Analytical methods for quantification of tranexamic acid in biological fluids: A review

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ABSTRACT

Tranexamic acid (TXA) is a synthetic derivative of the amino acid lysine with antifibrinolytic properties. There is still a lack of pharmacokinetic and pharmacodynamic data concerning variable age groups undergoing surgeries with high blood loss. The optimum dose and administration schedules of TXA are still subject of research, aiming at a safe inhibition of fibrinolysis in the perioperative period. Hence, effective methods for determination of TXA in biological samples are needed. The aim of this review is to discuss the required sample treatment procedures and the analytical methods applied for quantification of TXA, focusing on selected derivatisation agents and internal standards. Methods comprising a separative step (GC, LC or CZE) coupled to spectrophotometric, fluorimetric and mass spectrometry detection were considered, showing a tendency for implementation of MS/MS methods in more recent reports. Detection limits ranging from 0.01 to 0.5 μg mL^{-1} in blood plasma were so far attained using LC-MS/MS.

Keywords: Antifibrinolytic Pharmacokinetic Drug monitoring Mass spectrometry Chromatography

Abbreviations:
ACA, ε-aminocaproic acid;
ACN, acetonitrile;
BEH, ethylene bridged hybrid;
BTB, bromothymol blue;
CE, capillary electrophoresis;
CPB, cardiopulmonary bypass;
CZE, capillary zone electrophoresis;
DLLME, dispersive liquid-liquid microextraction;
DNS-Cl, dansyl chloride or 5-(dimethylamino)naphthalene-1-sulfonyl chloride;
L-DOPA, 3,4-dihydroxy-L-phenylalanine;
EDTA, ethylenediaminetetraacetic acid;
EC, electron capture;
EI, electron ionization;
FA, fluorescamine;
FL, fluorescence;
GC, gas chromatography;
HaCaT, human keratinocyte cells;
HPLC, high performance liquid chromatography;
IS, internal standard; LC, liquid chromatography;
LOD, limit of detection; LOQ, limit of quantification;
MS, mass spectrometry;
MS/MS, tandem mass spectrometry;
MRM, multiple reaction monitoring;
MW, microwave;
NDA, naphthalene-2,3-dicarboxaldehyde;
OAC, ofloxacin acyl chloride;
OPA, *o*-phthalaldehyde;
PE, paper electrophoresis;
PITC, phenyl isothiocyanate;
RP, reversed phase;
RSD, relative standard deviation;
SIM, selected ion monitoring;
SPME, solid-phase microextraction;
TXA, tranexamic acid;
UPLC, ultra-high performance liquid chromatography;
UV, ultraviolet;
UV–Vis, ultraviolet-visible.

1. Introduction

Tranexamic acid [*trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid] (TXA, Fig. 1, **1**) is a synthetic derivative of the amino acid lysine developed in 1960's and introduced into clinical practice >40 years ago. TXA is a biologically active compound with antifibrinolytic effect. It reversibly blocks the lysine binding sites on plasminogen through formation of a reversible complex of the drug with plasminogen molecules disrupting the action of plasmin and preventing the dissolution of the fibrin clot [1–4]. As a result, TXA is associated with reduction of bleeding due to its inhibitory effect on clot breakdown.

TXA was recently included in the World Health Organization (WHO) core list of essential medicines for use in adult trauma patients with ongoing significant haemorrhage, or at risk of significant haemorrhage within 8 h of injury [5]. This list contains minimum medicine needs for a basic health-care system which gives the most efficacious, safe and cost-effective medicines for priority conditions. Its usefulness has been reported in a wide range of clinical conditions to manage abnormal bleeding or bleeding tendencies in which local or systemic hyperfibrinolysis is considered to be involved [4,6–8]. TXA is employed to treat women suffering from menorrhagia, bleeding during pregnancy and for prevention and treatment of postpartum haemorrhage, in upper gastrointestinal bleeding, bleeding after cardiac surgery, to reduce blood loss and transfusion in trauma patients, etc. [9–12]. The therapeutic value of TXA has been also considered in the prevention of human ovarian carcinoma cell growth [13,14]. Other potential clinical and cosmetic applications have been proposed for TXA namely treatment of ultraviolet radiation-induced pigmentation and suppression of ultraviolet B eye irradiation-induced melanocyte activation [15–20].

The interest on TXA overwhelmingly grew after withdraw, in 2008, of the antifibrinolytic agent aprotinin, a serine protease inhibitor [21, 22]. TXA has been associated, however, with an increased incidence of postoperative seizures and has led to adverse neurological outcomes, longer hospital stays, and increased in-hospital mortality [23]. The lack of pharmacokinetic and pharmacodynamic data, in different age groups undergoing different surgeries, reinforce the difficulties to define the optimum therapeutic plasma concentration of TXA needed to safely inhibit fibrinolysis in the perioperative period. Some dosing schedules were based on doses previously determined to inhibit plasma fibrinolytic activity in different settings; while others were developed empirically. Clinical trials performed so far suggest a wide variability in response to TXA. Thus, a renewed attention to TXA has emerged in the literature as the pharmacokinetic, optimum dose and administrations schedules of this drug are still subject of research.

The present paper aims to provide an updated review of the analytical methods reported for the determination of this compound in biological material such as plasma, serum and urine. The review covers and critically addresses an extensive selection of instrumental analytical techniques ranging from liquid and gas chromatography to electrophoresis, automated and electroanalytical approaches. Attention is also paid on sample preparation protocols focusing on biological material.

2. Determination of TXA in biological fluids

In what concerns biological matrices, TXA has been mainly determined in human plasma and serum as summarized in Tables 1, 2 and 3. Because of the complexity of the biological matrices, most of the techniques require a careful and extensive sample pre-treatment, to remove potential interferences, namely proteins and branched-chain amino acids. As TXA has a structural resemblance to amino acids, the concomitant presence of these molecules in biological samples can interfere upon separative procedures prior to analysis.

The most commonly used methods associate reversed-phase HPLC to fluorescence [24–29] or UV [30–32] detection, as depicted in Fig. 2. Considering that TXA does not possess in its chemical structure a chromophore or fluorophore, exhibiting therefore a poor absorption at 220 nm, a derivatisation step is, in most cases, needed to increase the methods' sensitivity. More recently, several methods have been developed based on chromatographic techniques coupled to mass spectrometry [33–41]. MS detection enables straightforward analyte identification and quantification eliminating the need of any derivatisation procedure, which may introduce large assay variations, namely the stability of the newly formed derivative. Less common applications reporting the determination of TXA include techniques such as capillary electrophoresis and paper electrophoresis coupled to UV–Vis detection [42,43].

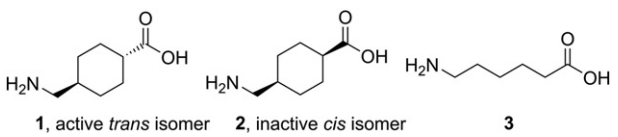

Fig. 1. Chemical structures of tranexamic acid (**1** and **2**) and ε-aminocaproic acid (**3**).

2.1. Sample preparation

Generally, the determination of TXA concentration in plasma or serum obtained from collected human blood requires firstly the deproteinization of the samples, after addition of the chosen internal standard (IS) if used (Tables 1, 2 and 3). A fluxogram of method analysis focused on sample treatment strategies used for TXA is presented in Fig. 3.

Several protein precipitation reagents have been applied to achieve this goal, specifically picric acid [42,44], heptafluorobutyric acid [33], perchloric acid [25,34,35,43], methanol [37,41], ethanol [26,30], and acetonitrile [24,28,31,39,45,46], which is in fact the most commonly used. Fiechtner et al. [28] reported the pre-treatment of plasma samples with leucine dehydrogenase prior to deproteinization by acetonitrile. This enzyme was used to minimize interferences by branched-chain amino acids since it is highly specific for these compounds [47].

Depending on the precipitation reagent used and if a derivatisation procedure is followed, pH adjustment might be necessary [24,26,30, 31,35,44]. If one chooses to use a mass spectrometer as detection system, the supernatant can, at this stage, be transferred into an auto-sampler vial and subsequently injected into, for example, the LC-MS or LC-MS/MS system [34,39]. In other cases, the supernatant is dried, the residue retaken in the mobile phase and injected [37].

Solid phase microextraction (SPME) has also been applied to the determination of TXA concentration in human plasma [36,38,40,48]. The viability of this technique in clinical use for the analysis of polar drugs such as TXA was first established by Bojko et al. [36] by analysing plasma samples from patients who underwent heart surgery with the use of cardiopulmonary bypass (CPB). For this, commercial thin-film microextraction (TFME) fibers coated with octadecyl carbon chains (C_{18}) were used after preconditioning overnight in a methanol:water (1:1, v/v) solution. Based on studies performed to evaluate the extraction time profile and to improve the method sensitivity, 300 μL of sample were exposed to the fiber for 90 min with vortex agitation at 1200 rpm, followed by rinsing with purified water for 30 s. The fiber was then placed, for desorption, in a mixture of ACN:water (4:1, v/v) with 0.1% (v/v) formic acid using the same type of agitation. The efficiency of this method was compared with standard techniques such as protein precipitation and ultrafiltration and the results revealed that the accuracy and precision obtained were equivalent. The linear ranges of TXA concentration in plasma samples obtained for this SPME procedure was 1.56–25 and 25–300 μg mL^{-1} [36].

Bojko et al. [40] latter developed an automated TFME procedure, where a polyacrylonitrile-C_{18} 60 μm thin-film was applied to cover miniaturized stainless steel blades. The amount of analyte extracted in this modified format was 12 times higher when compared to conventional fibers [36]. However, both procedures required an extensive time for extraction and desorption, around 2 h for each step. Considering the

Table 1
Summary of analytical methods using mass spectrometry detection for the quantification of tranexamic acid in biological fluids[a,b].

Sample	Separative technique	Ionization system/SRM	Sample preparation	Derivatisating agent	Column and eluent	IS	LOD and LOQ	Linear range	Ref.
Plasma	HPLC	ESI^+; *m/z* 157.8 > 95.2	SPME (C_{18} fibers); desorption with ACN:H_2O (4:1, v/v) plus 0.1% (v/v) formic acid by vortex agitation at 1200 rpm for 90 min	–	HILIC, ammonium formate buffer:ACN binary gradient, pH 3.5	–	0.5 and 1.5 μg mL^{-1}	1.56–300 μg mL^{-1}	[36]
Plasma	HPLC	ESI^+; *m/z* 157.8 > 95.2	Automated TF-SPME, polyacrylonitrile C_{18} 96 thin-film SPME coating; 20 min at 1000 rpm for extraction; blades washed for 10 s with purified H_2O; desorption using 1.5 mL of ACN:H_2O (4:1, v/v) with 0.1% v/v of formic acid at 1200 rpm	–	HILIC, ammonium formate buffer:ACN binary gradient, pH 3.5	–	0.5 and 1 μg mL^{-1}	1.0–300 μg mL^{-1}	[40]
Plasma	HPLC	ESI^+; *m/z* 158 > 95; IS 212 > 166	Deproteinization with perchloric acid (vortex 30 s; 16,000*g* × 10 min) and direct injection of supernatant	–	XTerra MS C_{18}, 2 mM ammonium acetate buffer:ACN (90:10, v/v), pH 3.5	Methyldopa	0.01 and 0.02 μg mL^{-1}	0.02–10.00 μg mL^{-1}	[34]
Plasma	HPLC	ESI^+; *m/z* 158 > 123; IS 144 > 109	WCX TF-SPME; desorption with MeOH:ACN:ammonium formate buffer (3:3:4, v/v/v) plus 1.5% formic acid for 10 min at 850 rpm	–	Bonus-RP, ammonium formate buffer:ACN plus 0.1% formic acid binary gradient, pH 3.0	*Cis*-4-aminocyclohexane carboxylic acid	0.04 and 0.1 μg mL^{-1}	0.25–300 μg mL^{-1}	[38]
Plasma	UPLC	ESI^+; *m/z* 158.15 > 140.68; IS 161.17 > 142.76	Deproteinization with ACN (vortex 30 s; 13,000 rpm, 4 min) and direct injection of supernatant	–	BEH amide, water:ACN both containing 0.1% formic acid (20:80, v/v)	$^{13}C_2$,^{15}N,*cis*-TXA	0.38 and 0.76 mg L^{-1}	0.8–200 mg L^{-1}	[39]
Plasma and CSF	UPLC	ESI^+; *m/z* 158.2 > 95.2; IS 132.1 > 114.0	Deproteinization with MeOH (vortex 30 s; 16,000*g* × 10 min)	–	BEH C_{18}, H_2O and MeOH gradient both containing 2 mM ammonium acetate and 0.1% formic acid	ACA	0.1 μg mL^{-1} (LOQ)	0.1–10.0 μg mL^{-1}	[37]
Serum	HPLC	ESI^+; *m/z* 158.0 > 122.7; IS 144.0 > 126.0	Deproteinization with perchloric acid (vortex 30 s; 14,000 rpm × 10 min); pH adjustment to 3–4 with sodium hydroxide	–	HyPurity C_{18}, 2 mmol/L NH_4COOH buffer:ACN (95:5, v/v), pH 3.8	*Cis*-4--aminocyclohexane carboxylic acid	0.05 and 1.0 μg mL^{-1}	1.0–200.0 μg mL^{-1}	[35]
Serum	GC	EI	Deproteinization with MeOH (3000*g* × 20 min)	ECF	HP-5	ACA	0.2 and 0.6 μg mL^{-1}	0.6–5.0 μg mL^{-1}	[41]
Serum	GC	EI	Deproteinization with heptafluorobutyric acid (10 min shaking by hand; 2000 rpm × 15 min)	HFBA	Chromosorb W-HP 80–100	TXA-d_6	NA	1–5 ng	[33]

ACA, ε-aminocaproic acid; BEH, ethylene bridged hybrid; CSF, cerebral spinal fluid; ECF, ethyl chloroformate; HFBA, heptafluorobutyric anhydride; LOD, limit of detection; LOQ, limit of quantification; NA, not available; WCX TF-SPME, weak cation exchange thin-film solid-phase microextraction.

[a] HPLC conditions are reverse phase unless stated otherwise.

[b] All samples were collected from humans unless otherwise indicated.

Table 2
Summary of analytical methods using UV–Vis detection for the quantification of tranexamic acid in biological fluids[a,b].

Sample	Separative technique	Detection wavelength	Sample preparation	Derivatisating agent	Column and eluent	IS	LOD and LOQ	Linear range	Ref.
Blood	HPLC	317 nm[c]	Deproteinization with ACN; pH adjusted to 7 with sodium acetate buffer	2-Hydroxy-1-naphthaldehyde	YMC-ODS, MeOH:H_2O:DCM:ACN (58:35:5:2, v/v/v/v)	–	3 ng	30–90 ng	[31]
Plasma	CZE	300 nm	Deproteinization with perchloric acid (vortex 30 s; 10,000 rpm × 10 min)	OAC	Uncoated fused-silica capillary, phosphate buffer at 25 °C, pH 3.00	4-(Dimethylamino) benzoic acid	2.5 μM (LOD)	10–200 μM	[43]
Plasma and HaCaT	UPLC	250 nm	Plasma: deproteinization with ACN (vortex; 14,800g × 6 min); DLLME HaCaT: 24 h incubation with TXA; washed with PBS and lysed with NP-40 lysis buffer for 30 min in ice; 10 min of ultrasonication and 3 cycles of flash freezing; deproteinization with ACN; DLLME	DNS-Cl[d]	ACQUITY UPLC BEH C_{18}, H_2O and ACN gradient both containing 0.1% formic acid	Ethyl paraben	Plasma: 3 pmol (LOD) HaCaT: 0.03 pmol (LOD)	Plasma: 5–500 μM HaCaT: NA	[45]
Serum	HPLC	254 nm	Deproteinization with ethanol (1500g × 10 min); supernatant pH adjusted to 9.2 with borax solution	Phenyl isothiocyanate	Cosmosil 5C_8, 20 mM phosphate buffer:ethanol (90:10, v/v), pH 7.0	3-Aminocyclohexane carboxylic acid	0.2 μg mL^{-1} (LOD)	0.22–22.5 μg mL^{-1}	[30]
Serum[e]	HPLC	205 nm	Deproteinization with ACN (vortex 60 s; 10,000 rpm × 10 min)	–	C_{18} Nucleosil, ACN:H_2O (50:50, v/v) adjusted with phosphoric acid, pH 2.6	Propylparaben sodium	12 and 40 ng mL^{-1}	40–10.000 ng mL^{-1}	[46]
Plasma and serum[f]	HPLC	NA	Deproteinization with picric acid (3000 rpm, 20 min); clean-up of supernatant with Dowex 2-X8; evaporation of solvent; pH adjustment to 2	Ninhydrin	Amberlite IR-120, eluent: NA	–	0.2–0.3 mg/100 mL (LOQ)	0.02–0.18 μmol mL^{-1}	[44]
Plasma and urine	PE	NA	Deproteinization with picric acid; pre-treatment on a cation exchange resin (Dowex in H^+ form)	Ninhydrin	–	–	0.5 μg mL^{-1} (LOQ)	NA	[42]
Urine	HPLC	245 nm	–	Phenyl isothiocyanate	Phenomenex Luna RP C_{18}; 10 mM phosphate buffer:ACN (65:35, v/v), pH 3.6 (adjusted using hydrochloric acid)	Heptaminol hydrochloride	0.0379 and 0.126 μg mL^{-1}	0.2–65 μg mL^{-1}	[32]

BEH, ethylene bridged hybrid; DLLME, dispersive liquid-liquid microextraction; DNS-Cl, dansyl chloride; HaCaT, human keratinocyte cells; LOD, limit of detection; LOQ, limit of quantification; NA, not available; OAC, ofloxacin acyl chloride; PBS, phosphate-buffered saline solution.

[a] HPLC conditions are reverse phase unless stated otherwise.
[b] All samples were collected from humans unless otherwise indicated.
[c] It is not clear if the optimum wavelength is 418 m or 317 nm.
[d] MW assisted.
[e] Simultaneous determination of TXA and losartan potassium.
[f] Rabbit plasma and serum were used.

Table 3
Summary of analytical methods using fluorimetric detection for the quantification of tranexamic acid in biological fluids[a,b].

Sample	Separative technique	Detection wavelength	Sample preparation	Derivatisating agent	Column and eluent	IS	LOD and LOQ	Linear range	Ref.
Blood[c,d]	HPLC	420/485 nm	Ion-pair extraction using BTB as counter-ion	FA (pre-column)	C_8 Nucleosil. ACN:phosphate buffer (55:45, v/v), pH 3	4-(Aminomethyl) cyclohexyl propionate	1–3 ng mL^{-1} (LOD)	10–100 ng mL^{-1}	[27]
Blood and plasma	HPLC	440/520 nm	PPP were obtained by centrifuging blood at 35,000g for 10 min at 25 °C; filtration through 3 kDa cut-off centrifuge filters	NDA/CN^-	C_{18} Atlantis, acetic acid/acetate buffer:ACN binary gradient, pH 5.5	*n*-Propylamine	0.5 and 1.5 μM	0.5–50 μM	[29]
Plasma	HPLC	410/450 nm	Deproteinization with perchloric acid (10 min occasional shaking; 3000g × 5 min) and direct injection of supernatant into HPLC system	*o*-Phthalaldehyde (post-column)	Nucleosil SA, 0.1 M trisodium citrate:MeOH (98:2, v/v), pH 4	AMBOC	10 μg L^{-1} (LOQ)	1–100 mg L^{-1}	[25]
Plasma	HPLC	NA	Deproteinization with ethanol (10 min agitation; 4000 rpm × 3 min); supernatant pH adjusted to 9.0 with buffer solution	DNS-Cl	C_{18} μ-Bondapak, ACN:H_2O:acetic acid glacial (45:65:1, v/v/v)	L-Lysine hydrochloride	0.2 μg mL^{-1} (LOD)	NA	[26]
Plasma	HPLC	NA	Pre-treatment of the sample with leucine dehydrogenase; deproteinization with ACN (2000g × 2 min); filtration with 0.45 μm PTFE filter	*o*-Phthalaldehyde	Microsorb-MV C_{18}, ACN:sodium phosphate buffer (19:81, v/v)	–	NA	0–40 μg mL^{-1}	[28]
Serum	HPLC	390/475 nm	Deproteinization with ACN (10,000g × 3 min); supernatant pH adjusted to 8 with phosphate buffer	FA	LiChrosorb RP-18, ACN:H_2O:CH_3COOH:THF (300:690:5:5) with 40 mmol/L sodium acetate	–	4 mg L^{-1} (LOD)	25–200 mg L^{-1}	[24]

AMBOC, 4-aminomethyl bicycle-(2,2,2)octane-1-carboxilic acid; BTB, bromothymol blue; DCM, dichloromethane; DNS-Cl, dansyl chloride; FA, fluorescamine; LOD, limit of detection; LOQ, limit of quantification; NA, not available; NDA, naphthalene-2,3-dicarboxaldehyde; PPP, platelet-poor plasma.

[a] HPLC conditions are reverse phase unless stated otherwise

[b] All samples were collected from humans unless otherwise indicated.

[c] A prodrug of TXA in the form of a propionic ester was used.

[d] Dog and rat whole blood samples were also used.

Fig. 2. Cumulative distribution of publications regarding assay of TXA in biological samples, emphasizing the application of UV–Vis + FL detection (light gray), MS (dark gray) and other (white) detection systems.

analysis of a single sample, this time frame is excessive but real sample analysis was performed under 96-well format, providing for instance 96 analysis in 35 min (mean sample preparation time of 22 s per sample) [38].

2.2. Separative methods

Liquid chromatography (LC) is by far the analytical technique most frequently chosen for the separation of TXA in biological samples, comprising generally a reversed phase column and a hydrophilic mobile phase. Various stationary phases have been used (Tables 1, 2 and 3) although the most extensively employed consists of C_{18} bonded to silica. Acetonitrile is usually the elution solvent in combination with water and/or a buffer solution (sodium phosphate, ammonium acetate) or acid (acetic acid, phosphoric acid, hydrochloric acid or formic acid) adjusting the pH from 2.6 to 5.5 (Tables 1, 2 and 3) [24,26,28,29,31,32,34,35,37,45,46]. TXA contains two ionizable groups in its structure, a carboxyl and an amino group (pK_a 4.3 and 10.6), and is therefore, a highly polar compound that exists as a zwitterion at physiological pH. Hence, the success of its separation is highly dependent on the pH value. For instance, the predominant form at pH 2.6 is the cationic species while at pH 5.5 the zwitterion will predominate.

Other chromatographic RP mode phases, namely C_8, were also applied [27,30]. Agilent Bonus-RP column was used as an alternative to C_{18} and C_8 alkyl bonded phases for the separation of TXA in biological fluids [38]. This stationary phase is composed of a polar amine group embedded in a long alkyl chain, thus reducing the interaction between basic compounds and the silica. Also, diisopropyl side groups provide steric protection against acid hydrolysis at low pH. Using HILIC technology Pawliszyn et al. [36,40] achieved a retention time of 4.52 min in a total run time of 12 min for TXA [40]. Delavenne et al. [39] reported that, among several different analytical columns tested for tranexamic acid, the BEH amide column (1.7 μm particles) showed the best performance and efficiency, presenting a retention time of 2.75 min using a mobile phase mixture of 20% aqueous + formic acid (0.1% v/v) and 79.9% of acetonitrile + formic acid (0.1% v/v).

GC analysis of TXA in biological samples (Table 1) has been also performed but, due to TXA low volatility, it requires previous derivatisation [33,41,49]. Miyazaki et al. [33] proposed a method based on the *N*-heptafluorobutyryl amine derivative (Fig. 4, **4**) using a Chromosorb WHP column while maintaining the temperature of column oven at 185 °C. An alternative GC approach was reported by Abbasi et al. [41] considering a non-polar HP-5 column using ethyl chloroformate as derivatising reagent and MS detection. The obtained derivative (Fig. 4, 5) was separated using a temperature ramp from 100 to 200 °C.

Electron capture detection coupled with GC column containing polar phase 1% OV-225 on Chromosorb G at 250 °C has been also used for the

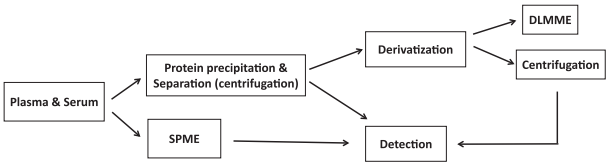

Fig. 3. Fluxogram of method analysis focus on sample treatment strategies used for plasma and serum prior to TXA determination.

quantification of TXA [49]. As TXA does not contain any functional group with a strong detector response (e.g. halogens, phosphorus, peroxides and nitro groups), derivatisation is mandatory with an electrophore, such as the esterified *N*-(2′-nitro)-4′-trifluoromethylphenyl derivative of TXA (Fig. 4, 6).

Capillary electrophoresis (CE) is often chosen to quantify drugs in biological fluids. It has several advantages over conventional chromatographic techniques since this method requires smaller sample volumes, minimal sample pre-treatment, low organic solvent consumption, and simplicity of equipment that can be streamlined through automation. This separation technique was applied to the analysis of TXA in plasma samples through an electrophoretic derivative (Fig. 4, 7) using ofloxacin acyl chloride as derivatising reagent. Capillary zone electrophoresis (CZE) was performed using an uncoated fused-silica capillary and samples were injected at the anode using a 3.45×10^3 Pa pressure during 3 s with applied voltage of 18 kV. The separation was achieved at 25 °C using a phosphate buffer solution at pH 3.00 (125 mM) with a total run time of 10 min [43]. Since TXA and TXA derivatives have both amino and carboxylic acid functionalities in their structures, special care should be taken in what concerns the applied pH. The extension of ionization and therefore the balance between the positively and negatively charged molecules may interfere with the migration speed and therefore, the efficiency of separation. At pH 3.00, the carboxylic group of TXA is predominantly protonated. An earlier publication by Eriksson et al. [42] reported the separation of TXA from human plasma and urine samples by paper electrophoresis using methanol as eluent and ninhydrin as staining agent.

2.3. Detection systems

Mass spectrometry detectors coupled to liquid chromatography are increasingly becoming the method of choice to determine TXA in biological samples [33–41]. Among the different ionization modes available for LC-MS and LC-MS/MS, ESI in positive mode was commonly employed for TXA determination in biological fluids (Table 1), using selected reaction monitoring (SRM) mode [34,36,37,39]. The precursor to product ion transitions employed for TXA quantification included the *m*/*z* transitions 158.2 > 95.2 [34,36,37] and 158.2 > 140.7 [39]. Moreover, the second most abundant product ions of the spectrum, corresponding to the *m*/*z* transitions 158.2 > 122.6, were used for identity confirmation in the work reported by Delavenne et al. [39]. Both full scan MS/MS and SIM modes have been likewise used for TXA quantification purposes [35,38]. The single transition chosen for monitoring corresponded to the *m*/*z* transition 158 > 123 [38]. Typically, for these methodologies, the detector response was found to be linear within a concentration ranging from 0.02 to 300 μg mL^{-1} (Table 1).

Internal standards (IS) are often used to improve the precision of quantitative analysis when using, for example, gas chromatography or mass spectrometry detection. IS can also be used to correct variability due to analyte loss in sample storage and treatment. When considering LC-MS or LC-MS/MS methods the use of an appropriate IS enables the control of the extraction procedure, LC injection, and ionization variability. The use of this standard is beneficial, especially when multiple sample preparation steps are used. For MS methods, the most suitable IS are isotope-labelled compounds because of their similar extraction recovery, chromatographic behaviour, and ionization response to that of the target analyte. In addition, isotope-labelled IS enable better compensation of the matrix effect on the ionization of the analyte.

The IS ε-aminocaproic acid (Fig. 1, **3**) was used by Abou-Diwan et al. [37] for the quantification of TXA in plasma and cerebral spinal fluid by LC-MS/MS. Although this is not an isotope-labelled compound, the retention times and *m*/*z* values for precursor ions (1.63 and 1.35 min; 158.2 and 132.1, for TXA and ACA, respectively) are similar enough to not extend the overall time taken for each analysis, because this is one of the most important parameters to take into account when bearing in mind routine clinical use.

Chang et al. [34] used the commercially available methyldopa (Fig. 5, 8), as internal standard, in the determination of TXA in human plasma by LC-MS/MS. Methyldopa is known for its instability due to potential oxidation of the 3,4-dihydroxypenyl group under neutral or basic pH conditions but this was circumvented by adding 0.1% formic acid. Once more the choice of this IS did not delay the chromatographic separation as typical retention times were 2.65 min for TXA and 2.60 min for IS.

A more structurally similar IS to TXA, *cis*-4-aminocyclohexanecarboxylic acid (Fig. 5, 9), was used to quantify TXA in human plasma and serum by LC-MS/MS [35,38]. The structural difference between TXA and this IS is a methylene group inserted between the cyclohexane ring and the amine function, and a relatively similar retention time was obtained for both. Delavenne et al. [39] used isotopically labelled TXA (Fig. 5, **10**) which enhanced the robustness of the LC-MS/MS method but is a more expensive alternative due to the cost of isotopically labelled standards.

GC–MS methods have also been applied to the determination of TXA in human biological fluids using electron ionization (EI) [33,41]. Miyazaki et al. [33] reported an ionization energy and trap current of 20 eV and 60 μA, respectively, with the ionization source at 250 °C and an accelerating voltage of 3500 V. The quantification was based on the monitoring of two characteristic fragment ions at *m*/*z* 308 (base peak) and *m*/*z* 336 that correspond to the loss of the *n*-butyl ether and carbonyl fragment, respectively, from the *n*-butyl ester *N*-heptafluorobutyryl derivative of TXA (Fig. 4, **4**). A GC–MS method in full scan mode using ε-aminocaproic acid (Fig. 1, **3**) as IS was proposed by Abbasi and coworkers [41] operating with a EI source at 70 eV. MS source was kept at 250 °C and the MS quadrupole at 230 °C.

UV detection coupled with liquid chromatography has been used for quantification of TXA on whole human blood, plasma, serum and urine

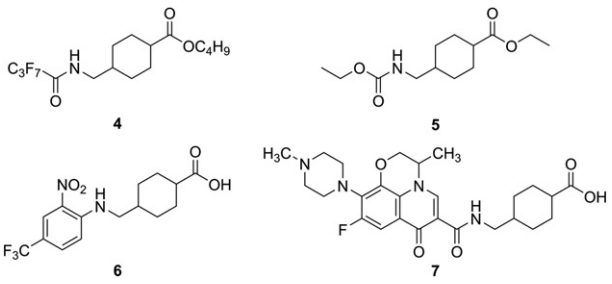

Fig. 4. Chemical structures of *N*-heptafluorobutyryl amine (**4**), ethyl chloroformate (**5**), *N*-(2′-nitro)-4′-trifluoromethylphenyl (**6**), and ofloxacin (**7**) derivatives of tranexamic acid.

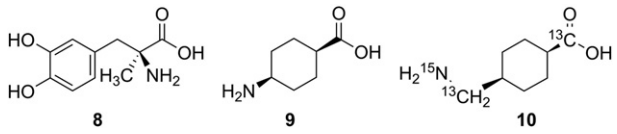

Fig. 5. Chemical structures of the internal standards used for TXA quantification in biological fluids by LC-MS/MS: methyldopa (**8**), *cis*-4-aminocyclohexanecarboxylic acid (**9**), $^{13}C_2$,^{15}N,*cis*-TXA (**10**).

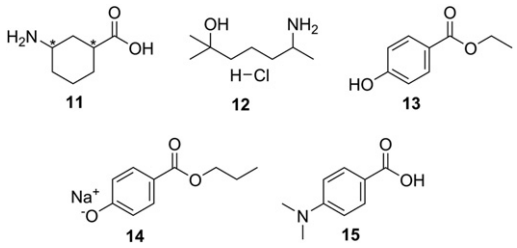

Fig. 6. Chemical structures of the internal standards used for TXA quantification in biological fluids by LC-UV: 3-aminocyclohexanecarboxylic acid (**11**), heptaminol hydrochloride (**12**), ethyl paraben (**13**), sodium propylparaben (**14**), and 4-(dimethylamino)benzoic acid (**15**).

(Table 2). Depending on the selected derivatising agent, and therefore, on the chromophore attached to the TXA, UV detection was performed using wavelengths ranging from 245 to 317 nm [30–32,45]. Typically, for these methodologies, the detector response was found to be linear within a concentration ranging from 0.2 to 79 μg mL^{-1} (Table 2). Arayne et al. reported the quantification of TXA, without any derivatisation, in human serum using UV detection at 205 nm. The detector response was found to be linear for 0.04–10 μg mL^{-1} [46]. UV-CZE has been developed and applied to the separation and determination of TXA in human plasma [43]. A phosphate buffer (125 mM, pH 3.00) was employed as background electrolyte, enabling UV detection of the ofloxacin acyl derivative of TXA at 300 nm using a photodiode array detector, with linearity in the range of 1.57–31.4 μg mL^{-1}.

In UV and FL determination, IS are also applied for sample extraction and LC injection control. Matsubayashi et al. [30] reported the use of compound 3-aminocyclohexanecarboxylic acid (Fig. 6, **11**), derivatised with PITC, as internal standard for the LC-UV determination of TXA in human serum. The authors reported that this IS raises concern in its use since it can exist in two diastereomeric forms because it has two chiral centres. These two chiral centres at the 1- and 3-positions of the cyclohexane ring (Fig. 6, compound **11** with chiral centres signalled with *) can occur in a mixture and produce, therefore, two peaks in the chromatographic analysis corresponding to the *cis*- and *trans*-isomer. Other IS have been used in the LC-UV quantification of TXA in human plasma, serum and urine namely heptaminol hydrochloride (**12**) [32], ethyl paraben (**13**) [45], and propylparaben sodium (**14**) [46] represented in Fig. 6. 4-(Dimethylamino) benzoic acid (Fig. 6, **15**) was used as IS in the quantification of TXA in plasma using CZE and UV detection.

Fluorescence detection has shown to be useful for the determination of TXA, upon derivatisation, in biological fluids [24–29]. Different excitation and emission wavelengths were applied upon the chosen derivatising agent (Table 3). For example, when using naphthalene-2,3-dicarboxaldehyde (NDA) labelling the post-column fluorescence detector is set with excitation and emission wavelengths of 440 and 520 nm, respectively [29]. Linearity was attained for 0.08–15.7 μg mL^{-1}.

Several compounds (Fig. 7) have been used successfully as IS for fluorescence detection. However, the use of 4-aminomethylbicyclo-[2.2.2]octane-1-carboxylic acid (Fig. 7, **16**) as IS raised problems because a partial interference of arginine with the IS peak was observed [25]. For pharmacokinetics and bioavailability studies of TXA in human plasma, L-lysine hydrochloride (Fig. 7, **17**) was chosen as IS

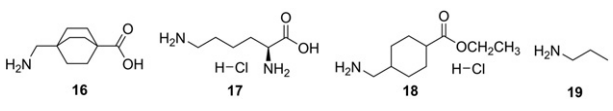

Fig. 7. Chemical structures of the internal standards used for TXA quantification in biological fluids by LC-FL: 4-aminomethylbicyclo-[2.2.2]octane-1-carboxylic acid (**16**), L-lysine hydrochloride (**17**), 4-(aminomethyl)cyclohexyl propionate hydrochloride (**18**), and *n*-propylamine (**19**).

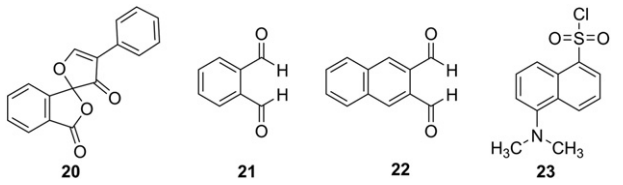

Fig. 8. Chemical structures of derivatising agents applied in fluorescence detection of TXA, including fluorescamine (**20**), *o*-phthalaldehyde (**21**), naphthalene-2,3-dicarboxaldehyde (**22**), and dansyl chloride (**23**).

[26] and for analysis of whole blood, 4-(aminomethyl)cyclohexyl propionate hydrochloride (Fig. 7, **18**) was employed as IS [27].

Huertas-Pérez et al. [29] tested several internal standards for quality control purposes when studying the LC-FL quantification of TXA in whole blood. Five commercially available amino compounds were evaluated for the derivatisation reaction with naphthalene-2,3-dicarboxaldehyde and cyanide, including *n*-propylamine (Fig. 7, **19**), isopropylamine, *n*-dodecylamine, cyclohexylamine, and L-DOPA (3,4-dihydroxy-L-phenylalanine). *n*-Propylamine was selected because all the other IS had one or more of the following problems: eluted too early which led to interferences from plasma components (L-DOPA); eluted too late, increasing the time of analysis (*n*-dodecylamine); high volatility which led to lack of precision upon IS addition to sample (isopropylamine); and poor recovery from plasma due to retention in filtering material (cyclohexylamine).

2.4. Derivatising agents

Many derivatisation reagents have been evaluated for detection of trace amounts of TXA in biological samples by fluorimetry, namely fluorescamine (FA) [24,27], *o*-phthalaldehyde [25,28], naphthalene-2,3-dicarboxaldehyde (NDA) and cyanide [29], and dansyl chloride (DNS-Cl) [26] (Fig. 8).

Two methods using fluorescamine (Fig. 8, **20**) as the derivatising agent for TXA or a pro-drug of TXA have been reported [24,27]. Fluorescamine has the advantage of reacting very fast with primary amino groups to produce a fluorescent product, while the non-fluorescent unreacted fluorescamine hydrolyses in a matter of seconds to non-fluorescent products being, therefore, effectively removed from the reaction. However, due to its poor water solubility and high reactivity, fluorescamine has to be added to samples dissolved in a water miscible nonhydroxylic solvent [50]. Hence, fluorescamine, dissolved in acetonitrile or acetone, was found suitable for derivatising TXA in serum samples and blood at room temperature at pH 7–8 [24,27]. It forms, with TXA, a strongly fluorescent product that can be detected at λ_{exc} = 390 nm and λ_{emis} = 475 nm [24].

Elworthy et al. [25] reported the use of *o*-phthalaldehyde (Fig. 8, **21**) as a derivatisation agent for TXA determination in plasma. In this case, after deproteinization of the sample spiked with IS, the supernatant is injected into the HPLC and the derivatisation occurs online by addition of the *o*-phthalaldehyde to the column eluent. The derivatives formed are detected using 410 nm and 450 nm as excitation and emission wavelengths, respectively. *o*-Phthalaldehyde (OPA) is one of the most sensitive fluorogenic compounds available for reaction with primary amines and the derivatives formed are somewhat unstable which

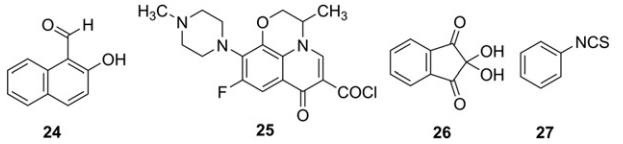

Fig. 9. Chemical structures of derivatising agents applied in UV–Visible detection of TXA, including of 2-hydroxy-1-naphthaldehyde (**24**), ofloxacin acyl chloride (**25**), ninhydrin (**26**) and phenyl isothiocyanate (**27**).

severely limits the use of this reagent [51]. Nevertheless, this gradual deterioration in buffer solution was circumvented by addition of EDTA. OPA was also used to tag TXA, in this case off-line, for TXA concentration monitoring during cardiopulmonary bypass [28].

Huertas-Pérez et al. [29] described a LC-FL for the quantification of TXA in platelet-poor plasma using naphthalene-2,3-dicarboxaldehyde (NDA, Fig. 8, **22**) and cyanide as derivatising reagents. NDA is widely used as a derivatising reagent for the determination of amino compounds and has significantly improved stability, when compared to the corresponding OPA, while retaining the high fluorescence quantum efficiencies [52]. Quantification of the NDA-labelled TXA is carried out after LC at $\lambda_{exc} = 440$ nm and $\lambda_{emis} = 520$ nm and is stable up to 24 h after derivatisation.

Dansyl chloride (Fig. 8, **23**) was another derivatising agent chosen for fluorescence detection of TXA in human plasma following RP-HPLC [26]. In this case, the reaction between the analyte and the derivatising agent has the disadvantage of requiring high temperatures (100 °C for 30 min).

Methods for the determination of TXA using UV–Vis spectrophotometry for detection also required previous labelling of the analyte, crucial to increase sensitivity for detection in biological fluids. In Fig. 9, the chemical structures of 2-hydroxy-1-naphthaldehyde [31], ofloxacin acyl chloride (OAC) [43], ninhydrin [42,44], and phenyl isothiocyanate (PITC) [30,32], which are the most frequently applied chromophores for tagging TXA, are presented.

The derivatisation of TXA in blood samples with 2-hydroxy-1-naphthaldehyde (Fig. 9, **24**) was achieved by adding the derivatising reagent in aqueous ethanol at neutral pH in a 1:1 ratio [31]. The implementation of this method is cumbersome as a large amount of blood (5 mL) is required for derivatisation with adequate sensitivity.

Ofloxacin acyl chloride (OAC, Fig. 9, **25**) was chosen by Lin et al. [43] as derivatisation reagent for TXA detection in human plasma. The resulting TXA derivative displays a tertiary amino function and therefore an additional ionizable moiety is added which makes the derivative suitable for separation by CE. OAC has, however, to be prepared through a one-step reaction between the commercially available ofloxacin and thionyl chloride, a very toxic and reactive reagent. Also, due to high reactivity of the acyl halide obtained, the residue is only dissolved in acetonitrile and used without any purification being assumed that the residue is constituted by OAC.

Ninhydrin (Fig. 9, **26**) is one of the most common compounds employed to label α-aminoacids for detection by colorimetric assays. TXA reacts with ninhydrin in the presence of phosphate buffer via oxidative deamination followed by condensation to form a deep purple compound which is then detected [53]. Ninhydrin reacts selectively with free alpha-amino groups and does not react, for example, with tertiary or aromatic amines. This derivatising agent was used by Okamoto et al. [44] in rabbit plasma samples and Eriksson et al. [42] in human urine and plasma samples.

Phenyl isothiocyanate (PITC, Fig. 9, **27**) was first used by Matsubayashi et al. [30] to introduce a chromophore in TXA occurring in human serum to be analysed by HPLC-UV. The serum sample was initially spiked with IS (Table 2) and after sample treatment, the supernatant was treated with 10 mM borax solution (pH 9.2) and PITC. After a laborious LLE procedure the final extract was resuspended in mobile phase to be injected into the chromatographic system. This derivatisation agent was applied, almost 20 years later, by Hadad et al. [32] to determine TXA concentration in human urine samples and to investigate the pattern of urinary excretion of this drug by a healthy male. In this case, phosphate buffer was added first to the urine sample, followed by PITC in a methanol solution (1% v/v). The reaction conditions used with PITC are slightly harsher and the derivatisation time is longer (20–30 min at 40–60 °C) when considering this type of samples and the conditions applied to other derivatising reagents. A more modern approach was taken by Liao et al. [45] using accelerated microwave assisted derivatisation and dispersive liquid-liquid microextraction (DLLME). Dansyl chloride (Fig. 8, **23**) was chosen to derivatise TXA present in human plasma, among other matrices, followed by LC-UV. The time of derivatisation reaction was reduced to 4 min (at 400 W).

Volatility of sample is a requirement for GC analysis. Derivatisation must provide highly polar compounds sufficiently volatile so that they can be eluted at reasonable temperatures avoiding thermal decomposition or molecular re-arrangement. Also, in particular for TXA, derivatisation can improve resolution and can reduce tailing because this molecule contains quite polar functional groups (—COOH and —NH_2), which are known to contribute to these effects through the formation of intermolecular hydrogen bonds.

Different derivatisation procedures have been applied for the detection of TXA in biological matrices by GC–MS. An early work of Miyazaki et al. [33] on the determination of TXA in human serum considers a simultaneous amidation and esterification of the drug (Fig. 4) using heptafluorobutyric anhydride as derivatising agent. The derivatisation procedure is rather complex and time consuming and entails purification by column chromatography over silica gel. Ethyl chloroformate was also used as derivatising reagent for the determination of TXA by a gas chromatographic procedure (Fig. 4, **5**) [41]. In this case, after the derivatisation procedure, a simple extraction with chloroform is required to collect the carbamate derivatives in the organic layer.

The insertion of an *N*-(2′-nitro-4′-trifluoromethylphenyl) moiety into TXA (Fig. 4, **6**) enhanced its detectability by electron capture [49]. This derivatisation process is rather long and several parameters should be controlled, namely the competing hydrolyses of the derivatising reagent and the reaction rate that is dependent on the type and composition of solvent used, reagent concentration and pH [49].

3. Quantification of TXA

Concerning the practical application of the described quantification techniques to clinical medicine, TXA concentration has been monitored mainly in cardiopulmonary bypass surgery [28,36,37,40]. Pharmacokinetic studies have also been performed in patients undergoing orthopaedic surgery and after intravenous administration to normal volunteers [39,42]. The bioavailability of TXA after administration of an intramuscular dose was also studied in healthy male volunteers [26]. The usual tranexamic acid plasma concentration observed for cardiac surgery 1 h after discontinuation of TXA infusion, reported in the literature, was 17.7 μg mL^{-1} [28]. Bojko et al. [40] reported that, patients undergoing heart surgery with the use of CPB showed, after discontinuation of TXA infusion, an average concentration of 72 μg mL^{-1}.

Methods based on LC-MS/MS applied to the TXA quantification in biological fluids, particularly blood plasma, allowed detection limits ranging from 0.01 to 0.5 μg mL^{-1} [34–40]. The lowest LOD and LOQ values (0.01 and 0.02 μg mL^{-1}) were attained for TXA extracted from a 200 μL of plasma sample [34]. The lower the LOQ value, more suitable is the method developed for the measuring of TXA in plasma collected to determine the clearance of the drug from the system after surgery or even its concentration during the clinical procedure. TXA recovery from plasma, determined for four levels of concentration, ranged from 99.07% to 102.09% while the IS recovery averaged 97.93%. This method, developed by Chang et al. [34], showed linearity over a concentration range of 0.02–10.00 μg mL^{-1} with the calibration curve being split in two concentration ranges. The authors reported that matrix components in plasma did not cause significant changes in the MS/MS response of TXA with the percent of ion suppression being <2.09% across QC sample levels. Also, studies on the inter-lot matrix variability, using TXA at 1.5 μg mL^{-1}, showed no significant variation in the peak area ratio (RSD < 3.84%) [34].

The concomitant quantification of tranexamic acid with rocuronium bromide in human plasma using a LC-MS/MS method provided higher LOQ (0.1 μg mL^{-1}) and LOD value (0.04 μg mL^{-1}) values [38] despite the larger sample volume (800 μL). Linearity was obtained over the

range 0.25–300 μg mL^{-1} and the extraction recovery values ranged between 1.1% and 1.4%. This recovery values are typical of a non-exhaustive SPME pre-treatment of the sample.

There is only one report considering the quantification of TXA in serum by application of a LC-MS/MS methodology. This method, developed by Delyle et al. [35], showed linearity over a concentration range of 1.0–200.0 μg mL^{-1} which makes it suitable for quantification of TXA in clinical studies. The LOD and LOQ values obtained were of 0.05 and 1.0 μg mL^{-1}, respectively. The mean overall recovery of TXA was 85.8% while the mean matrix effect was 102.0% which indicates the absence of any matrix effect in this method. The amount of serum used in this method was small (100 μL) when compared to other reported methods which makes it suitable when considering paediatric patients. The method was applied to a pharmacokinetic study which comprised two adult patients that were subjected to cardiac surgery and two different dose regiments. For the highest dose administered (total dose of 34 mg/kg), serum concentrations after baseline were between 142.6 and 198.5 μg mL^{-1}, while the lowest dose (total dose of 12 mg/kg) provided values between 19.0 and 53.6 μg mL^{-1} under similar conditions [35].

For LC-FL methods, higher LOD and LOQ were obtained. The limit of detection and quantification reported by Huertas-Pérez et al. [29] was 0.08 and 0.2 μg mL^{-1}. Linearity was observed for concentrations ranging from 0.08 to 7.86 μg mL^{-1}. Recovery assays performed in whole blood samples revealed a constant negative bias, which was not observed for plasma. Puigdellivol et al. [26] reported a LOD value 2.5 times higher for the same type of biological samples using a different sample treatment procedure and another derivatising agent (Table 3). For serum samples, the LOD obtained was of 4 μg mL^{-1} and linearity was observed over a concentration range of 25 to 200 μg mL^{-1} [24].

The lowest limit of detection value obtained when using fluorescence detection was achieved for the determination of a prodrug of TXA in dog's whole blood [27], which was ca. 0.001–0.003 μg mL^{-1} using a 500 μL sample volume. This value was dependent on the volume of blood collected and how much of the organic layer could be taken and used for further re-extraction into the aqueous phase. This method showed linearity over a concentration range of 0.01–0.1 μg mL^{-1}.

The simultaneous determination of tranexamic acid and losartan potassium concentration in human serum samples was performed after protein precipitation and without any derivatisation step [46]. LOD and LOQ of 0.012 and 0.040 μg mL^{-1} were attained, with good linearity in the concentration range between 0.04 and 10 μg mL^{-1}, showing recoveries higher than 97.8%.

In what concerns TXA determination in plasma samples using a LC-UV system, the method developed by Liao et al. [45] showed a low LOD at 0.02 μg mL^{-1}. This same method applied to HaCaT cells led to a lower LOD of 0.0002 μg mL^{-1}. The method showed linearity over a concentration range of 0.8–78.6 μg mL^{-1}.

A less studied biological matrix, for the quantification of TXA, is urine with only two reports found in the literature [32,42]. Hadad et al. [32] reported a LOD and LOQ of 0.04 and 0.13 μg mL^{-1}, respectively, using LC-UV. For this matrix, mean recovery of TXA was 100.18%.

Serum samples containing TXA were also analysed by GC-EI leading to LOD and LOQ of 0.2 and 0.6 μg mL^{-1}, with linearity in the concentration range between 0.6 and 5.0 μg mL^{-1}. The method showed recovery of tranexamic acid added to deproteinized serum of 99.6% [41].

4. Conclusions

Although TXA has been in use for several years in clinical practice, there is a large variation in what is the recommended dose and standard protocols for the determination of plasma concentration of this drug during clinical procedures. Therefore, for the study of TXA pharmacokinetics in humans, a simple, fast, cheap and validated method for determination in biological fluids, such as plasma, is still required.

This review has outlined conventional and more up-to-date separation and detection techniques used for the quantification of tranexamic acid in biological fluids. While it has been shown herein that many studies have traditionally used spectrophotometric detection methods coupled to liquid chromatography, current research is tuning to liquid chromatography couple to tandem mass spectrometry. LC-MS/MS has been, over recent years, the technique of choice since it allows increased sensitivity and the ability to measure, in complex matrices such as plasma or serum, down to the ng mL^{-1} range. However, application of this technique still requires attention to ion suppression/enhancement, studies on the efficiency of analyte ionization, and the need for a suitable internal standard.

There is also an increased need for method development in what concerns application of described methods to a large number of samples. This necessarily relates to automated sample preparation that would allow shorter analysis time. Therefore, automated sample preparation with direct sample injection into the LC system could be an important step forward in TXA sample pre-treatment. This will improve, not only the analytical throughput, but also reduce significantly the need for manipulation of biological materials leading to the improved safety of laboratory personnel involved in clinical practice routines.

Acknowledgments

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