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Fluorimetric determination of aminocaproic acid in pharmaceutical formulations using a sequential injection analysis system

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Abstract

A sequential injection analysis (SIA) methodology for the fluorimetric determination of aminocaproic acid in pharmaceutical formulations is proposed. The developed analytical procedure is based on the derivatisation reaction of the aminocaproic primary amine with *o*-phthalaldehyde (OPA) and *N*-acetylcysteine (NAC) and fluorimetric detection of the formed product ($\lambda_{ex} = 350$ nm; $\lambda_{em} = 450$ nm). The implementation of a SIA flow system allowed for the development of a simple, fast and versatile automated methodology, which exhibits evident advantages regarding the US Pharmacopoeia 24 (USP 24) reference procedure. By combining the SIA time-based sample insertion with a subsequent zone sampling approach, which permitted to select for detection of a well-defined sample zone, it was possible to implement an on-line dilution strategy that enabled the expansion of the analytical working range of the methodology, and thus its application in dissolution studies, without manifold re-configuration.

Linear calibration plots were obtained for aminocaproic acid concentrations up to 6×10^{-5} mol l^{-1}. The developed methodology exhibit a good precision, with a R.S.D. < 2.0% ($n = 15$) and the detection limit was 2.5×10^{-7} mol l^{-1}. The obtained results complied with those furnished by the reference procedure with a relative deviation lower than 1.2%. No interference was found.

Keywords: SIA; Fluorimetric detection; Dissolution test; Aminocaproic acid; On-line dilution; *o*-Phthalaldehyde (OPA)

1. Introduction

Aminocaproic acid is an antifibrinolytic agent used in the treatment and prophylaxis of haemorrhagic states associated to excessive fibrinolysis [1]. With the exception of the reference methodologies proposed by several pharmacopoeias, which are based on non-aqueous titrations, almost all of the available methodologies for aminocaproic acid determination involve either a non-specific potentiometric titration [2] or the utilisation of time-consuming and expensive chromatographic techniques [3–5].

The derivatisation of an amino group with *o*-phthalaldehyde (OPA) [6–8] in the presence of a thiol donator, followed by fluorimetric detection of the formed product, has been used as an expeditious analytical approach for the determination of several species, taking advantage of the sensitivity and selectivity of fluorimetric measurements and extending its range of application to the determination of compounds devoid of native fluorescence. Among the available thiol compounds mercaptoethanol (ME) [6] and *N*-acetylcysteine (NAC) [7,8] are the most commonly used, although thiofluor (TF) has recently emerged as a valuable alternative [9].

Aminocaproic acid is a drug originally non-fluorescent but the presence of an amino group in its molecular structure anticipates the possibility of derivatisation with OPA and, in accordance, the development of a fast and simple analytical procedure for its determination. When implemented with all the automation facilities provided by sequential injection analysis (SIA) technique it combined the advantageous features exhibited by fluorimetric measurements, re-enforced by the reaction simplicity, and the robustness, reliability and easy of operation of SIA [10], guaranteeing a noteworthy

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analytical potential for application in the analysis of pharmaceutical preparations. The effective control of the most relevant analytical parameters at run-time assured a great operational flexibility, which allowed the assessment of distinct analytical strategies without physical reconfiguration of the flow set-up and facilitate system optimisation. In this sense, it was possible to extend the analytical working range of the methodology by implementing an on-line dilution approach based on a time-based selection of defined sample zones of the initially inserted sample plug, that were sent to detection. The developed methodology enabled the analysis of samples within a wide range of concentrations without manifold re-configurations and with the consumption of a low sample volume, which permitted its application in the monitoring of the drug concentration in dissolution studies.

The main goal of this work is the development of an automated methodology for the determination of aminocaproic acid in pharmaceutical formulations, which, supported by its simplicity, versatility, low reagent consumption, robustness and easy of operation could constitute an advantageous alternative to the available procedures.

2. Experimental

2.1. Reagents

All solutions were prepared using analytical grade chemicals and deionised water with specific conductance <0.1 $\mu S\,cm^{-1}$.

A 5×10^{-3} mol l^{-1} aminocaproic acid (minimum 99%) stock solution was prepared in deionised water. Working standard solutions were daily prepared by appropriately diluting the above solution with water.

10 mmol l^{-1} *o*-phthalaldehyde (OPA) and 0.8 mmol l^{-1} *N*-acetylcysteine (NAC) solutions were prepared in deoxygenated borate buffer, pH 9.3, with 4% of methanol. Both reagents were protected from light and kept in ice throughout use. The solutions of thiofluor (TF) and mercaptoethanol (ME) used in the evaluation of the thiol-donator compounds were prepared in the same conditions of NAC.

The solutions of the commercially available aminocaproic acid pharmaceutical formulations were prepared by dissolving the required amounts of each pharmaceutical formulation in water.

2.2. Instruments

Fluorescence measurements (λ_{ex} = 350 nm; λ_{em} = 450 nm) were carried out using a LabAlliance Fluorescence detector LC 305 equipped with an 8 μl flow-cell.

The SIA system (Fig. 1) consisted on a Gilson Minipuls 3, peristaltic pump, equipped with a PVC pumping tube (1.0 mm i.d.) and a 10-port multiposition Vici Valco selection valve.

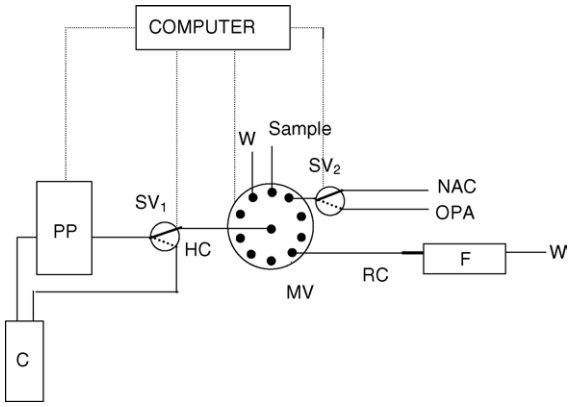

Fig. 1. SIA system used for the determination of aminocaproic acid in pharmaceutical preparations: PP, peristaltic pump; C, carrier (deionised water); SV_1, SV_2—3-way Solenoid valves; HC, holding coil (4 m; 0.8 mm i.d.; straight); MV, multiposition selection valve; RC, reaction coil (1 m; 0.8 mm i.d.; figure eight); F, fluorescence detector; W, waste.

Manifold components were connected by means of PTFE tubing, 0.8 mm i.d., which was also used for the holding and reaction coil (4 and 1 m, respectively).

A 3-way solenoid valve (NResearch 161 T031, W. Caldwell, NJ, USA) (SV_2, Fig. 1), placed at one of the inlets of the selection valve, was used to process the online addition of OPA and NAC. A second solenoid valve (SV_1, Fig. 1) and a contact device (on the peristaltic pump), similar to the one described before by Araújo et al. [11], controlled the pump starting point in order to guarantee reproducibility in the solution aspirated or propelled volumes.

Analytical system control, including the operation of the peristaltic pump, selection valve and solenoid valves, was achieved by means of an Advantech PCL 711B interface card and a Pentium-I based microcomputer. Software was developed in Microsoft Quick-Basic and permitted to control flow rate, flow direction, valve position, sample and reagent volume and data acquisition and processing.

During optimisation the analytical signals were also recorded on a Kipp & Zonen BD 111 strip chart recorder.

Dissolution studies were carried out by coupling the developed SIA system to a stirring basket Erweka DT dissolution apparatus. Sample solutions were aspirated at runtime through an inline filter (Schleicher & Schuell, 1.3 cm diameter).

2.3. SIA manifold

Aminocaproic acid was determined by fluorimetry (λ_{ex} = 350 nm; λ_{em} = 450 nm) upon reaction with OPA and NAC. The analytical cycle started when the reagents were alternately aspirated into the holding coil (HC, Fig. 1), by binary sampling, actuating sequentially solenoid valve SV_2 between position 1 and 0 (on/off). This solenoid valve was placed at position 10 of the multi-port selection valve MV. Each reagents insertion sequence consisted on three cycles of an intercalation time of 1 s (each reagent solution was

aspirated during 1 s at a flow rate of 0.5 ml min^{-1}), which permitted to intercalate three slugs of 8 μl of OPA with three slugs of 8 μl of NAC creating a well-homogenised reagents zone of, approximately, 50 μl. The sample plug was subsequently aspirated into the holding coil at a sampling time of 3 s, which, at the referred flow rate (0.5 ml min^{-1}), corresponded to a sample volume of about 25 μl. By flow reversal the developing reaction zone was, after that, directed towards detection, at a flow rate of 1.5 ml min^{-1}, and an analytical signal was obtained at approximately 40 s.

The analytical cycle programming to carry out the on-line dilution approach, used during the execution of the dissolution studies, was similar to the one referred with just a slight difference. When the flow was reversed the entire developing reaction zone was not immediately sent towards detection but was instead directed to waste. After a pre-defined time interval (16 s at a flow rate of 0.5 ml min^{-1}), when a significant part of the initially inserted reaction zone was already discharged, the multi-port valve was placed in the detector position and only the tailing edge of the sample zone that remained in the holding coil was sent to detection.

2.4. Reference method

Aiming at a comparative evaluation, the samples analysed by the developed procedure were also analysed with the methodology recommended by the US Pharmacopoeia 24 [12] consisting on a non-aqueous titration in glacial acetic acid with 0.1 mol l^{-1} perchloric acid (in glacial acetic acid) using crystal violet as end-point indicator.

2.5. Dissolution studies

Dissolution studies were carried out according to the USP 24 [13]. A stirring-equipped (100 rpm) basket apparatus was connected to the analytical system through the sample feeding line. Water at 37.0 ± 0.5 °C was used as dissolution medium. Sample aliquots were collected at pre-set time intervals through an in-line filter and analysed without further treatment.

3. Results and discussion

The thiol-donator compound played a fundamental role in the two-stage derivatisation reaction of aminocaproic acid with OPA. Previous works involving the analysis of distinct amino compounds referred NAC, ME and TF [6–9] as the mostly used alternatives dictating their selection for the subsequent experiments. By assessing increasing aminocaproic acid concentrations and by using an OPA concentration of 2 mmol l^{-1} and a thiol compound concentration of 0.4 mmol l^{-1} [6] it was verified that the highest analytical signals were obtained with NAC ($IF = 1 \times 10^7 C + 19.47$; where IF is the fluorescence intensity and *C* the concentration of aminocaproic acid in mol l^{-1}) twice over the fluorescence intensity obtained with TF ($IF = 6 \times 10^6 C + 10.99$; where IF is the fluorescence intensity and *C* the concentration of aminocaproic acid in mol l^{-1}).

The evaluation of NAC was carried out for concentrations ranging from 0.25 to 20 mmol l^{-1} at an OPA concentration of 2 mmol l^{-1} [6]. The obtained results showed that a NAC concentration of 0.4 mmol l^{-1} yielded the highest fluorescence intensity. This NAC concentration was consequently used to assess OPA at a concentration range between 1 and 6 mmol l^{-1}. In this case the best results were obtained for 5 mmol l^{-1} OPA. It was also verified that as the NAC/OPA ratio increased the reaction kinetics decreased and consequently the analytical signals were lower. Ultimately, for a NAC concentration of 20 mmol l^{-1} no fluorescence was detected at all.

The stability of the NAC/OPA reagent solution was a major problem in the early stage of the experiments. It was observed that for the same concentration the obtained fluorescence intensity changed markedly throughout the working day as it was affected by multiple factors including dissolved oxygen, light and heat. In an attempt to overcome this situation the borate buffer used in its preparation was deoxygenated and the resulting NAC/OPA solution was kept on ice and protected from light. However, despite this careful conditioning the analytical signals obtained immediately after the preparation of the solution were higher than those obtained after several hours, which seemed to indicate that the poor solution stability was not only affected by external factors but could be also a consequence of the combined presence of both reagents. In view of that, it would be advisable to keep the reagents apart and mix them in-line only when the reaction takes place. Doing so a complementary aspect would have to be considered: since reagents were introduced immediately before the sample, reagent mixing would have to be achieved promptly in order to guarantee an adequate sample zone homogenisation during transportation to detection. To accomplish this objective the reagents (OPA and NAC) were introduced through the same port of the multi-position valve, by binary sampling [14]. At this port was connected a three-way solenoid valve conveying the two reagent solutions (OPA, NAC, Fig. 1). The alternate actuation of the solenoid valve between the two positions holding OPA and NAC solutions allowed the establishment, by aspiration, of a reagents string consisting on very small segments of OPA intercalated with very small segments of NAC, the number of segments determined by the insertion cycle. The very small volume of the inserted reagents segments, which coalesced during transportation through the multi-position valve into the holding coil, facilitate mixing and the formation of a homogenised reagents zone. The evaluation of the insertion cycle showed that improved analytical signals were obtained when the intercalation time for each reagent was 1 s (intercalation of about 8 μl for a flow rate of 0.5 ml min^{-1}) and the total number of intercalation cycles was 3, which resulted in the insertion of approximately 25 μl of each reagent solution (50 μl for the total reagents volume). The utilisation of an

inferior number of intercalation cycles (one or two cycles, corresponding to a total volume of 16 or 32 μl, respectively) yielded lower analytical signals, which could be explained by a reagent shortage. A similar result was obtained with the utilisation of four or five intercalation cycles (corresponding to a whole reagent's volume of 64 or 80 μl, respectively), in this case probably a consequence of an increase in dispersion due to an excessive reagent volume. Although lower intercalation times could have been used, an intercalation time of 1 s assured the aspiration of solution volumes low enough to provide an adequate mutual dispersion without affecting reproducibility. For the same aminocaproic acid concentration the obtained analytical signals remained stable throughout the working day, the consumption of reagents was low (25 μl each per assay) and since the reagent solutions were kept apart their activity was unaffected.

The assessment of the influence of the inserted sample volume was carried out in terms of sampling time that was the period of time of sample aspiration at a flow rate of 0.5 ml min^{-1}. Sampling times between 1.5 and 6 s were evaluated, which corresponded to sample volumes of approximately 12.5–50 μl. It was verified that the analytical signal increased with the sampling time until a value of 3 s (25 μl) above which no measurement was obtainable due to detector's saturation.

As it was previously referred, an important feature affecting system performance was the sample/reagent mixing and reaction zone homogenisation. In a SIA system these aspects are, to a great extent, determined by the sequence of insertion (or aspiration) of sample and reagents into the holding coil, which dictates how the different zones will mutually inter-disperse or which zone will endure a higher degree of dispersion. The finest results, in terms of mixing efficiency and signal magnitude, were obtained when the mixture of reagents was first introduced in the holding coil followed by the sample plug, which could be explained by the lower sample dispersion and the greater reagents zone penetration.

The influence of flow rate was evaluated considering two complementary instances: sample and reagents aspiration for insertion and reaction zone propelling for detection. The flow rate during solutions aspiration was evaluated between 0.3 and 1.5 ml min^{-1} and it was verified that a flow rate of about 0.5 ml min^{-1} produced superior results. Lower flow rates affected sampling rate while higher values, although assuring an increased sample throughput, exhibited a poorer repeatability (R.S.D. > 2%). The optimisation of the solutions propelling flow rate was more demanding because it had also to take into account the length of the reaction coil. Since reaction rate was high a compromise had to be establish between flow rate and coil length in order to assure an adequate residence time, thus convenient sample/reagent mixing and reaction development, but avoiding an undue dispersion of the reaction products that would affect sensitivity. The assessment of flow rates between 1 and 3 ml min^{-1} together with reaction coils of up to 1-m length showed that flow rates above 1.5 ml min^{-1} resulted in too short residence times that give rise to low analytical signals, while reaction coils above 0.5-m length lead to unwanted sample dispersion. Distinct reaction coil configurations (straight, figure eight and coiled reactors) were also tested and figure eight reactors were considered the most convenient because they produced higher and narrower peaks.

Following optimisation of parameters such as sample volume, reagent volume, flow rate and reaction coil length the developed methodology was evaluated for aminocaproic acid concentrations up to 6×10^{-5} mol l^{-1} as well as in the analysis of commercially pharmaceutical formulations of aminocaproic acid. A characteristic of the developed procedure that was clearly evident was its sensitivity. However, this feature, which most of the time is a desirable advantage, could sometimes work as a drawback especially when the working analytical range of the methodology is relatively restricted or the detector is easily saturated. In such a situation a manual dilution step is often required to adjust sample concentration. However, the versatility of the developed flow system provided the means for the implementation of an automated on-line dilution approach that would permit to easily overcome this difficulty. The reduction of the inserted sample volume or flow rate, or the increment of the reactor's length could act as immediate solutions but they have limited applicability not only in terms of the dilution level attainable but also due to its negative impact on sampling rate. Moreover, the insertion of very low sample volume is more prone to errors and usually exhibits poorer repeatability. A more feasible strategy was the implementation of a sampling zone approach [15]. This was more versatility, assuring a wide range of dilution levels, and did not require any physical modification of the flow manifold. This way, all of the optimised parameters (sample volume, reagent volume, flow rate and reactor length) remained unchanged and only an extra line was added to the system control programming.

For the implementation of the sampling zones approach the sample/reagents insertion stage was maintained and the only modifications were introduced in the subsequent solutions propelling after flow reversal. In this stage, instead of being immediately directed to detection, the developing reaction zone was carried out towards waste (Fig. 1) by selecting the appropriate position of the multi-port valve. After a predefined transport time the multi-port valve was placed in the detector position and the tailing edge of the highly dispersed reaction zone, which remained in the holding coil, was detected. This way, under a simple time-based control it was possible to determine which portion of the initially inserted sample plug was removed to waste or sent to detection, providing a suitable dilution level in a controlled and reproducible process.

By inserting a 1×10^{-6} mol l^{-1} aminocaproic acid standard solution increasing transport times were assessed. As it was expected, the obtained results showed that the analytical signal decreased as the transport time increased (Fig. 2) since decreasing fractions of the initially inserted sample reached detection. Ultimately, if an excessive transport time was used

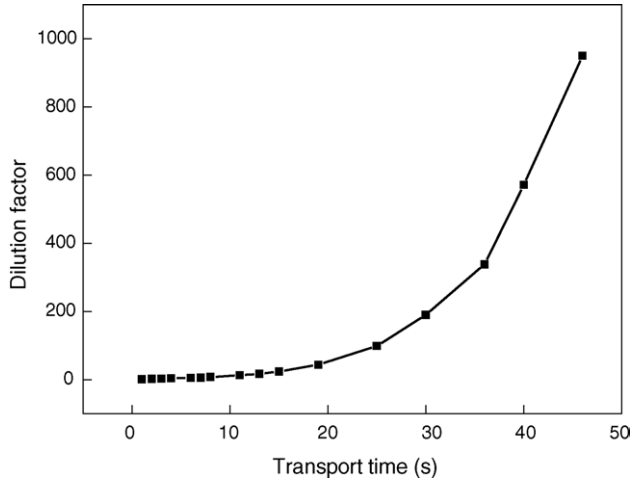

Fig. 2. Influence of transport time on the obtained dilution factor. Dilution factor: dilution level attained by calculating the ratio between the analytical signals obtained with and without dilution. Transport time (s): time interval during which the sample zone was transported to waste prior to re-directing to detection.

then the entire sample plug would be discharged and no analytical signal would be obtained. For the assessed transport times the dilution level was about 100-fold for 25 s, 200-fold for 30 s, 600-fold for 40 s and 1000-fold for 47 s, meaning that the versatility of this dilution approach permits to adjust the dilution level to the sample concentration range just by selecting an appropriate transport time.

It was also verified that a transport time of 16 s (at a flow rate of 0.5 ml min^{-1}) enabled the analysis of aminocaproic acid concentrations of about 3.8×10^{-2} mol l^{-1}, which was perfectly adequate for carrying out the dissolution studies according to USP 24 [12] taking into account the aminocaproic acid dosage of each pharmaceutical formulation.

4. Dissolution studies

For carrying out the dissolution studies, and after the estimation of the most convenient transport time, it was also necessary to evaluate the performance of the developed flow manifold after its connection to the stirring basket dissolution apparatus and the filter support. The sample feeding tubing was immersed into the dissolution medium in such a way as to restrain the occurrence of hydrodynamic disturbances and the influence of the filter support was also studied since it could impose a resistance to the flowing stream affecting the aspiration flow rate. It was observed that the performance of the developed system, including the aspiration and propelling flow rates, was unaltered and that all of the previously optimised analytical parameters were adequate and did not require any adjustment.

The tablets dissolution profiles resulting from periodic measurements showed an increase of the aminocaproic acid concentration, in the dissolution medium with time (Fig. 3)

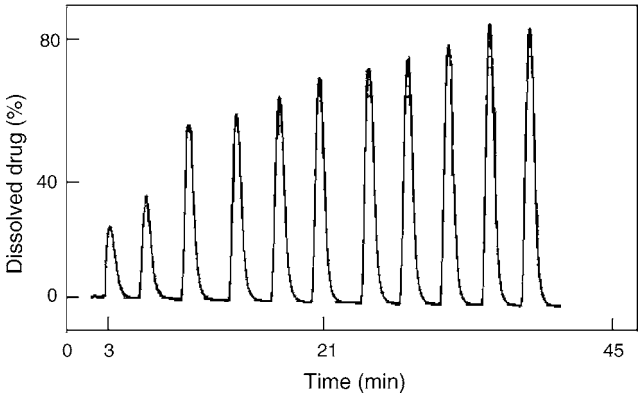

Fig. 3. Analytical signals record obtained in dissolution studies of aminocaproic acid solid dosage formulations.

that approached stabilisation. For all the formulations tested the final results were in accordance with the criterion imposed by USP 24, which refers that not less than 75% of the labelled amount of aminocaproic acid should be dissolved in 45 min.

The low solutions consumption characteristic of SIA systems was an important advantage in these experiments since for each analysis only a very small sample volume was required, which did not significantly alter the total volume of the dissolution medium. This way it was possible to carry out multiple successive determinations without affecting the concentration of the dissolution vessel.

4.1. Interferences

Considering that the developed methodology was to be applied in the analysis of pharmaceutical preparations it was important to assess the potential interfering effect of several compounds commonly used as excipients. Standard solutions with a fixed amount of aminocaproic acid (1×10^{-6} mol l^{-1}) and increasing concentrations of the excipients (glucose, lactose, sucrose, fructose, borate and starch) were analysed by the SIA methodology. A specie was considered as non-interfering when the analytical signal variation regarding the one obtained in its absence was lower than 3%. The obtained results showed that up to a 100 molar ratio aminocaproic/excipients (highest value tested) no interfering effect was noticeable.

5. Analysis of pharmaceutical formulations

Following system optimisation linear calibration plots for aminocaproic acid concentrations up to 6×10^{-5} mol l^{-1} were obtained, the detection limit [16] being 2.5×10^{-7} mol l^{-1}. The analytical curve was represented as $IF = 10^7\, C + 6.1921$, where IF = fluorescence intensity and C = aminocaproic acid concentration expressed in mol l^{-1} with a correlation coefficient of 0.9995 ($n = 6$). The developed methodology was applied in the determination of aminocaproic acid in pharmaceutical formulations

Table 1
Results of the analysis of the pharmaceutical preparations by SIA and reference methods

Samples	SIA (g/formulation)	Reference method (g/formulation)	Relative error (%)
Epsicaprom, 3 g			
With dilution	3.066 ± 0.002	3.024 ± 0.006	+1.4
Without dilution	3.072 ± 0.003		+1.6
Epsicaprom, 2.5 g			
With dilution	2.480 ± 0.007	2.505 ± 0.009	−1.0
Without dilution	2.501 ± 0.005		−1.0
Ipsilon, 0.5 g			
With dilution	0.490 ± 0.005	0.497 ± 0.002	−1.4
Without dilution	0.496 ± 0.005		−0.2

commercially available in Portugal and Brazil. Sample solutions of distinct concentration were analysed. To evaluate the developed dilution approach more concentrated samples were prepared and analysed. These samples were then manually diluted and analysed by the developed direct procedure. No significant differences (R.S.D. < 2%) were obtained in the repetitive analysis ($n = 15$) of samples by both the dilution procedure (samples within the 3.9×10^{-2} and 7.4×10^{-3} mol l^{-1} concentration range) and direct procedure (samples within the 2×10^{-7} and 4.2×10^{-5} mol l^{-1} concentration range), which confirmed the repeatability of the developed procedure. The sampling rate was about 40 and 50 samples per hour for the dilution and direct procedure, respectively. In order to evaluate the accuracy of the proposed SIA methodology the obtained results were compared with those furnished by the reference procedure. No significant divergence was verified in the results obtained by both methods with a relative deviation (expressed in percentage) lower than 1.7% (Table 1). These results were further confirmed by a paired *t*-student, which, for a 95% ($n = 6$) confidence level, showed no statistical discrepancy between both methodologies (*t*-value estimated = 0.739 while *t*-value tabulated = 2.570).

6. Conclusions

The developed flow methodology combines the well-known advantages of SIA, like simplicity and robustness with the promptness, selectivity and sensitivity of fluorescence measurements provided by the derivatisation reaction. The obtained results confirmed that it represents a valuable alternative to the method recommended by USP 24, allowing the routine determination of aminocaproic acid in pharmaceutical formulations in a fast, automated, low reagent consumption and low residues production style. Moreover, it is also more expeditious and requires less manipulations and less expensive equipment that the alternative chromatographic methods, exhibiting as well a wider working analytical range.

The developed system demonstrated that by combining the SIA technique with the sampling zones approach, and without reconfiguration of the flow manifold, a very efficient on-line dilution strategy is implemented, which is able to analyse samples with assorted concentrations at high sampling rate and to carry out dissolution studies. At the same time it provides results characterised by good precision and accuracy and statistically similar to those furnished by the reference procedure.

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