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Direct determination of adamantanamine in plasma and urine with automated solid phase derivatization

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ABSTRACT

A simple, highly sensitive and selective method is described for adamantanamine determination in plasma and urine by high-performance liquid chromatography with fluorescence detection. The method involved a simultaneous extraction and derivatization of biological fluids with a 9-fluoreneacetate (9-FA) solid-phase derivatization reagent. This approach eliminated tedious sample preparation steps and provided automatic derivatization with selective and efficient sample clean-up for direct injection of biological fluids. Derivatized adamantanamine was separated under conventional reversed-phase conditions and determined by fluorescence detection. The optimization and validation of the derivatization method with the 9-FA solid-phase reagent is described.

INTRODUCTION

Biological samples of urine and plasma are usually analyzed for therapeutic drug monitoring. The low concentration and the lack of a distinctive chromophore in the drug, as well as the complexity of the sample matrix, all put high demands on sample pretreatment and the analytical method to be used. Surfactant-containing mobile phases, internal reversed-phase (ISRP) packings or shielded hydrophobic stationary phases are three available approaches used for direct injection analysis of biological fluids in reversed-phase high-performance liquid chromatographic (RP-HPLC) systems [1–4]. However, poor detectability of the analytes limits the application of all these direct injection methods. Direct solution derivatization of biological samples usually fails, as the organic solution of the derivatization reagents, such as 2,4-dinitrofluorobenzene, 9-fluorenylmethoxycarbonyl (9-FMOC) chloride or dansyl chloride, all readily precipitate proteins in biological fluids and will deteriorate the analytical system.

Direct analysis of 1-adamantanamine in biological fluids by HPLC is also difficult due to poor detectability of this drug. The classical analytical method for 1-adamantanamine in biofluids has involved organic solvent extraction at a basic pH. Proteins precipitated under these conditions [5–10]. The 1-adamantanamine in the protein-free extract was then determined by gas chromatography or derivatized with a chromo-

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phore-containing reagent and detected by HPLC. Adamantanamine may co-precipitate with the proteins or only be partially extracted from the biological fluids, causing potential inaccuracy using this procedure.

The use of solid-phase extraction (SPE) to accomplish selective extraction, clean-up and preconcentration, with minimum sample manipulation, was previously demonstrated [11]. Toluene, which was successfully used to extract adamantanamine from basic urine or plasma samples, can be easily replaced by crosslinked porous polystyrene with an SPE technique [8].

Solution derivatization has been widely used in HPLC to analyze samples containing amines. Pre-column derivatization of analytes improves both HPLC separation and detection limits via a variety of detection techniques [UV fluorescence (FL), electrochemical, etc.] [12]. Only the free amine exhibits significant nucleophilicity. To ensure a fast reaction rate and an high yield, the pH of the derivatization medium should be adjusted 1–2 pH units above the pK_a value of the particular amine [5]. This requirement greatly limits the effectiveness of using acid chloride-type reagents, due to the large amount of hydrochloric acid produced by its hydrolysis, which can overwhelm the buffer capacity of the reaction media.

Over the past decade, solid-phase reagents (SPRs) have been developed for achiral and chiral derivatizations of nucleophilic analytes [13]. This paper describes the direct determination of 1-adamantanamine in plasma and urine by an SPR containing a covalently bound activated ester of 9-fluoreneacetate (9-FA) (Fig. 1). Simultaneous extraction and derivatization of the amine were performed by an automated, on-line precolumn reaction. The controlled pore size of the polymeric reagent restricted any access to the inner pore surface by high-molecular-mass components, where the bulk of the activated 9-FA reagent was immobilized [14]. This diffusion-restricted reagent also provided selective extraction of the unmodified hydrophobic adamantanamine. The protein components of the plasma and urine samples are excluded and did not interfere with the derivatization or separation/extraction

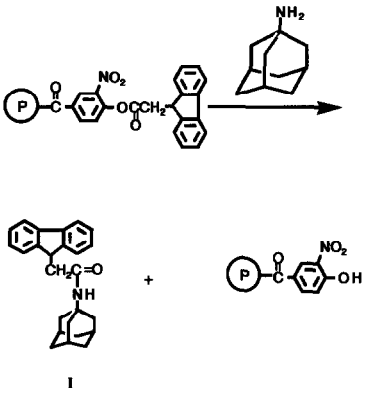

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Fig. 1. 9-Fluoreneacetate (9-FA) derivatization of adamantanamine by solid-phase reagent (SPR).

of the analyte. The low detection limits were achieved by combining selective extraction, preconcentration, and derivatization procedures. Reproducibility and accuracy of the analysis were shown by repeated injections and single-blind, spiked testing.

EXPERIMENTAL

Reagents

9-Fluoreneacetic acid (99%), triethylamine (TEA, 98%), ethyl acetate and dichloromethane (99%) were obtained from Aldrich (Milwaukee, WI, USA). HPLC solvents were donated by EM Science (Gibbstown, NJ, USA) as their Omnisolv HPLC brand. HPLC mobile phases were used after filtration through a 0.45-μm HVHP-type solvent filter (Millipore, Bedford, MA, USA) and degassed under vacuum with stirring. 1-Adamantanamine and lyophilized human plasma were obtained from Sigma (St. Louis, MO, USA) and reconstituted in 50 m*M* NaOH solution before spiking. Human urine sample was adjusted with 50% NaOH to make a 50 m*M* NaOH solution and filtered before spiking.

Apparatus

Automated on-line derivatization was performed on a Gilson HPLC system (Gilson Medical Electronics, Middleton, WI, USA), consisting of a Gilson 232 autosampler, two Gilson 203 HPLC pumps, a Gilson 121 fluorescence detector

with excitation at 254 nm and emission from 305 to 395 nm, a Gilson 811B dynamic mixer (1.5 ml), a Gilson 621 DataMaster, and an AST Premium 286 computer (AST Research, Irvine, CA, USA). Thermo-strip wrapping was purchased from Upchurch Scientific (Oak Harbor, WA, USA).

Preparation of authentic standard FA-adamantanamide derivative

The N-(1-adamantyl)-9-fluoreneacetamide (I) standard was prepared through a reaction between 9-fluoreneacetyl chloride and 1-adamantanamine in the presence of TEA. 9-Fluoreneacetic acid (1.3 g) was reacted with 0.9 ml of thionyl chloride in 20 ml of benzene at 60°C for 1 h. The solvent was evaporated under vacuum, and 1.7 g of 1-adamantanamine free base, 0.8 ml of TEA and 20 ml of benzene were added. The reaction mixture was held for 1 h at room temperature. After evaporating the benzene, the residue was dissolved in 20 ml of ethyl acetate and washed with 150 ml of 0.2 *M* aqueous HCl followed by 150 ml of 0.2 *M* aqueous NaOH. The organic layer was filtered and dried to yield a light yellow solid. The residue was recrystallized from methanol. Elemental analysis results of this compound are: C%, 83.67 (83.80); H%, 7.51 (7.54); N% 4.01 (3.92), the numbers in parentheses are the calculated values for $C_{25}H_{27}NO$.

On-line, pre-column derivatization

9-FA-tagged reagent was prepared on irregular resin of 12% crosslinked polystyrene with 60 Å pore size and 16–20 μm particle size, as previously described [14–16]. The resin was slurried in acetonitrile and packed into a stainless-steel derivatization column (27 mm × 2.1 mm I.D.) and installed on the loop position of the Gilson autosampler (Fig. 2). The derivatization column was continuously heated at 75°C by a thermo-strip. The column was washed with 400 μl of acetonitrile, 400 μl of 40% acetonitrile–water–1 m*M* sodium dodecyl sulfate (SDS) and 400 μl of 5 m*M* SDS–water before sample injection. Then a 50-μl sample was injected and slowly pushed (360 μl/min) into the SPR by 50 μl of 5 m*M* SDS–

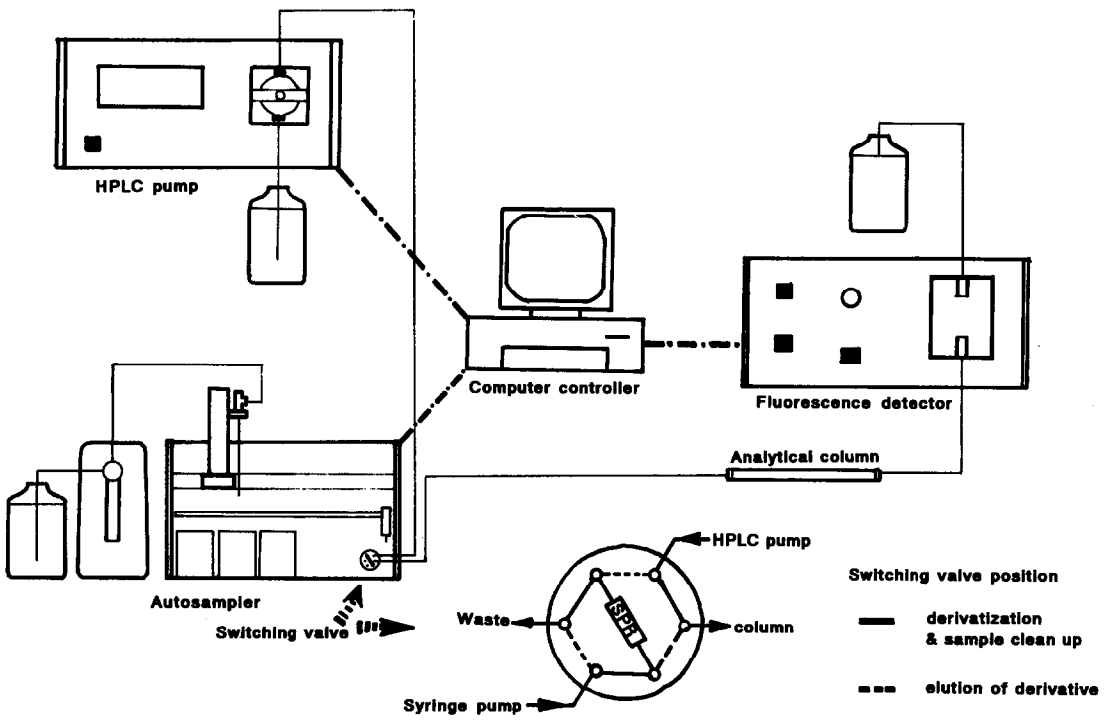

Fig. 2. Diagram of the automated HPLC system for the on-line, pre-column solid-phase derivatization.

water. Derivatization was held for a certain time and washed with 400 μl of 5 m*M* SDS–water. The derivatized adamantanamine (I) was eluted to the analytical column with back flushing for 15 s by 55% acetonitrile mobile phase (1.5 ml/min). The derivatization column was then switched back to load position and washed with 400 μl of acetonitrile. The autosampler was held in the waiting position for 11.5 min until the next run. Total separation gradient time was 13.47 min, with the following gradient composition: at 0.00, 1.50, 5.50, 10.50, 11.50, and 13.47 min 55.0, 55.0, 85.0, 85.0, 55.0, and 55.0% acetonitrile, respectively.

RESULTS AND DISCUSSION

Simultaneous extraction and derivatization with the solid phase reagent (SPR) derivatization column

Sample clean-up in biological analysis involves two major goals: removal of interfering components and sample preconcentration. Determination of adamantanamine in biological fluids has been performed by tedious, time-consuming and expensive liquid–liquid extraction methods. The SPR derivatization column, on the other hand, provides a more hydrophobic phase for efficient extraction and its pore size excludes interfering proteins from its reactive interior surface. The bulk of the immobilized reagent resides within the small pores, but some amount of reagent is also on the surface of the polymeric resin. A reactive, fluorescence detection tag (9-FA) has been immobilized on this SPR for nucleophilic derivatization of hydrophobic analytes. The extent of hydrolysis of the immobilized tag was suppressed by limiting the accessibility of the aqueous base to the tag by its hydrophobic surroundings, and by limiting the presence of the basic buffer only to the derivatization step. This approach was achieved in the following stepwise manner: (1) inject biofluid sample in an SDS-containing solution; (2) rinse out hydrophilic and high-molecular-mass components; (3) hold for a certain time to conduct derivatization; (4) clean any residual proteinaceous composition again with an SDS-containing solution, while the derivatized adamantanamine is held by the porous hydrophobic polymer; (5) backflush to a conventional RP column using the starting analytical mobile phase for 15 s; and (6) wash the derivatization column with 100% acetonitrile solution to clean the SPR and remove water for better reagent stability. In the derivatization step, a basic reaction medium will ensure that the adamantanamine is in the free basic form, easily extracted by the hydrophobic SPR and derivatized by the activated 9-FA-tagged reagent.

Improving sensitivity and selectivity

The controlled pore size of the SPR and use of an SDS-containing solutions avoid protein precipitation inside the hydrophobic pores and allow modification of specific analytes. Here, a 12% crosslinked polystyrene-based 9-FA SPR was evaluated by derivatizing a bulky and sterically hindered primary amine drug, adamantanamine (Fig. 1). The actual available loading of the 9-FA tag on the SPR was 0.60 ± 0.05 mmol/g (n = 3), as determined by total hydrolysis of a freshly prepared reagent [16]. Improvement in sensitivity after solid-phase derivatization was realized by sensitive FL detection. Analysis of even lower concentrations of adamantanamine can be performed by simply increasing the sample size, together with on-line trace enrichment on the same polymeric reagent. The selectivity of this SPR was obtained by eliminating many endogenous interfering compounds and selectively retaining only part of the sample components, especially the adamantanamine. Exogenous interferences are, at times, due to the SPR and primarily from hydrolytic byproducts, but they had little interference at the retention time of the adamantanamide derivative. Since the 5 m*M* SDS washing solution has a relatively weak elution power, the hydrophobic adamantyl-9-FA amide was retained in the polymeric SPR column, while hydrophilic interferents were washed away, prior to back-flushing the reaction mixture to the analytical column. Extraction efficiencies of a 40-ng standard of adamantanamine in water at 75°C and room temperature were 80.2 ± 7.2 and 96.7 ± 4.5% (n = 4), respectively, using a 27 mm × 2.1 mm I.D. derivatization column.

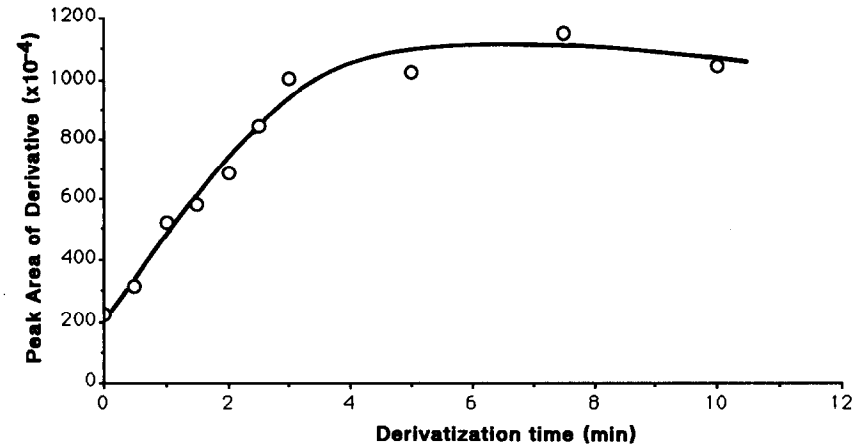

Fig. 3. Optimization of derivatization time. 9-FA-tagged reagent on 12% DVB substrate in a 27 mm × 2.1 mm I.D. reactor; derivatization temperature, 75°C; sample, 50 μl of 1.36 μg/ml adamantanamine in 50 m*M* NaOH. Protocol: wash reactor with 400 μl of 100% acetonitrile, inject 50-μl sample and 50 μl of 10% acetonitrile, wait reaction time, wash reactor with 200 μl of 10% acetonitrile, switch to inject and backflush on-line for 15 s, switch back to load position, wash reactor with 400 μl of 100% acetonitrile, wait 11.50 min. Chromatographic conditions: mobile phase A, 100% water; mobile phase B, 100% acetonitrile; mobile phase events at 1.5 ml/min; total separation gradient time; 13.47 min. at 0.00, 1.50, 5.50, 10.50, and 11.50 min 55.0, 55.0, 85.0, 85.0, and 55.0% B, respectively.

Time optimization for derivatization

At room temperature, almost no derivatization of the sterically hindered adamantanamine occurred, even after 20 min. Based on previous results with amphetamine, on-line derivatization of spiked adamantanamine samples in 50 m*M* NaOH was conducted at 75°C [14]. As shown in Fig. 3, 3 min is the minimum time required for derivatization, without unnecessary hydrolysis of the tag on the SPR.

pH Value influence of derivatization medium

It has been shown that derivatization of adamantanamine requires strong basic conditions, and maintaining a pH of approximately 11 has been needed in order to conduct efficient derivatizations [5]. In solution derivatization, keeping a pH 11 in the presence of a large excess of the reagent presents a problem. By using an SPR, adamantanamine is a free base at pH 11 and is extracted by the porous, polystyrene-based re-

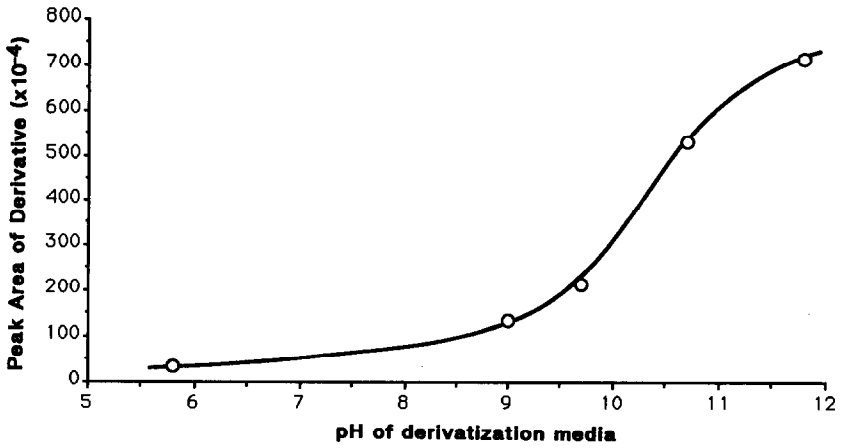

Fig. 4. Effect of solvent pH on the derivatization yield in on-line derivatizations of 0.9 μg/ml adamantanamine in aqueous solution. Conditions as in Fig. 3, derivatization at 75°C for 3.0 min.

agent. Here, there are no major problems in keeping a large excess of the reagent and maintaining the basic pH. To evaluate the pH effect on the adamantanamine derivatization by the SPR, five aqueous solutions having different pH were prepared. They were: distilled water (pH 5.8), 50 m*M* $Na_2B_4O_7$ (pH 9.0), 50 m*M* Na_2CO_3–$NaHCO_3$ (1:1, pH 9.7), 50 m*M* Na_2CO_3 (pH 10.7), and 50 m*M* NaOH (pH 11.8). These solutions were spiked with 0.91 μg/ml adamantanamine and derivatized by the SPR at 75°C for 3 min. Of the five solutions, 50 m*M* NaOH was found to provide the largest N-(1-admantyl)-9-fluorenacetamide peak area (Fig. 4). Although higher concentrations of NaOH may provide better derivatization efficiency, the solid-phase derivatization reagent will be more quickly depleted and on-line reproducibility will be sacrificed. Typical chromatograms of adamantanamine derivatizations in plasma and urine are shown in Fig. 5a and b.

Limit of quantitation and linear range of calibration plot for adamantanamine determination

Calibration curves were prepared by plotting derivative (I) peak areas *versus* concentration of adamantanamine of spiked plasma and urine standards. The correlation coefficients of the calibration curves were 0.999 and 0.988 for concentration of 0.2–4 μg/ml in plasma and urine samples, respectively. The line equations were derived as: $y_1 = 507.9\ x_1 - 12.8 (r^2 = 0.999)$ and $y_2 = 514.6\ x_2 - 24.3 (r^2 = 0.988)$ where x_1 and x_2 were the concentrations (in μg/ml) of adamantanamine in urine and plasma, respectively. The

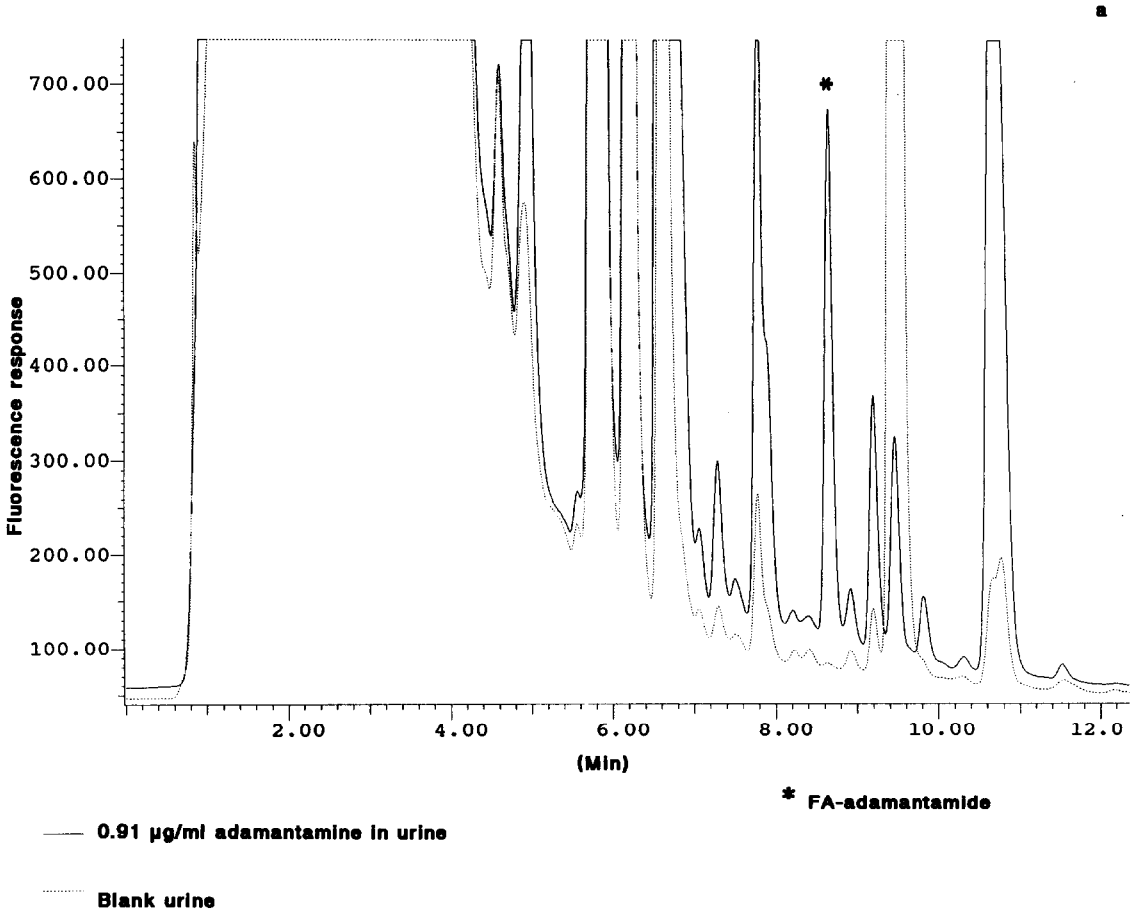

Fig. 5.

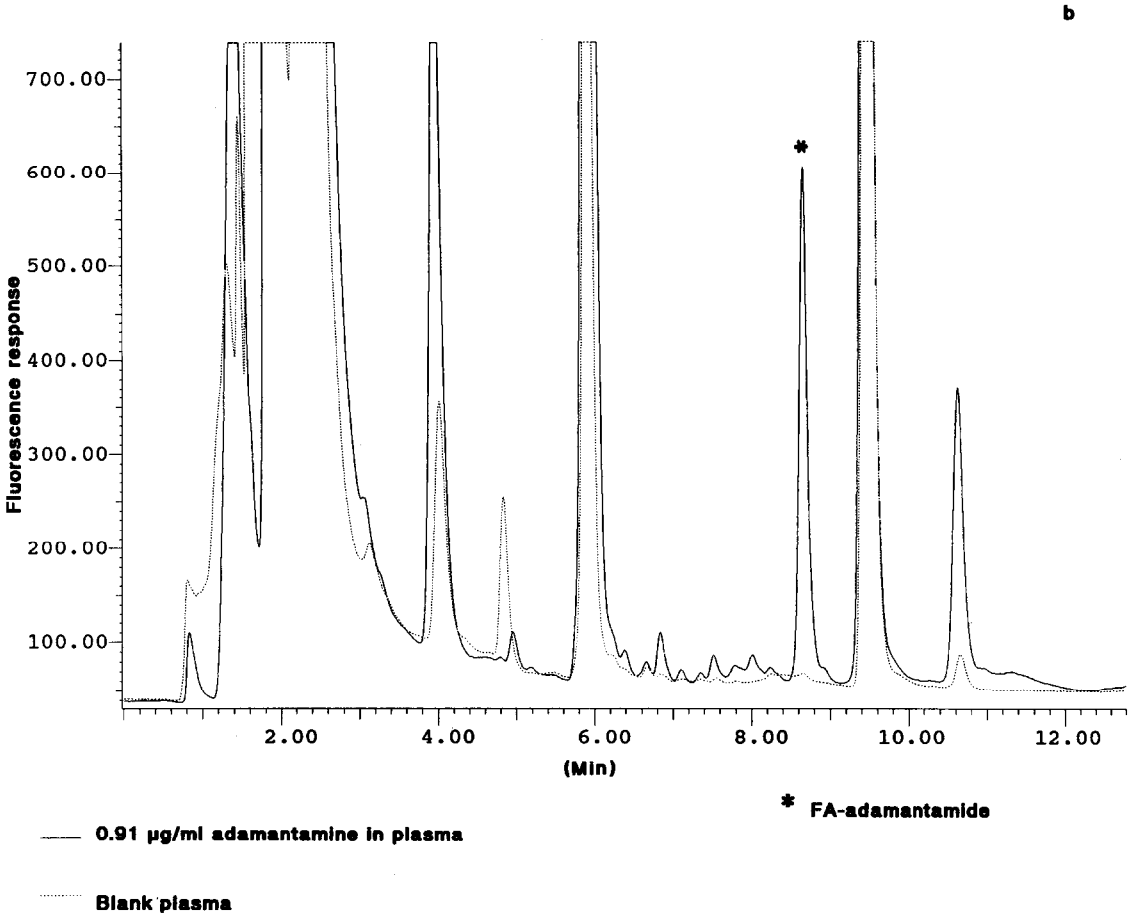

Fig. 5. Typical chromatograms of adamantanamine derivatization in urine and plasma. Separation conditions as in Fig. 3, derivatization at 75°C for 3.0 min. (a) Urine sample; (b) plasma sample.

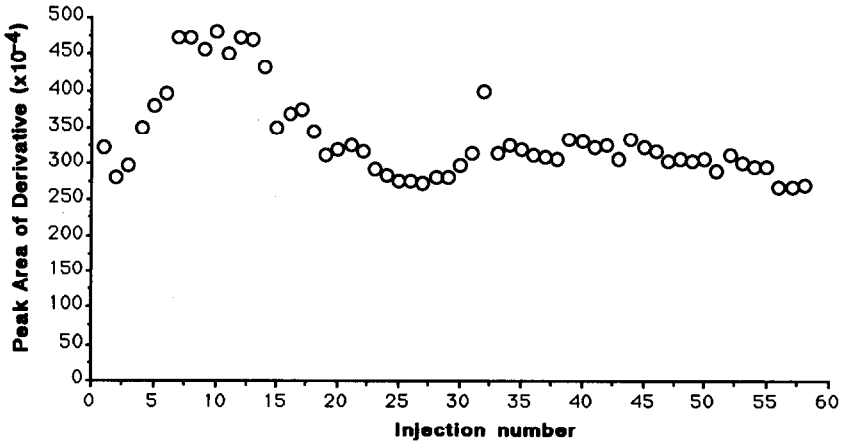

Fig. 6. Derivatization performance of repeated injections by on-line derivatizations of 0.91 µg/ml adamantanamine in urine. Separation conditions as in Fig. 3, derivatization at 75°C for 3.0 min.

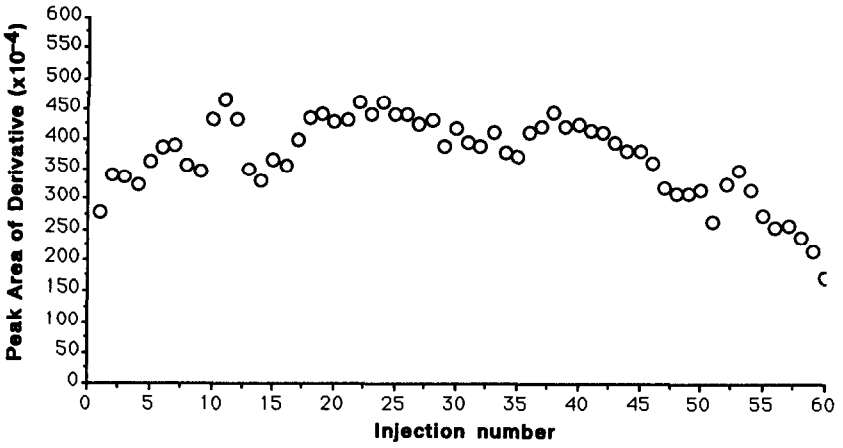

Fig. 7. Derivatization performance of repeated injections by on-line derivatizations of 0.91 μg/ml adamantanamine in plasma. Separation conditions as in Fig. 6.

value y correlates to the peak-area counts (arbitrary units $\times 10^{-4}$) of I obtained from solid-phase derivatization. Standard deviations of slope were 9.6 and 19.9 and those of intercept are 3.2 and 10.9 for urine and plasma derivatization, respectively. The minimum detectable amounts of adamantanamine in urine and plasma were 0.74 and 0.79 ng (50-μl injections), respectively, by normalizing signal-to-noise to 3:1 from the corresponding chromatograms.

Reproducibility of on-line solid-phase derivatization

Unlike solution phase derivatization, one solid-phase derivatization column can be used repeatedly for a series of efficient, on-line derivatizations. Under the optimized derivatization conditions, the freshly prepared 9-FA-tagged SPR was evaluated for derivatization reproducibility of adamantanamine-spiked urine and plasma (Figs. 6, 7). Good reproducibility of on-line derivatization was obtained.

Single blind spiked detection

Three spiked urine and two spiked plasma samples were analyzed by using their respective calibration curves. The results of adamantanamine concentrations are shown in Table I. The detected concentrations were in good agreement with the spiked levels.

TABLE I

DETERMINATION OF SPIKED ADAMANTANAMINE IN PLASMA AND URINE

Spiked concentration (ng/ml)	Concentration found (mean ± S.D., $n=3$) (ng/ml)	R.S.D. (%)	Relative error (%)
Urine			
378	416 ± 14	3.4	10.1
786	767 ± 46	6.0	−2.4
952	1028 ± 47	4.6	7.9
Plasma			
236	251 ± 27	10.8	6.4
910	943 ± 58	6.2	3.6

CONCLUSIONS

This paper describes a simple and rapid procedure for determination of adamantanamine in urine and plasma. A 9-FA SPR based on a porous polystyrene resin was used for simultaneous extraction and derivatization. This reagent was used for the direct injection determination of adamantanamine in plasma and urine without

any sample pretreatment. This approach is quantitative, reproducible and sensitive, using conventional RP-HPLC separation in a direct adamantanamine on-column mode to analyze biological fluids. Totally automated procedures should have a wide range of applications in any direct drug determination in biological fluids.

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REFERENCES

1 D. Bentrop, F. V. Warren, Jr., S. Schmitz and B. A. Bidlingmeyer, *J. Chromatogr.*, 535 (1990) 290.
2 R. A. Grohs, F. V. Warren, Jr. and B. A. Bidlingmeyer, *Anal. Chem.*, 63 (1991) 384.
3 I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
4 D. J. Gisch, B. T. Hunter and B. Feibush, *J. Chromatogr.*, 433 (1988) 264.
5 F. A. L. van der Horst, J. Teeuwsen, J. J. M. Holthuis and U. A. Th. Brinkman, *J. Pharm. Biomed. Anal.*, 8 (1990) 799.
6 V. Schwarz, Z. Deyl and K. Macek, *J. Chromatogr.*, 340 (1985) 401.
7 P. M. Belanger and O. Grech-Belanger, *J. Chromatogr.*, 228 (1982) 327.
8 A. Siouffi and F. Oimmier, *J. Chromatogr.*, 183 (1982) 33.
9 M. J. Stumph, M. W Noall and V. Knight, *Clin. Chem.*, 26 (1980) 295.
10 N. Narasimhachari, E. Helgeson and U. Prakash, *Chromatographia*, 12 (1979) 523.
11 R. Huber and K. Zech, in R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in HPLC, Part A*, (*J. Chromatogr. Lib.*, Vol. 39A), Elsevier, Amsterdam, 1988, Ch. 2.
12 S. T. Colgan and I. S. Krull, in I. S. Krull (Editor), *Reaction Detection in Liquid*, Marcel Dekker, New York, 1986, Ch. 5.
13 C.-X. Gao and I. S. Krull, *Biochromatography*, 4 (1989) 222.
14 F.-X. Zhou, I. S. Krull and B. Feibush, *J. Chromatogr.*, 609 (1992) 123.
15 B. J. Cohen, H. Karoly-Hafeli and A. J. Patchornik, *J. Org. Chem.*, 49 (1984) 924.
16 A. J. Bourque, I. S Krull, and B. Feibush, *Biomed. Chromatogr.*, (1993) in press.