement with previous observations [19], Fig. 5 demonstrates that the

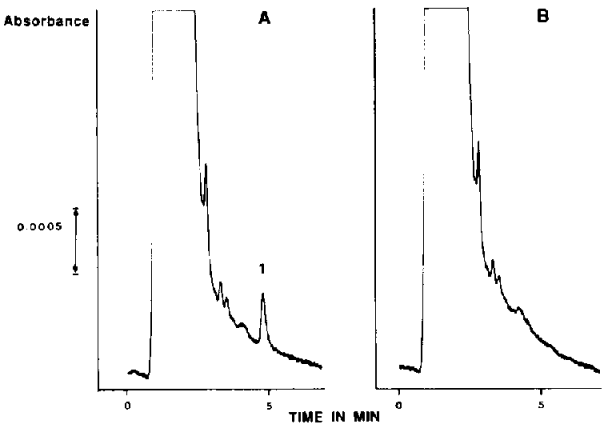

Figure 5
RP–HPLC chromatograms of (A) urine spiked with 150 ng ml^{-1} amantadine; and (B) blank urine, after the on-line derivatization with DNFB in the presence of 90 mM CTAB for 4.2 min at 60°C and pH 11. Peak 1 is the amantadyl–DNP derivative. RP–HPLC conditions: see Experimental.

performance of the chromatographic system is not adversely affected by the presence of micelles in the sample. It should be noted that acidification of the acetonitrile–water mobile phase to pH 2.5 is necessary to diminish the UV absorption of 2,4-dinitrophenol at the monitoring wavelength of 350 nm, while the width of the injection peak was reduced to some extent by the addition of tetramethylammonium bromide to the mobile phase.

Conclusions

The present study demonstrates that a cationic micellar system can be used for the automated on-line derivatization of amantadine in urine prior to RP–HPLC analysis. The use of micellar derivatization systems offers several advantages over traditional techniques. Firstly, due to the presence of the cationic micelles, the derivatization rate of amantadine with DNFB is reduced from 20 to 4 min at 60°C. This means that a simple on-line combination with the subsequent RP–HPLC separation can readily be obtained. An additional advantage is that the reaction products are satisfactorily solubilized in the micellar solution. In aqueous solutions severe precipitation occurs which impairs the analytical performance.

In summary, the present study demonstrates that micelle-mediated derivatization is a technique with good potential for the bio-analysis of amines.

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