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Analysis by liquid chromatography of desipramine in aqueous solutions and plasma application of dichlorotriazinylaminofluorescein (DTAF) as a pre-column fluorescent derivatising agent for selected secondary amines

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Abstract: Dichlorotriazinylaminofluorescein (DTAF) has been proposed as a fluorescent derivatising agent for secondary aliphatic amines. In this report, its applicability as a pre-column reagent is demonstrated by its use in the analysis of aqueous solutions and plasma samples of the tricyclic antidepressant, desipramine. Samples were processed by liquid–solid extraction onto XAD-2 resin, followed by DTAF derivatisation of the fraction of the column effluent containing analyte. The reaction mixture was then subjected to HPLC using a dual column switching configuration that was designed to (a) separate excess DTAF from the reaction product, and to (b) achieve the necessary analytical resolution. Fluorometric detection of the analyte in column effluent afforded a sensitivity of 150 fmol of desipramine injected on-column, a 2–3 order of magnitude increase in sensitivity relative to previously described methods. DTAF appears to be a convenient reagent for fluorescence derivatisation of *N*-methyl secondary amines prior to their HPLC analysis.

Keywords: *Desipramine; fluorescence; pre-column derivatisation; DTAF.*

Introduction

The trace analysis of secondary amines from biological fluids has posed significant analytical challenges, particularly in situations where a chemical derivatisation step is needed to enhance sensitivity. Dichlorotriazinylaminofluorescein (DTAF) was recently identified as an analytical reagent capable of converting selected secondary aliphatic amines to intensely fluorescent fluoresceinyl derivatives [1]. In this paper a sensitive method is described for analysis of desipramine from aqueous solution and plasma as an illustration of the reagent's utility in the analysis of a pharmaceutically-relevant secondary amine. Desipramine is a highly protein bound material. Because pharmaco-

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logic and toxicologic response is related to the non-protein bound fraction, it was deemed desirable to develop a method which responded specifically to that "free" fraction. A number of articles [2–7] have previously appeared which describe procedures for total desipramine analysis from plasma. These involve an extraction step followed by HPLC, carried out in the normal-phase, reversed-phase, or ion pair modes, with spectrophotometric or amperometric monitoring of the column effluent. The best of these methods afforded detection limits of 50 pmol of analyte injected on-column or 15 pmol/ml of plasma. The method described here involves pre-column derivatisation of an extract of an aqueous solution or of a plasma sample with DTAF followed by an HPLC step using a column-switching configuration to remove excess reagent and provide analytical separation of the derivatised product. Fluorescent monitoring of the eluent provides for trace detection of desipramine as its fluoresceinyl derivative.

Experimental

Apparatus

The chromatographic system consisted of two Beckman Model 110A pumps, both adapted for use with the SLIC 1400 controller (Sys-Tec Corp., New Brighton, MN). The detector was a Schoeffel FS-970 fluorescence monitor equipped with a Corning 7–51 primary filter and a 500 nm cutoff secondary filter. A Rheodyne Model 7125 fixed loop injector equipped with a 50 μl loop was used throughout these studies. A Rheodyne Model 7030 switching valve, equipped with a 7001 pneumatic actuator, was used for column-switching purposes. Columns of ODS-Hypersil (Shandon; 4.6 × 50 mm, 5 μm particles) packed in-house were utilised. Chromatograms were obtained on a Houston Instrument Omniscribe recorder. The switching valve and both pumps were connected to a Sys-Tec SLIC 1400 for control and timing purposes. The configuration of the chromatographic system is described in Fig. 1. A Sys-Tec Model ED-1413 accessory keypad was used to signal the SLIC 1400 when an injection was made.

Sample preparation and derivatisation

Desipramine was isolated from plasma by passing samples through Pasteur pipettes packed with 100–200 mesh Amberlite XAD-2 (Sigma, St. Louis, MO) resin. The columns were prepared by placing a glass wool plug in the bottom of a Pasteur pipette and then filling with a methanol slurry of approximately 250 mg of XAD-2 resin. The packing was washed with 4 ml each of acetonitrile, water, and 1×10^{-4}M NH_4OH solution. The samples were eluted with 2 ml of acetonitrile containing 0.01% (v/v) glacial acetic acid. The eluent was dried under a nitrogen stream and the sample reconstituted in 1 ml of 0.01 M, pH 9.5, carbonate buffer. Then, 100 ml of a DTAF suspension (0.8 mg ml^{-1} of acetone) was added, and the vial immersed for 45 min in a bath maintained at 30°C by a Haake circulating temperature controller.

Chromatographic analysis

The samples were analysed on the chromatographic system described in Fig. 1. The mobile phase used on both columns was 50% acetonitrile:50% 0.1 M ammonium acetate (v/v) that was 5 mM overall in heptanesulphonic acid (Eastman Chemical). The SLIC was notified of an injection by the manual pressing of an interrupt key on the Sys-Tec keypad. The switching valve's initial state was such that Pump A (1 ml min^{-1}) eluted Column 1 to waste, while Pump B (1 ml min^{-1}) was used to elute Column 2 through the

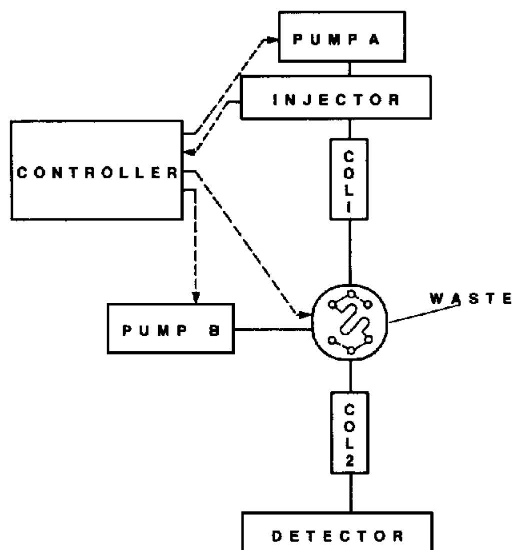

Figure 1
Schematic representation of the apparatus used in the column-switching assay for desipramine.

detector. After 1.50 min the eluent from Column 1 was diverted, by actuation of the switching valve, to Column 2, Pump B was shut down, and Pump A continued pumping at 1 ml min^{-1} (all under control of the Sys-Tec SLIC 1400). At an elapsed time of 2.40 min, the whole system was returned to its initial state by the SLIC 1400. Column 2 was the analytical column in this configuration and was connected to the fluorescence detector.

Recoveries

The recovery of desipramine from XAD-2 micro-columns was determined from aqueous solutions. A stock solution of desipramine (4.07×10^{-3} mg ml^{-1} in water) was prepared and a 1 ml aliquot was passed through the column as per the plasma method described above. The reconstituted sample was injected on an HPLC system consisting of a single 5 cm column with a mobile phase of 60% acetonitrile:40% 0.1 M ammonium acetate (v/v) that was 5 mM overall in heptanesulphonic acid. Detection was via an Altex Model 153 Analytical UV detector. The peak height obtained for the sample was compared to that obtained from injection of the original stock solution of desipramine.

Standard curve

Standard curves were prepared by passing aqueous solutions or plasma samples containing desipramine the XAD-2 columns and assaying the samples as described above. The samples were made from dilutions of a single stock solution of desipramine (4.07×10^{-4} mg ml^{-1} in water). The concentration range of desipramine studied was 3–117 pmol/ml. Peak height versus the concentration of desipramine was plotted in each sample and a linear regression expression was calculated. These curves were subsequently used for the analysis of the plasma samples.

Results and Discussion

The kinetics of reactions of DTAF with secondary amines (e.g. *N*-methylbenzylamine and *N*-butylbenzylamine) and the spectral properties and chemical stability of the

resulting fluoresceinyl derivatives have been previously described [1]. Based on that information, DTAF was considered a viable candidate reagent for fluorescent labelling of secondary amines.

Initially, to assess the viability of DTAF as an analytical reagent, methodology for trace analysis of the model compound, *N*-methylbenzylamine from aqueous solutions has now been developed. Following reaction with DTAF in carbonate buffer (0.01 M; pH 9), the fluoresceinyl derivative was extracted into chloroform:hexane (75:25). At this composition of the binary solvent mixture, maximal differential extraction is achieved, i.e. 97% of the derivatised amine is recovered in the organic layer, and only 3% of unreacted DTAF is extracted. Subsequent HPLC analysis of processed extract with fluorescent monitoring of column effluent formed the basis for an analytical method. To validate the procedure, aqueous solutions of *N*-methylbenzylamine were prepared at six different known concentrations of analyte over the range 4.7–70.4 ng ml^{-1}. The DTAF assay was applied to these samples and a calibration curve constructed. The curve was linear over this concentration range as defined by the regression line:peak height = $3.0x + 5.5$ (correlation coefficient = 0.999). A detection limit of 140 fmol ml^{-1} was determined by analysing samples containing this amount of material ($S/N > 3$; a coefficient of variation of 7.5% was determined from multiple analyses at this concentration). Because of the success derived in applying this analytical approach to *N*-methylbenzylamine, attention was subsequently directed at a more pharmacologically relevant secondary amine, desipramine, and in extending this analysis from aqueous solutions to include biological fluid (i.e. plasma).

Spectral monitoring of reaction mixtures containing desipramine and DTAF demonstrated consumption of reagents. However, to adequately monitor the course of the reaction and eventually provide a suitable analytical method, a strategy was required to separate excess DTAF reagent from derivatised product, since these two species are difficult to separate chromatographically in these systems where DTAF is present in considerable excess. Although partial separation of reagent and derivative was achieved by liquid–solid extraction followed by HPLC on a single column, chromatographic resolution was insufficient to form the basis for an analytical method. Accordingly a multiple column strategy was adopted.

Development of the column-switching system

The first step in the development of the column-switching assay was the establishment of a single column method for the partial separation of the analyte from the derivatising agent.

The initial elution systems using acetonitrile:ammonium acetate mobile phases produced broad, badly tailing peaks. Desipramine is a highly hydrophobic amine that suffers severe tailing on reversed-phase HPLC systems [8]. The tailing of desipramine peaks could be overcome by the addition of triethylamine or sodium heptanesulphonate to mobile phases used in its analysis. The addition of 5 mM sodium heptanesulphonate to the mobile phase was found to eliminate the tailing of the peak representing the product formed from the reaction of DTAF with desipramine and was used for subsequent chromatography.

Difficulties in chromatographically resolving DTAF-derivatised products were caused by the overloading of the column by the excess DTAF used in the derivatisation. A column-switching strategy was considered to allow shunting of the majority of the excess DTAF to waste from the primary column. Sufficient capacity should then exist on the

second (analytical) column to resolve the residual DTAF from the analyte. To achieve desired chromatographic resolution, two identical ODS-Hypersil columns (4.6 × 150 mm) were used, employing identical mobile phases for elution of both columns. A schematic of the system is shown in Fig. 1; Fig. 2 is a flow chart showing the sequence and timing of events for the column-switching assay for desipramine.

An improvement in the analysis was achieved through the use of shorter columns, 4.6 × 50 mm, which decreased the analysis time and permitted reduction in the concentration of acetonitrile used in the mobile phase resulting in an enhanced fluorescence signal from the derivative. A comparison of the chromatograms for the single column analysis and for the column-switching analysis of desipramine derivatised with DTAF is shown in Fig. 3. In going from single column analysis to a column switching mode, analysis time was reduced from 10 min to 4 min, and detection limits were reduced by two orders of magnitude.

Sample preparation

Recoveries of 90% have been reported for the clean-up of plasma samples containing desipramine utilising XAD-2 microcolumns [9]. Application of the reported procedure in which drug was eluted from XAD-2 columns with chloroform:methanol [3:1 (v/v)] resulted in an 80% recovery of desipramine from aqueous solutions. The substitution of acetonitrile for the chloroform:methanol eluent gave equivalent recoveries of drug (75.0 ± 4.8%).

Addition of a small amount of base or acid to the organic eluent eliminated the excessive peak tailing which contributed to incomplete recovery of the drug. The

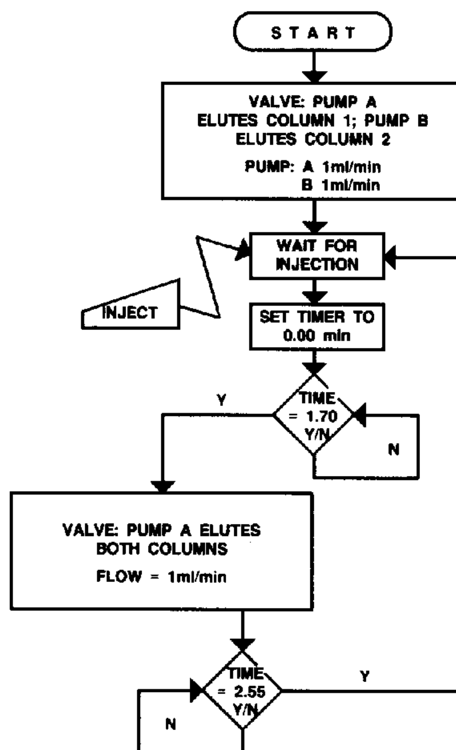

Figure 2
Flow chart for the column-switching assay for desipramine.

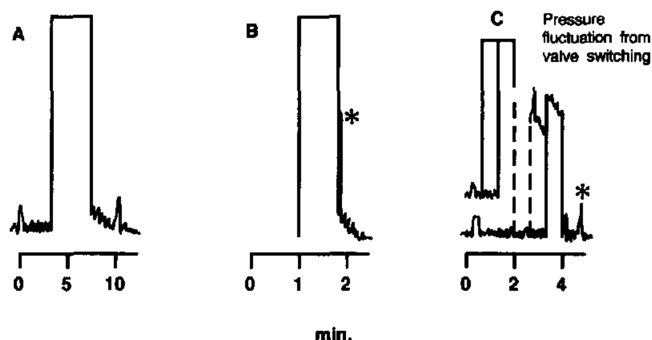

Figure 3
Chromatograms comparing the single column and column-switching assays for desipramine following derivatisation with DTAF. A. Single column detection of desipramine (365 pmol ml^{-1}); B. detection of desipramine (*) from column one (unswitched); C. detection of desipramine (*) from analytical column following column-switching (3.85 pmol ml^{-1}).

inclusion of glacial acetic acid (0.02% v/v) in the acetonitrile eluent increased desipramine recovery from an aqueous solution to 97.8 ± 3.5%. When this procedure was extended to plasma samples, however, a small amount of protein precipitate was contained in the effluent. To eliminate the protein precipitate (which tended to clog the frits at the head of the HPLC column), the first 250 μl of eluent from the XAD-2 columns was discarded. When these conditions were adopted they afforded a recovery of desipramine of 92.5 ± 1.8% from aqueous samples.

Reproducibility of the analysis of desipramine derivatised with DTAF, using the column switching procedure

Ten 1 ml aliquots of a single aqueous solution containing desipramine (30 pmol ml^{-1}) were applied to ten identical XAD-2 columns. The processed desipramine samples (after elution, drying under a nitrogren stream, and reconstitution in 0.01 M, pH 9.5 carbonate buffer) were derivatised with DTAF and analysed by the column-switching system. The mean height was determined to be 33.5 ± 1.2 mm (3.5% coefficient of variation). When this experiment was repeated with plasma samples, at a desipramine concentration of 33.6 pmol ml^{-1}, the coefficient of variation of mean peak height was 6.7%. This reproducibility is well within the limits expected for a column-switching analysis of samples of biological origin.

Standard curve for the analysis of desipramine from aqueous solution

Aqueous solutions of desipramine were derivatised with DTAF after isolation from XAD-2 micro-columns, and analysed using the column-switching procedure outlined above. A plot of the peak height versus the concentration of desipramine at 9 concentrations of drug over the concentration range of 3–117 pmol ml^{-1} was linear [regression line:peak height = $2.1x - 1.5$ (correlation coefficient >0.998)]. A detection limit of 3 pmol ml^{-1} was determined based on analysis of aqueous samples at this concentration (S/N > 3) and represents 5-fold better sensitivity than other assays for desipramine reported in the literature. The actual amount of desipramine detected, 150 fmol injected, is two to three orders of magnitude better than other assays reported to date.

Assay of desipramine in plasma

Plasma samples containing desipramine in the range of 33–330 pmol ml^{-1} of plasma were similarly analysed. A plot of desipramine concentration measured versus the concentration of desipramine added to the plasma samples is shown in Fig. 4, and was also found to be linear, although a significant suppression in slope was observed. The suppressed slope of the plot (0.066 relative to the slope of the curve generated from desipramine analysis from aqueous solution) is believed to represent the fraction of non-protein bound desipramine. This interpretation of the data is based on the observations that (a) similar recoveries of analyte are obtained from plasma when samples are prepared in glass containers or polypropylene vessels suggesting that loss is not likely due to adsorption to container surfaces; (b) the loss in recovery is noted with plasma samples, but not with aqueous samples containing drug at similar concentrations; and (c) desorption of drug and restoration of high recoveries is easily achieved and appears concentration independent (over the range 33–330 pmol of analyte per ml). If this interpretation is correct, it suggests that desipramine is 86% protein bound. To desorb the drug from plasma proteins, plasma samples were acidified and proteins precipitated with percholoric acid prior to applying the analytical scheme previously described. The recovery of desipramine from these samples increased to 92.0 ± 5.2% and a standard curve generated over a similar concentration range was linear. While the recovery was less than 100%, this experiment supports the assumption that the slope does indeed represent the amount of non-protein bound desipramine. This estimate of desipramine–protein binding (86%) is in agreement with the value (90.2 ± 0.8%) determined independently [10] by micropartition techniques. Thus, if measurement of total drug levels is desired, prior acidification of plasma samples achieves desorption and allows efficient recovery of analyte in a matrix applicable to the described analytical scheme. Alternatively, an indication of free circulating levels of drug can be obtained by carrying out the analysis without the initial acidification step.

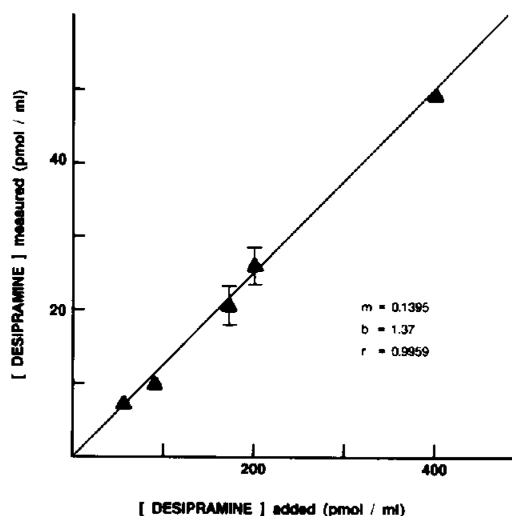

Figure 4

Plot of desipramine concentration measured versus the desipramine concentration added to human plasma. The slope represents the fraction of non-protein bound desipramine.

The method developed for the analysis of desipramine from plasma utilising derivatisation with DTAF requires minimal sample handling (eliminating the need for multiple extractions [2, 4, 5]) and offers satisfactory reproducibility (±6%) at desipramine concentrations ≥33 pmol ml^{-1} of plasma (based on assays of 5 spiked samples).

The most significant advantages of this procedure are: (a) the improved sensitivity achieved, as measured in terms of the amount of desipramine injected on-column; and (b) the ability to measure the non-protein bound fraction of desipramine in plasma (total drug concentration can be determined by initial sample treatment with perchloric acid). Having the ability to monitor both total plasma levels of a drug as well as the free circulating fraction may have wide clinical applicability, since generally only the free fraction is biologically active or available for renal clearance. In the specific case of tricyclic antidepressant therapy, this method may be valuable in understanding the differences in therapeutic effect [11] associated with nominally the same plasma concentrations of drug (if such differences can be explained in terms of variability in protein binding).

The procedure developed for desipramine eliminates the pre-concentration steps utilised previously to achieve low detection limits [3, 4]. Elimination of the need to concentrate samples also avoids the concentration of potential interferences and loss by adsorption of drug to container surfaces. The potential exists for further improvements in the detection limit of our method via pre-concentration steps, by increasing the size of plasma samples used beyond 1 ml and/or reconstitution of effluent from micro-columns in <1 ml of carbonate buffer. Decreases in the detection limit of desipramine were not investigated as part of this study since this would not improve the absolute on-column detection limit for desipramine. The method described here (illustrated using *N*-methylbenzylamine and desipramine as model analytes), however, demonstrates the value and viability of the DTAF derivatisation/column-switching strategy, and suggests its broader applicability to the analysis of unhindered secondary aliphatic amines.

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