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Simple, rapid, and sensitive liquid chromatography-fluorescence method for the quantification of tranexamic acid in blood☆

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Abstract

Tranexamic acid (TA) is a synthetic antifibrinolytic agent that is being considered as a candidate adjuvant drug for site-specific pharmaco-laser therapy of port wine stains. For drug utility studies, a high-performance liquid chromatography (HPLC)-fluorescence method was developed for the quantification of TA in blood. Platelet-poor plasma was prepared, size-separated using 3 kDa cut-off centrifuge filters, and derivatized with naphthalene-2-3-dicarboxaldehyde (NDA) and cyanide. The excess of NDA was quenched after 2 min by adding tryptophan. The derivatives were separated on a 2.1 mm C18 column using an acetate buffer/acetonitrile gradient. Excellent separation from plasma background was obtained at pH 5.5. Quantification was carried out at 440/520 nm. The limit of detection was 0.5 μM and the mean ± SD recovery from whole blood was 81.7 ± 10.9%. Derivatized TA samples were stable for at least 36 h at 4 °C. The method was successfully applied to a heat-induced TA release study from thermosensitive liposomes.

Keywords: Antifibrinolytic drug; Fluorogenic reagent; NDA; Pre-column derivatization; Plasma; Whole blood; Thermosensitive liposomes

1. Introduction

Tranexamic acid [trans-4-(aminomethyl) cyclohexanecarboxylic acid] (TA) is the most potent antifibrinolytic lysine analogue [1,2] used in a broad spectrum of peri- and post-operative interventions and bleeding disorders [3–7]. The administration of TA is associated with a reduction in bleeding due to its inhibitory effect on clot breakdown (fibrinolysis). TA occupies the lysine binding sites of plasminogen, thereby inhibiting the formation of a molecular complex required for fibrinolysis [8,9]. It has recently been proposed [10] that synthetic antifibrinolytic agents such as TA may also constitute suitable candidates for site-specific pharmaco-laser therapy, a development-stage treatment modality for vascular anomalies (e.g.,(Fig. 1A) refractory port wine stains) in which laser irradiation is combined with the concomitant administration of a prothrombotic and/or antifibrinolytic-encapsulating drug delivery system. The objective of this modality is to induce occlusion of the pathological blood vessels through laser-mediated hemostatic processes, whereby the accumulated drug carrier progressively releases its content at the target site. The release of TA during blood coagulation will impart stability and integrity onto the clot by inhibiting its breakdown. Complete occlusion of the blood vessel, initiated by laser irradiation and facilitated by the presence of prothrombotic and/or antifibrinolytic pharmaceuticals in the circulation, will lead to local hypoxia and the consequent removal of the irradiated vasculature. This ultimately results in a reduction in blood volume, and therefore the redness of the port wine stain [10]. The aim of this work is to develop a suitable method for TA quantification in blood and plasma samples, which will be used in drug release

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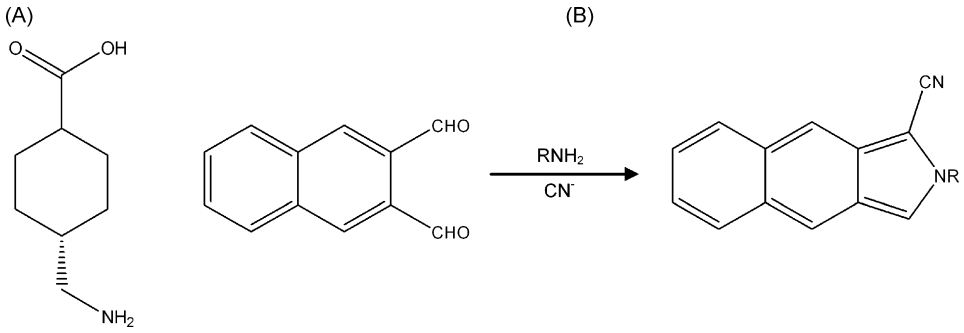

Fig. 1. (A) Chemical structure of tranexamic acid; (B) derivatization reaction of naphthalene-2-3-dicarboxaldehyde (NDA)/CN^- with primary amines.

and pharmacokinetics studies during the development phases of site-specific pharmaco-laser therapy. Although drug concentrations inside such carriers can be very high, the relatively slow release kinetics (minutes to hours) and instantaneous dilution effects will require a highly sensitive and selective chemical analytical approach to quantify TA release rates and half-lives in plasma and blood samples.

In order to develop a simple yet effective quantification method for TA over the 10^{-4} to 10^{-7} M range, several hurdles inherent to TA's molecular structure must be overcome. First, TA lacks the electronic configuration (i.e., π-electrons) to behave as a chromophore or fluorophore, necessitating a derivatization step prior to spectroscopic detection. Secondly, TA shares structural similarity to amino acids with its amino and carboxylic acid groups. High levels of bloodborne amino acids and possibly small peptides are therefore expected to cause major interferences during separation, which is required for assaying TA in biological samples. The chemical analytical techniques for TA determination published to date are summarized in Table 1. Limit of detection (LOD) values in the low-to-sub-μM range have been reported for pharmaceutical preparations and buffered solutions [12,15,16,25,27,28,30], but these techniques are not suitable or have not been investigated for biological samples. In Ref. [11], a prodrug of TA with more convenient chromatographic properties was analyzed. The majority of the other published methods, including ion-exchange-, gas-, and reversed-phase high-performance liquid chromatography (RP-HPLC), and electrophoresis coupled to spectroscopic or mass spectrometric (MS) detection are either not sensitive or selective enough for our purposes [13,17–24,26,29,31]. An excellent method based on HPLC–tandem MS, although requiring expensive instrumentation, was recently reported by Chang et al. [14].

Here we describe a simple, sensitive, and highly selective RP-HPLC method based on pre-column derivatization with naphthalene-2-3-dicarboxaldehyde (NDA) for the quantification of TA in the 5×10^{-7} to 1×10^{-4} M range in blood and plasma samples. NDA has been widely used as a derivatization reagent for the determination of amino compounds in biological samples by fluorescence detection after liquid chromatography [32] and capillary electrophoresis [33]. The reaction of NDA with a primary amine in the presence of CN^-, which proceeds facilely and quickly due to the high reactivity, is depicted in Fig. 1B. The derivatives are relatively stable and the reagent blank signal is low inasmuch as the starting reagent itself emits negligible fluorescence [32]. Our present work focuses on sample preparation, optimization of the derivatization reaction, chromatographic separation from large excesses of amino-containing plasma components, sample stability, and the use of internal standards. The method was validated for plasma and whole blood samples that were anticoagulated with citrate, ethylenediaminetetraacetic acid (EDTA), or heparin. Finally, the optimized method was applied to a heat-induced drug release study in which the amount of TA released from thermosensitive liposomes was quantified in plasma.

2. Experimental

2.1. Chemicals and reagents

Ultrapure water (Milli-Q-UF Plus, Millipore, Bedford, MA, USA) was used in all assays. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-polyethylene glycol (DSPE-PEG2000) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). HPLC-grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands), and acetic acid and ammonium acetate were acquired from Riedel-de-Haën (Seelze, Germany). TA (>96%, Fluka, Buchs, Switzerland) was prepared at a concentration of 20 mM in water, diluted to the desired concentrations, and stored at 4 °C. Cyclohexylamine (Sigma–Aldrich, Steinheim, Germany), *n*-propylamine (Baker Chemicals, Deventer, The Netherlands), isopropylamine (Sigma–Aldrich), *n*-dodecylamine (Baker Chemicals), and 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) (Sigma–Aldrich) were tested as internal standards (I.S.). A 1 mM solution of each I.S. as well as the stock of 40 mM NaCN (>97%, Merck, Darmstadt, Germany) and 10 mM tryptophan (Sigma–Aldrich) were prepared in water and stored at 4 °C. The 20 mM NDA stock solution (Molecular Probes, Eugene, OR, USA) was prepared in acetonitrile and stored at 4 °C in the dark for up to 4 weeks. BD Diagnostics (Franklin Lakes, NJ, USA) glass Vacutainer tubes containing buffered sodium citrate, K_2EDTA, or Li/Na heparin were used for blood collection. D-Phe-Pro-Arg-chloromethylketone HCl (PPACK) was obtained from Calbiochem (Merck Biosciences) and aprotinin was purchased from Bayer (Mijdrecht, The Netherlands).

Table 1
Summary of available chemical analytical techniques for the quantification of tranexamic acid (TA)

Source	Method	Column	Derivatization	Detection	LOD (μM)	Sample; remarks
[11][a]	HPLC	C8 Nucleosil	Pre-column; fluorescamine	fluor. (280/470)	0.04	Blood; analyte was prodrug of TA
[12]	HPLC	LiChrosorb RP-18-5	Pre-column; *o*-phthalaldehyde	chemilum.	0.05	Buffer
[13]	cat HPLC	Nucleosil SA	Post-column; *o*-phthalaldehyde	fluor. (410/450)	0.064	Plasma; higher LOD due to plasma interferences
[14]	HPLC–MS/MS	Xterra MS C18	–	Tandem MS; m/z 158 → 95	0.064	Plasma
[15]	Spectrofluorometry	–	7-Chloro-4-nitrobenzofurazan	fluor. (462/520)	0.064	H_2O/methanol
[16]	Spectrophotometry	–	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	abs. (470)	1.2	Pharmaceutical preparations
[16]	Spectrophotometry	–	7,7,8,8-Tetracyanoquinodimethane	abs. (750)	0.76	Pharmaceutical preparations
[17]	HPLC	C18 μ-Bondapak	Pre-column; 5-(dimethylamino)-naphthalenesulfonyl chloride	fluor. (N.L.)	1.3	Blood
[18]	HPLC	Cosmosil 5C8	Pre-column; phenylisothiocyanate	abs. (254)	1.3	Serum
[19]	HPLC	YMC-ODS	Pre-column; 2-hydroxy naphthaldehyde	abs. (317)	2	H_2O/ethanol, blood; LOD refers to standard solution
[20]	CE	Uncoated fused silica	Pre-CE; ofloxacin acyl chloride	abs. (300)	2.5	Buffer, plasma
[21]	PE	–	Post-PE; ninhydrin	abs. (500)	3	Plasma, urine
[22]	GC	CHDMS or Chromosorb G	Pre-column; 4-fluoro-3-nitrobenzene trifluoride, ethylation	Electron capture	6	Serum, urine; extra TLC step could lower LOD
[23][b]	HPLC	Microsorb-MV C18	Pre-column; *o*-phthalaldehyde	fluor. (N.L.)	10	Blood
[24]	cat HPLC	Amberlite IR-120	Post-column; ninhydrin	abs. (570)	13	Blood
[25]	Spectrophotometry	–	Ninhydrin	abs. (565)	13	Pharmaceutical preparations
[26]	HPLC	LiChrosorb RP-18	Pre-column; fluorescamine	fluor. (390/475)	25	Blood
[27]	Spectrophotometry	–	Chloranil in alcohol	abs. (346)	N.L.	Pharmaceutical preparations
[28]	Spectrophotometry	–	Tetracyanoethylene	abs. (330)	N.L.	Pharmaceutical preparations
[29]	GC–MS	Chromosorb W-HP 80/100	Pre-column; dry hydrogen chloride-*n*-butanol, heptafluorobutyric anhydride	MS	N.L.	Blood
[30]	ip HPLC	LiChrospher SI-100; Nucleosil 100	Pre-column; naphthalene-2-sulfonate	abs. (254)	N.L.	Buffer
[31][c]	cat HPLC–GC	LC: AmberLite GC-120; GC: Chromosorb W80/100	Post-column; CF_3COOH, CH_2Cl_2	MS	N.L.	Serum, cerebrospinal fluid

HPLC conditions are reversed-phase, unless otherwise indicated. *Abbreviations*: LOD, limit of detection; cat, cation exchange; ip, ion-pair; GC, gas chromatography; MS, mass spectrometry; CE, capillary electrophoresis; PE, paper electrophoresis; fluor., fluorescence (excitation/emission wavelength); chemilum., chemiluminescence; abs., absorbance (absorption wavelength); TLC, thin layer chromatography; N.L., not listed.

[a] A propionic ester of TA was used.

[b] LOD value deduced from text.

[c] TA was employed as internal standard.

2.2. Preparation and characterization of TA-encapsulating liposomes

Large unilamellar vesicles were prepared from DPPC and DSPE-PEG at a 96:4 molar ratio. Phospholipids were dissolved in chloroform and mixed at the desired ratios. The solution was desiccated by evaporation under a stream of N_2 gas and exsiccated for 20 min. The resulting lipid film was hydrated with 318 mM TA in 10 mM HEPES buffer [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] (Sigma–Aldrich), pH 7.4, to a final lipid concentration of 5 mM, and bath sonicated for 10 min. The mixture was subjected to 10 freeze-thaw cycles and extruded five times through 0.2 μm filters (Anotop, Whatman, Brentford, UK) at 55 °C. Unencapsulated TA was removed from the liposome suspensions by size exclusion chromatography (Sephadex G-50 fine, GE Healthcare, Chalfont St. Giles, UK). Phospholipid concentrations were determined by the phosphorous assay according to Rouser et al. [34], and encapsulated TA was quantified spectrofluorometrically after treatment of liposome suspensions with 1% (final concentration) Triton-X 100 (Sigma–Aldrich) and derivatization with fluorescamine (Sigma–Aldrich) [35]. Liposome size and polydispersity were measured by dynamic light scattering at a 90° angle using unimodal analysis (Zetasizer 3000, Malvern Instruments, Malvern, UK), and the phase transition temperature (T_m) of the formulation was determined by differential scanning calorimetry (MicroCal, Northampton, MA, USA). Liposomes were stored in the dark at 4 °C.

2.3. Sample preparation and derivatization

Blood was collected by venipuncture from healthy volunteers into Vacutainer tubes containing buffered sodium citrate, K_2EDTA, or Li/Na heparin. PPACK (25 μM final concentration) and aprotinin (200 KIU/mL) were added to inhibit clotting factors and serine proteases such as plasmin, respectively. The tubes were immediately centrifuged at 3500 × *g* for 10 min at 25 °C to obtain platelet-poor plasma (PPP), which was isolated and stored in the dark at −20 °C until further use. Throughout this work, PPP was used instead of platelet-rich plasma, mostly for reasons of long-term stability.

PPP samples (typically 200 μL) were spiked with TA (where applicable) and I.S., and water was added to a final volume of 400 μL. For the recovery studies from blood, whole blood was spiked directly with TA at three concentration levels. A volumetric correction factor, which corrects TA concentrations for volumetric differences between PPP and whole blood, is given by V_P/V_{WB}, where V_P is the plasma volume and V_{WB} is the volume of whole blood prior to centrifugation. The average plasma volume for adult males is approximately 52% [36], and this value was used in all recovery computations from whole blood. All experiments were carried out independently; i.e., each assayed sample was individually derivatized prior to HPLC so as to attest the validity of the method in retrospect.

The spiked PPP samples were filtered through 3 kDa cut-off centrifuge filters (Millipore) at 14,000 × *g* and 25 °C until a sufficient volume of the filtrate was obtained (usually within <20 min). Subsequently, 100 μL of standard solution (TA + I.S. in water) or filtered sample was added to 140 μL of 100 mM borate buffer (pH 9.3), followed by 30 μL of CN^- (40 mM) and 30 μL of NDA (20 mM) to a total volume of 300 μL. The mixture was thoroughly vortexed. It is important to note that the order of reagent addition is crucial for minimizing the possibility of benzoin condensation reactions [37,38]. The resulting solution was allowed to stand for 2 min at room temperature (RT), after which the reaction was quenched by the addition of 50 μL of tryptophan (10 mM) to avoid slow side reactions of unused NDA/CN^-. The samples were stored at 4 °C in the dark for a maximum of 24 h prior to HPLC.

2.4. Heat-induced TA release from liposomes

Active drug release from TA-encapsulating DPPC/DSPE-PEG liposomes was quantified following heat exposure at the T_m at predefined time intervals. Prior to heat treatment the gel filtered suspensions were diluted 10× with PPP that had been kept at 4 °C. Following 5 min equilibration at 4 °C, 160 μL of the diluted sample was suspended in 0.2 mL ultra-thin PCR tubes and incubated at 4 °C for 10 min before thermally induced drug release, which was carried out in a thermal cycler (Biozym, Oldendorf, Germany). Samples were heated for 0.5, 1.5, and 3.0 min, after which they were immediately submersed in an ice bath. The samples were then centrifuged at 355,000 × *g* for 60 min at 4 °C to pellet the liposomes. Fifty microliters of the supernatant was withdrawn, of which 20 μL was diluted five-fold with water, derivatized as described above for PPP samples, and assayed for TA concentration by HPLC.

2.5. HPLC

The HPLC system consisted of a master high-pressure piston pump equipped with a manometer module, a second high-pressure piston pump as slave, and an autoinjector (Models 305, 805s, 302, and 234, respectively, Gilson Medical Electronic, Villiers Le Bel, France), and polyetheretherketone (PEEK) connections with zero-dead volume.

The reversed-phase separation was carried out on a C18 Atlantis column (particle size 3 μm, 2.1 mm × 100 mm, Waters, Dublin, Ireland) in conjunction with an Alltech k852-00 C18 guard column (Keystone Scientific, Bellefonte, PA, USA). The column oven temperature (Model 7971, Jones Chromatography, Cardiff, UK) was maintained at 40 °C, and the sample injection volume was 5 μL. A binary gradient was used for elution; solvent A consisted of 20 mM acetic acid/acetate buffer (pH 5.5) containing 1% (v/v) acetonitrile, and solvent B was comprised of 20 mM acetic acid/acetate buffer (pH 5.5) containing 90% (v/v) acetonitrile. The selected gradient was 30% of solvent B during the first 2 min, which increased linearly to 72% B in 21 min and then to 100% B in 1 min. One hundred percent of B was sustained during 3 min for column cleaning. A programmed loop to initial conditions was carried out after 10 min. The mobile phase flow rate was kept at 0.2 mL/min.

NDA-derivatized compounds were analyzed post-column with a fluorescence detector (λ_{ex} = 440 nm, λ_{em} = 520 nm, Jasco FP-1520, Tokyo, Japan). Detector signal output was processed online with a PC and Chromeleon software (Dionex, Sunnyvale, CA, USA). Recoveries of TA from plasma samples were calculated by dividing the integrated peak area by the respective nominal TA concentration for calibration and PPP samples (F_{CAL} and F_{PPP}, respectively) and expressed as the quotient (in %) of these fractions: $(F_{PPP}/F_{CAL}) \times 100\%$. Statistical analysis (two-tailed, homoscedastic Student's *t*-test) was performed where applicable as indicated in the text. *p*-Values < 0.05 were considered significant.

3. Results and discussion

3.1. Optimization of the HPLC separation

After removal of high molecular weight compounds (>3 kDa) by centrifuge filtration, plasma samples still contain relatively high levels of small peptides and monomeric amino acids that are also derivatized with NDA and could hence interfere with TA determination. Several HPLC parameters, such as pH, gradient profile, and column temperature were tested in order to achieve the best possible separation of TA from the plasma constituents within a minimum elution time. The optimization experiments were carried out using a 10% (v/v) plasma solution, diluted with 100 mM borate buffer (pH 9.3), spiked with TA (0.02 mM final concentration), and derivatized with NDA/CN^- (0.07 and 0.2 mM final concentrations, respectively).

Different linear gradients at different pH values were tested using a binary solvent system: solvent A, 1% (v/v) acetonitrile in 20 mM acetic acid/acetate buffer and solvent B, 90% (v/v) acetonitrile in 20 mM acetic acid/acetate buffer. At pH 3, the carboxylic acid groups of TA, amino acids, and small peptides are predominantly protonated (neutral), which makes these molecules rather lipophilic after NDA labeling. Under these conditions, separation was not possible with any of the tested linear gradients. In contrast, when similar experiments were carried out at pH 6.5, all derivatized components eluted early (as deprotonated anions) and no separation could be achieved either. The effect of pH on the resolution was therefore investigated using the same solvents in a pH range of 3.5–6, with two different fixed gradient profiles (from 0% B to 100% B in 15 and in 30 min). Adequate separation was achieved with both gradients in a pH range of 4.0–5.5, probably due to differences in pK_a values between TA and plasma amino acids. It is well known that the pK_a of the carboxylic acid group in amino acids is significantly lower than that of an isolated alkyl-COOH such as in TA, and apparently the same holds for their NDA derivatives. Fig. 2 shows the separation of NDA-labeled TA in plasma; the derivatized plasma components give rise to major peaks at the beginning of the chromatogram. These are not observed when derivatizing water blanks (NDA itself is non-fluorescent). There is practically no interference at the longer elution times, i.e., in the vicinity of the NDA–TA peak. The best resolution from minor plasma peaks was obtained at pH 5.5. The slower gradient provided better resolution between TA and plasma components, but the efficiency and symmetry of the peaks were worse and the analysis time longer. Thus, the steeper gradient at pH 5.5 was selected for further experiments.

The influence of column temperature was also evaluated since changes in temperature could have an effect on retention, selectivity, and efficiency. Complete separation was achieved at 40 °C. The gradient program was further optimized in order to shorten the analysis time. The best results were achieved when an initial isocratic step was ensued by a solvent gradient after 2 min. The optimized elution program was as follows: from 0 to 2 min a constant 30% B followed by a linear gradient from 30% B to 72% B from 2 to 23 min. Under these optimum chromatographic conditions TA was well resolved from plasma interferences with a 12 min elution time.

3.2. Selection of an internal standard

For quality control purposes, several internal standards (I.S.) were tested. Five commercially available amino compounds were chosen as a model of primary amines for the reaction with NDA/CN^- and were evaluated as I.S., namely: *n*-propylamine (*n*PA), isopropylamine (iPA), *n*-dodecylamine (DA), cyclohexylamine (CHA), and L-DOPA. Using the selected mobile phase, L-DOPA eluted too early (interference from plasma components) and DA eluted very late, prolonging the analysis time. The high volatility of iPA would affect the precision with which iPA could be added to the samples; the further use of this compound was therefore discontinued. *n*PA and CHA both eluted shortly after TA in a clean zone of the chromatogram (see Fig. 2), and throughout most of the work both I.S.s were used. However, later experiments showed poor recovery of CHA from plasma, presumably as a result of binding to large proteins (e.g., albumin) and consequent retention on the 3 kDa filter. For the more polar TA and the smaller *n*PA no such losses were observed; *n*PA was therefore selected as the most appropriate I.S.

3.3. Optimization of the labeling reaction

Different reaction parameters were considered to optimize the derivatization: pH, excess of reagents, and reaction time.

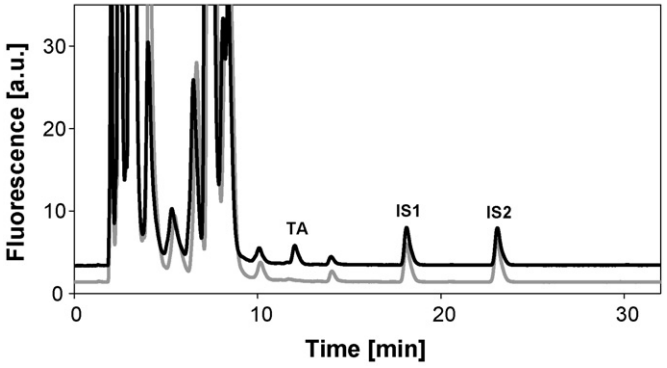

Fig. 2. Chromatogram of platelet-poor plasma before (gray) and after (black) spiking with 10 μM TA, illustrating the separation at pH 5.5 of NDA–TA from the large excess of all major derivatized plasma components and internal standards (I.S.1 = nPA; I.S.2 = CHA).

A 100 mM borate buffer (pH 9.3) was selected as derivatization medium [32], since the amino group should be electrically neutral for the nucleophilic addition reaction. To ensure a rapid and complete reaction of TA and I.S. in PPP, an excess of NDA is desirable (especially when plasma components use up a large fraction of the available NDA), but overabundances must remain balanced insofar as side reactions may result in precipitation or the formation of interfering compounds. NDA concentrations in TA and I.S.-spiked plasma samples were therefore assayed from 0.2 to 2.3 mM with steps of 0.3 mM (final concentrations, with a proportional increase in the CN^- concentration from 0.6 to 6.9 mM) until no further increase of the peak heights was observed. Consequently, a 2 mM NDA concentration was selected.

The reaction of NDA with TA and I.S. proceeds very quickly (in <2 min), whereas the reaction of NDA with some of the plasma components is slower. Increases in plasma peaks were observed with increasing NDA concentrations above 0.7 mM, while the TA and I.S. signals remained constant in the range 0.7–2.3 mM. These results were corroborated spectrofluorometrically (SPEX Fluorolog, HORIBA Jobin Yvon, Edison, NJ, USA) in batch experiments during which fluorescence emission by NDA-conjugated TA, I.S., and diluted PPP was monitored as a function of time. In the case of TA and I.S. the maximum fluorescence yield was reached in less than 2 min (results not shown).

When excess concentrations NDA were used, slow precipitation of yellow crystals could be observed and the solutions would turn red after several hours. The favorable reaction kinetics of NDA towards TA and I.S.s were utilized to resolve these effects. By adding 50 μL of 10 mM tryptophan to the reaction mixture after 2 min, the slow side reactions were quenched through the consumption of any remaining NDA by tryptophan and precipitation would not occur. The resulting major peak of labeled tryptophan eluted early with the other plasma components and did not interfere with TA determination. It should be noted that for standard TA solutions in buffer (in the absence of plasma components), the NDA/CN^- concentrations must be reduced by a factor 2 so as to prevent degradation of NDA reaction products in the presence of excess NDA.

In the abovementioned experiments, the concentration ratio between CN^- and NDA was 3. Once the concentration of NDA had been fixed, the ratio between CN^- and NDA was studied in the range 1–5. Although it has been reported before that the optimal ratio between CN^- and NDA is 10 [38,39], we observed no significant differences in signal heights or areas when this ratio was ≥1.5. A ratio [CN^-]/[NDA] of 2 was therefore selected for further experiments. The use of fresh CN^- is recommended: older batches of NaCN or KCN were found to result in lower yields.

3.4. Calibration curve and performance characteristics

Under the optimal conditions described above, a calibration curve was established by assaying single injections of TA standard solutions (in water) at different concentrations (0.5, 1, 2.5, 5, 10, and 50 μM). Linearity was confirmed using least square regression analysis, with the analytical signal (integrated peak area) plotted as a function of TA concentration. In order to check the influence of both the centrifuge filtration protocol and the matrix effect when TA is analyzed in PPP samples, a calibration curve was established for plasma as well using the same TA concentrations. The obtained regression equations were $y = 1.3926x - 0.2083$ ($R^2 = 0.9997$) and $y = 1.4453x + 0.8487$ ($R^2 = 0.9996$) for, respectively, TA standard and TA plasma solutions. Only a small difference (3.4%) exists between the slope coefficients, suggesting that there are neither substantial losses of analyte throughout the procedure nor matrix effects on detector sensitivity. These results substantiate the direct use of the external calibration curve for quantification purposes in plasma, and therefore no recovery corrections were applied to the TA measurements in recovery studies from blood and liposomes.

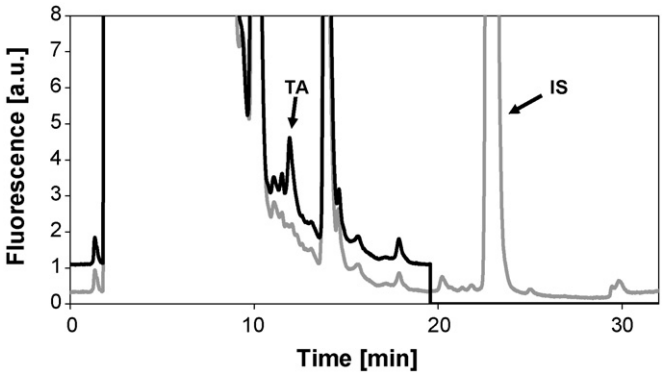

Fig. 3. Chromatogram of platelet-poor plasma before (gray) and after (black) spiking with 0.5 μM TA and cyclohexylamine as internal standard (I.S.), illustrating the background from derivatized minor plasma components and the intensity of the NDA–TA peak at the limit of detection level.

The method detection and quantification limits, calculated using an S/N ratio of 3 and 10, respectively, were 0.5 and 1.5 μM (Fig. 3). Variations in the levels of minor plasma interferences, and not detector noise, determined the LOD. It is therefore recommended that when low TA levels are expected, a blank blood sample should be collected from the patient or animal prior to TA treatment in order to establish the specific baseline.

3.5. Stability of the derivatized samples

In preliminary experiments we found that during long sequences the signal intensity of derivatized TA would slowly decrease after several hours, and that this effect was more pronounced for *n*PA and CHA. The stability of the derivatized products was therefore examined more thoroughly. The labeling reaction of spiked plasma was carried out as described above at pH 9.3 and quenched after 2 min with tryptophan. Aliquots of the sample were transferred into several autoinjector vials and kept at RT or at 4 °C in the dark and analyzed at various time points up to 36 h. The resulting signal intensities are plotted in Fig. 4. The stability plots clearly indicate that cooling helps to retard degradation, particularly with respect to NDA-I.S. products, and that a cooled autosampler should be employed for long chromatographic sequences.

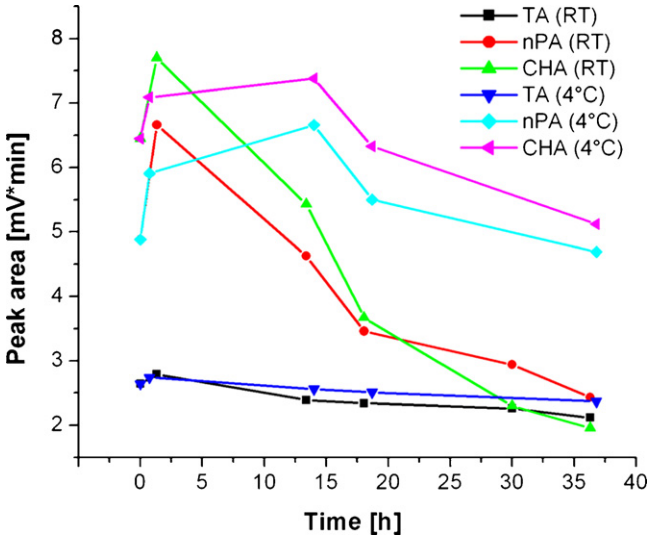

Fig. 4. Stability curves of NDA-derivatized TA and two internal standards, *n*-propylamine (*n*PA) and cyclohexylamine (CHA), at room temperature (RT) and at 4 °C.

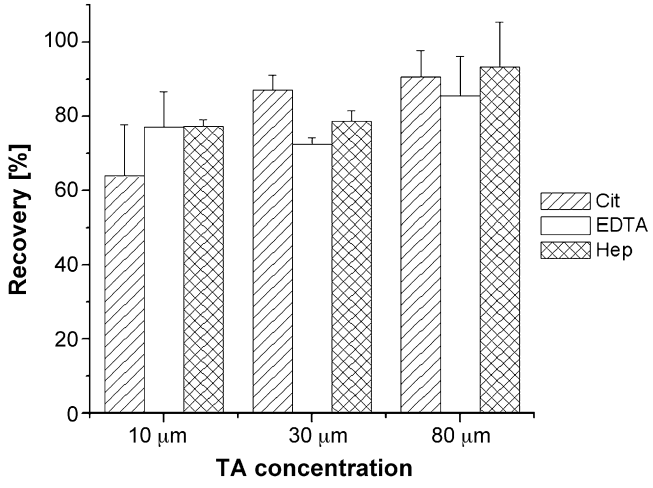

Fig. 5. Recoveries of different concentrations tranexamic acid (TA) from whole blood anticoagulated with citrate (Cit, striped), EDTA (blank), or heparin (Hep, checkered). Recoveries were calculated by dividing the integrated peak area by the respective nominal TA concentration for calibration and whole blood samples (F_{CAL} and F_{WB}, respectively) and expressed as the quotient (in %) ±SD of these fractions: $(F_{WB}/F_{CAL}) \times 100\%$. Data from three independent experiments per group per concentration, total of 27 independent measurements.

Due to the unavoidable instability of the derivatized *n*PA it was decided not to divide the TA peak areas by those of *n*PA, as this would introduce unjustified error and inaccurately reflect actual TA concentrations. Nevertheless, the use of *n*PA as a control standard is warranted for assessing the validity of the method by means of logging the peak areas from continued experiments in Shewhart-type charts, which function to monitor experimental outcomes (i.e., fluctuations) over a longer period of time.

3.6. TA recovery from whole blood

In order to evaluate the applicability of the proposed method in the context of whole blood, citrate-, EDTA-, and heparin-anticoagulated blood samples were spiked with TA at different concentration levels (10, 30, and 80 μM) and independently assayed in triplicate. The blood samples were treated in accordance with the protocol described above, with the exception of a two-fold dilution step for the 80 μM TA samples so as to stay well within the detection range. The final TA concentrations were corrected for a reduction in volume following centrifugation (see Section 2.3). This correction factor must be taken into account when comparing TA levels in blood and in PPP. The obtained recoveries ± SD are depicted in Fig. 5. Statistical analysis showed that no significant differences exist between the mean recoveries of citrate- versus EDTA- versus heparin-anticoagulated blood samples (when mean recoveries per anticoagulant group are averaged over the respective TA concentrations), suggesting that the validity of the method is not influenced by the type of anticoagulant used during blood collection. However, when comparing the mean recoveries per TA concentration (i.e., disregarding the type of anticoagulant used), an increasing trend in mean recoveries was found with higher TA spiking levels (72.8 ± 10.2% for 10 μM, 79.4 ± 6.9% for 30 μM, and 89.9 ± 9.4% for 80 μM). The difference in mean recoveries between the 10 and 80 μM as well as the 30 and 80 μM TA concentrations was statistically significant ($p = 0.005$ and 0.016, respectively). Since this effect was not present during the plasma recovery measurements (Section 3.4) it cannot be due to the membrane filtration process. Therefore, these results imply that part of the TA molecules must be lost during the centrifugation of TA-spiked whole blood samples. The data indicate a constant loss, but the effect is relatively more pronounced for lower TA concentrations. The exact reason is currently unknown and will be investigated in future experiments.

The reproducibility of the whole blood assays was evaluated by comparing the results obtained for different TA spike levels on different days. The relative standard deviations (calculated from peak areas) were 9–13% ($n = 9$ per concentration, measured on three different days with different calibration curves). The mean ± SD elution time was 11.87 ± 0.10 min ($n = 88$).

3.7. Heat-induced TA release from liposomes

Sterically stabilized thermosensitive DPPC/DSPE-PEG liposomes with a mean TA:lipid ratio of 0.91, a mean ± SD external diameter of 153 ± 2 nm at a polydispersity index of 0.083, and a T_m of 43.3 °C were heated at the T_m for 0, 0.5, 1.5, and 3 min and assayed for TA release as described in Section 2.4. Hyperthermia has been employed for numerous liposomal formulations in vitro and in vivo [40–48] to initiate a thermotropic alteration in membrane permeability that will lead to triggered release of the loaded molecules. Phospholipids undergoing a phase transition from the relatively ordered gel phase to the relatively disordered liquid-crystalline phase will introduce packing order defects in the lipid bilayer (i.e., liposomal membrane) which makes it easier for small hydrophilic or amphipathic molecules to transgress the highly hydrophobic bilayer core [49–51]. Thus, upon heating of a drug-containing liposomal formulation to the T_m, the release of encapsulated drug should progress in a sigmoidal-type fashion, with the first phase being the result of thermal diffusion,

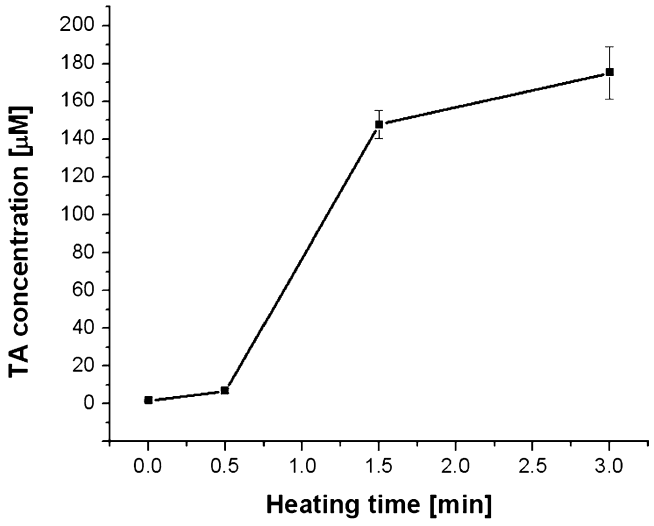

Fig. 6. Heat-induced tranexamic acid (TA) release from thermosensitive DPPC/DSPE-PEG liposomes (96:4 molar ratio). Liposomes were added to platelet-poor plasma and heated to their phase transition temperature (43.3 °C). TA release was quantified by HPLC-fluorescence (based on integrated peak area) as a function of heating time ($n = 3$).

followed by steep release at T_m, and finally an asymptotic phase in which the system gradually moves towards equilibrium (i.e., equal distribution of the drug inside and outside the liposomes). In agreement with expectations, Fig. 6 displays a sigmoidal-type progression of the release curve as a function of time. The release capacity of the system, i.e., the maximum number of molecules released from a liposomal formulation, appears to be reached within ~3 min. A Student's *t*-test confirmed that all data points are statistically different from each other ($p < 0.04$). These results demonstrate that the method is also suitable for the determination of liposomal TA in plasma samples.

4. Conclusions

A novel RP-HPLC method with fluorescence detection was developed for the quantification of TA in blood and PPP samples. In an attempt to eliminate potential chromatographic interferences from plasma components, a rapid and easy centrifuge filtration step was introduced in addition to optimizing the HPLC separation (eluent pH, gradient, and column temperature). The derivatization reaction was optimized for maximum yield (pH, excess of reagents, and reaction time); the addition of tryptophan after 2 min to quench the derivatization reaction helped to stabilize the sample. Several internal standards were explored for quality control purposes. Ultimately, *n*PA was found to be most appropriate. Moreover, the samples proved to be stable for 24 h when stored at 4 °C in the dark. For large RP-HPLC sequences a cooled autosampler is required to maximize sample stability.

The proposed method is affordable, reliable, simple, and fast. One of the major advantages of the method is that TA can be determined in whole blood and PPP samples at concentrations not reported before using similar techniques. The method was reproducible (RSD of <12% for whole blood samples) and linear in the range tested ($R^2 = 0.9996$ for PPP samples). Moreover, the results were independent of the type of anticoagulant used during blood collection. An LOD of 0.5 μM was obtained. Because the LOD is determined by plasma interferences, it is recommended to establish a plasma baseline for every subject prior to assaying TA samples at near-threshold concentrations. Lastly, it was shown that the method is suitable for in vitro and potentially in vivo experiments with TA-encapsulating drug delivery systems. In the near future we intend to use this method in the development of a TA-based, site-specific pharmaco-laser modality for the treatment of port wine stains and possibly other vascular anomalies.

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