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Validation of a Quantitative Determination Method of Paramino-Salicylic Acid by High-Performance Liquid Chromatography and Its Application in Rat Plasma

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1. Introduction

The non steroid anti-inflammatory drugs (NSAID) are among the most prescribed because of their analgesic and anti-inflammatory properties [1].The derivatives of the acid aminosalicylic (4ASA and 5-ASA) are used since many years in the treatment of the intestinal chronic inflammatory diseases: Crohn disease and colitis. Aminosalicylic acids exert a direct anti- inflammatory action on the intestinal mucous membranes. They do not have any link with the drugs of cortisone family, or with traditional NSAID used for pain and rheumatism. They are also different from the acetylsalicylic acid (Aspirine®).
4ASA also known under the name of paramino benzoïc acid (PABA) or paramino salicylic acid (PASA) (figure 1) is employed for the treatment of the ulcerative colitis and Crohn disease. These diseases are characterized by an ignition of the colorectal mucous membrane [2].

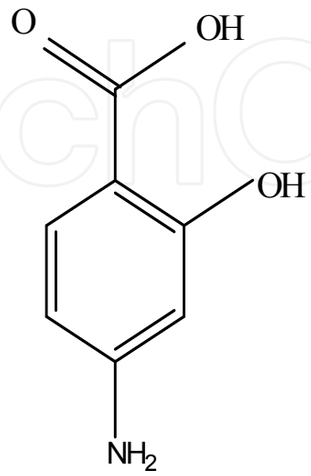

Fig. 1. Molecular structure of 4ASA

In the literature, several analytical methods primarily based on separation were described. Therefore, quantitative methods to assay, for instance a drug substance or its impurities, have needed to be fully validated after development. Depending on the type of analytical method and its intended use, a check for linearity, precision, accuracy, specificity, range, limits of detection and quantification, and/or robustness, is required [3-7], gas chromatography coupled with the mass spectrometry were the most usually used methods for analytes separation [8]. A capillary method of electrophoresis was developed for 4ASA determination and its metabolites: acid N-acetyl-p-aminosalicylic (N-acetyl-PASA) in urine. Good separation of analytes was carried out with a 12 min retention time for15min [9]. 4ASA is also quantified by HPLC in the biological fluids [10, 11]. In this present article, we describe the development of a new RP-HPLC method for the determination of 4ASA at the same time in both aqueous medium and plasma.

2. Material and methods

4ASA was supplied by Merck (Hohenbrunn, Germany). Formic acid (99-100%) was provided by Prolabo. Potassium dihydrogene phosphate (KH2PO4), acetonitrile (MeCN) and potassium hydroxide (KOH) were provided by SDS (Peypin, France). Alpha cyclodextrine was purchased from Waker and Gelucire® from Gattefossé (France). Sodium citrate was obtained from Sigma chemical Co (USA). Water purified on Milli-Q system (Millipore, USA) was used. All other chemicals were of analytical grade.

2.1 Instrumentation

The liquid chromatographic system used in the present study, consisted of a pump (880-Jasco, Japan), an automatic injector (autosampler HPLC -360Kontron, Brehme, Germany) and a detector (Jasco 875-UV). All the parameters of HPLC were controlled by the Azur software: version 3.0 coupled to an acquisition box (Azur PAD).

2.2 Chromatographic conditions

The mobile phase was prepared by mixing water, formic acid and acetonitrile in varied proportions. The optimum mobile phase used in the validation studies consisted of water-formic acid-acetonitrile 67:3:30, (v/v/v). Before analysis the mobile phase was filtered through a 0.45µm membrane and degassed by ultra sonication (Transsonic 950, Prolabo). Solvent delivery was employed at a flow rate of 1.0 ml min^{-1}. A Kromasil column: C-18µm, 250mm ×4.6mm (Chromato, France HAS) was used and maintained at room temperature. Detection of the analytes was carried out at 300nm.The appropriate wavelength for the detection of the drug was determined by wavelength scanning over the range of 200-400nm. Injection volume of the analytes was set to a constant volume of 50µl.

2.3 Validation of the method

The described method has been validated in terms of specificity, precision, linearity, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

2.3.1 Precision

Precision was expressed with respect to the intra and inter-day variations in the expected drug concentrations.

2.3.2 Linearity

Data were obtained using a stock solution of 4ASAS at 0.5 $mg.ml^{-1}$ as concentration. This solution was then diluted using highly purified water to obtain the standard solutions from 1 to 125µg/ml concentrations. Five injections were made for each concentration. The linearity of the calibration curves was determined on three different days for intra or inter-day variation.

2.3.3 Accuracy

Accuracy of the proposed method was established by recovery experiments using standard addition method. This study was employed by addition of a standard reference (pure marketed product whose purity was tested beforehand by Differential Scanning Calorimetry (DSC) and Fourier transformed Infrared Spectrometry (FT-IR).

2.3.4 Robustness

An experimental design was used to evaluate the influence of selected factors. Two-level factorial design was used (Table **1**).

	1	2	3	123
	MeCN (%)	Wavelength (nm)	Flow rate (ml/min)	pH
1	-	-	-	-
2	+	-	-	+
3	-	+	-	+
4	+	+	-	-
5	-	-	+	+
6	+	-	+	-
7	-	+	+	-
8	+	+	+	+

Low level	-	27	297	0,8	2
High level	+	33	303	1,2	2,4

n=5

Table 1. Influence of selected factors: MeCN (%), Wavelength (nm), Flow rate (ml/min) and pH on the robustness of the method:

For this method only factors were examinated: acetonitrile ratio, wavelength, flow rate and pH.

2.3.5 Limit of detection and limit of quantification

Several approaches were used to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio, the use of standard deviation of the response and the slope of the calibration curve.

In the present study, the LOD was related to signal / noise of the system and was defined as a peak whose report/ration signal background noise was at least equal to 3/1. The LOQ was defined as a peak whose report/ ratio signal background noise was at least equal to 10/1.

The determination of these values was made starting from the line of the range standard. These two values were calculated from the Response Factor (Rf) according to formulas (1) and (2): the initial concentration corresponds to the intersection of the right-hand side to the y-axis [12].

$$\mathrm{Fr} = \frac{\text{Standard area}}{\text{Initial Concentration}} \quad (1)$$

$$\text{Sample concentration} = \frac{Sample\ area}{Fr} \quad (2)$$

2.3.6 Stability

Experiments were carried out under the same conditions as the samples analysis.

2.3.7 Recovery test

Recovery test was carried out by weighing three batches of pellets (containing 50% of 4ASA) compared with a powder sample of pure active ingredient. Amounts representing 120,100 and 80% of 4ASA were used as equivalent concentrations. Each batch is crushed using a mortar then filtered before being solubilized in 250ml water with respect of sink conditions. The release properties of the active ingredient from the extruded formulations matrix were studied by using a process of the delayed release. The medium of dissolution was maintained at 37°C with 100 rpm stirring. After 6 hours, three samples of 1 ml were taken in each sample of buffer solution of phosphate potassium (0.2 M) whose pH was adjusted to 7.5 using a potassium hydroxide buffer (KOH 1M).

3. Application to biological samples

The proposed method was applied to the determination of 4ASA in plasma samples from the bioequivalence study. *In vivo* studies of 4ASA blood concentration were carried out by using established methods of the literature [10]. Male Sprague-Dawley rats weighing 200–250g (n = 4) were purchased from Charles Rivers Enterprise (France) and kept in laboratory conditions before experiments.

3.1 Linearity and precision

Linearity was assessed by analyzing seven standards with concentrations over the range of 10-100µg/ml in plasma (n=5). Precision and accuracy were tested comparing to the values obtained previously in aqueous solution. Aliquot of 1000µl plasma were mixed with 500µl of standard solution of 4ASA. The mixture was vortexed for 2 min and transferred into the vial for HPLC analysis (n=8).

3.2 Recovery test in plasma

For recovery test, three samples of 1000µl plasma were mixed with 250µl of standard solution (25, 50 and 100µg/ml) of 4ASA. The mixture was vortexed for 2 min and transferred into the vial for HPLC analysis (n=5).

3.3 Stability test in plasma

Drug stability during sample collection and processing is an important factor for clinical bioanalysis. Three samples of 1000µl plasma were mixed with 500µl of standard solution of 4ASA and analyzed at 0 and 24hours at room temperature and at 4°C (n=5).

3.4 Blood-plasma partition ratio

Blood-plasma partition ratio would predict plasma concentration-time courses following 4ASA oral doses to rats.

Partition test was carried out using three fresh samples of blood (1ml) mixed with 500µl of 100µg/ml standard solution of 4ASA. The mixture was maintained in constant stirring at room temperature for ten minutes and analyzed by HPLC.

3.5 Bioavailability

Each rat received an oral dose of minigranules containing 25mg of 4ASA. Three hours after administration of minigranules, the rats were anesthetized with an intra peritoneal pentobarbital 10% (ABBOT, Ringis, France) at the dose of 100µl/100g (re-injected with 10µl/100g as necessary). The internal carotid artery was cannulated via 4cm paratracheal incision, and blood samples were taken at 0.5, 1, 2, 3 and 4 hours after anesthesia.

Rats were killed at the end of experiments by exsanguinations. These samples were collected into eppendorf tubes containing 500µl of citrate buffer (0.129M) and kept on ice for at least 30 min.

Plasma was obtained by centrifugation (Eppendorf centrifuge, Germany) at 3500 rpm for 10 min and transferred to clean test tubes. After vortexing for 1 min using a Heidolph Top-mix 94323 (Bioblock scientific, Germany), plasma samples were analyzed by HPLC method. Analyte concentrations were calculated by using an account of the factor of dilution.

4. Results and discussion

4.1 Chromatographic conditions

Experiments were carried out with 10 up to 90% of acetonitrile and water as a mobile phase. The best peak shape and the maximum of separation were obtained with a binary mixture: water - acetonitrile (70% and 30% respectively). A minimum of retention time (Rt=4.3-4.8) with good peak resolution was obtained under a flow of 1ml.min^{1}.

The optimum wavelength for analyte detection with adequate sensitivity was found to be 300nm.

Retention time was very sensitive to the freshness or ageing of mobile phase.

4.2. Validation of the method

4.2.1 Specificity/selectivity

A chromatogram was obtained from blank samples. No peak corresponding to the endogenous compounds was detected at the analyte retention time (4.3-4.8min) at 300nm. It means that the developed method is selective in relation of the used carrier for pellet formulation.

4.2.2 Linearity

Seven-point linearity curve was constructed for three consecutive days. Samples were quantified using

concentrations – peak area relationships and were calculated by the simple regression analysis. The plots of peak area ratios versus concentrations of all analytes were found to be linear within the concentration range (Figure **2**).

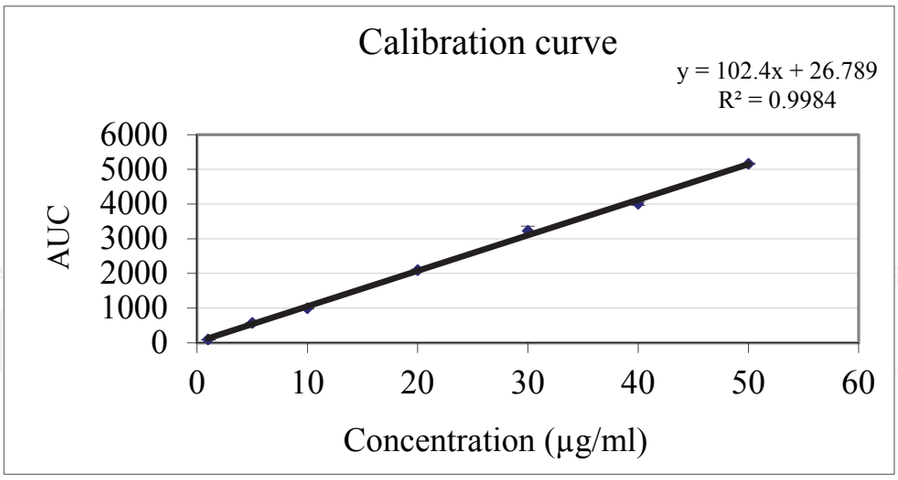

Fig. 2. Linearity of quantitative method by HPLC. Bars represent standard deviations of the mean (n = 5)

4.2.3 Accuracy and precisions

The results were expressed as percent recoveries of the particular components in the samples. The average percent recoveries (±RSD) were respectively in the range of 97.3±0.9 - 99±1.7 (Table **2**).

Concentration (mg/ml)	**0,005**	**0,0075**	**0,01**
Mean of AUC	**329,747**	**489,049**	**725,458**
RSD (%)	**0,740**	**1,874**	**0,51**

Table 2. Accuracy of the proposed method (n =10). AUC: Area Under Curve

The results of intra-day and inter-day precision experiments are indicated according to the averages and relative standard deviations (RSD). Inter-day and intra-day precision given by relative standard deviation (%R.S.D.) of quality control samples were ≤ 3.3%. The calculated values are presented onto (Table **3**).

Concentration (µg/ml)	Intra-day		Inter-day	
	Area (UA)	RSD (%)	Area (UA)	RSD (%)
1	98,47	0,98	94,57	2,20
5	551,00	0,60	563,31	3,35
10	998,01	0,61	990,82	0,76
20	2058,66	0,18	2090,11	0,51
30	3189,39	2,29	3246,26	1,19
40	4059,34	3,19	4046,40	1,26
50	4843,45	1,01	4939,88	3,24

Table 3. Intra and inter-day precision of 4ASA quantification with area (UA) andRelative Standard Deviation (RSD) (n=3).

4.2.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The results showed a less significant limit of detection (105ng/ml) compared to the limit of quantification (305ng/ml).

4.2.5 Recovery test

The overall mean recoveries calculated were 80, 100 and 120 for low, medium and high quality control samples respectively. A linear curve was obtained meaning proportional analyte amount (Figure **3**).

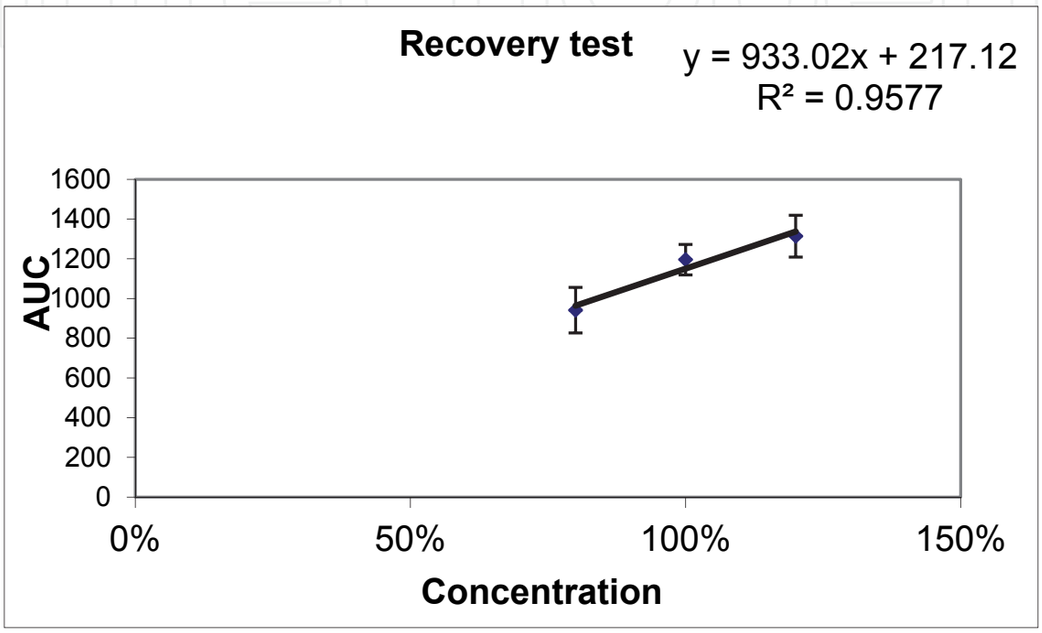

Fig. 3. Chart of the test of recovering: Bars represent standard deviations of the mean (n=9).

4.2.6 Robustness

Two–level fractional factorial design were applied in this method. The operational parameters do not lead to essential changes of the performance of the chromatographic system except the wavelength. Only the wavelength seemed to influence the robustness of the method. The interaction between the acetonitrile ratio and the flow rate didn't seem to have any effect on the robustness (Figure 4).

The obtained values were accurate and RSD values didn't exceed 3.3%. On the other hand the retention time varied quickly from 2.98 to 5.28 min for each modified parameter. Acetonitrile change ratio would change the polarity of the mobile phase while pH could modify the analyte protonation.

4.2.7 Study of 4ASA stability

Three aliquots were used during this experiment which lasted 6 hours. The medium of dissolution was maintained with 37°C, in constant stirring with 100 rpm to simulate gastrointestinal conditions. The results showed a relative stability of 93.5% (Figure **5**).

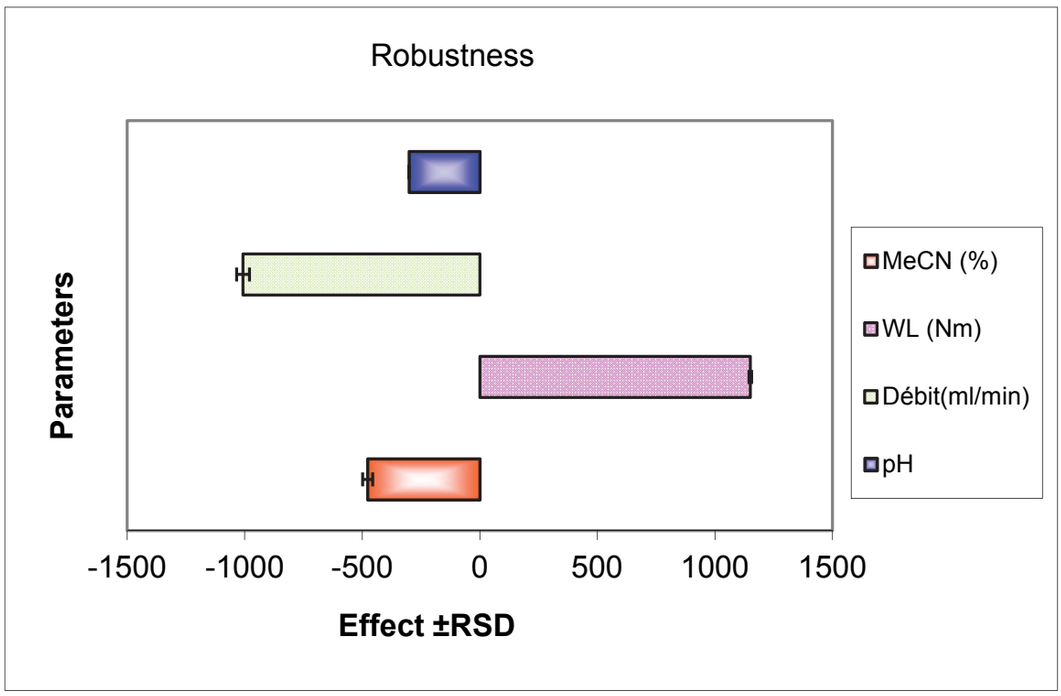

Fig. 4. Histogram representing robustness test of the method. Bars represent standard deviations of the mean (n=5).

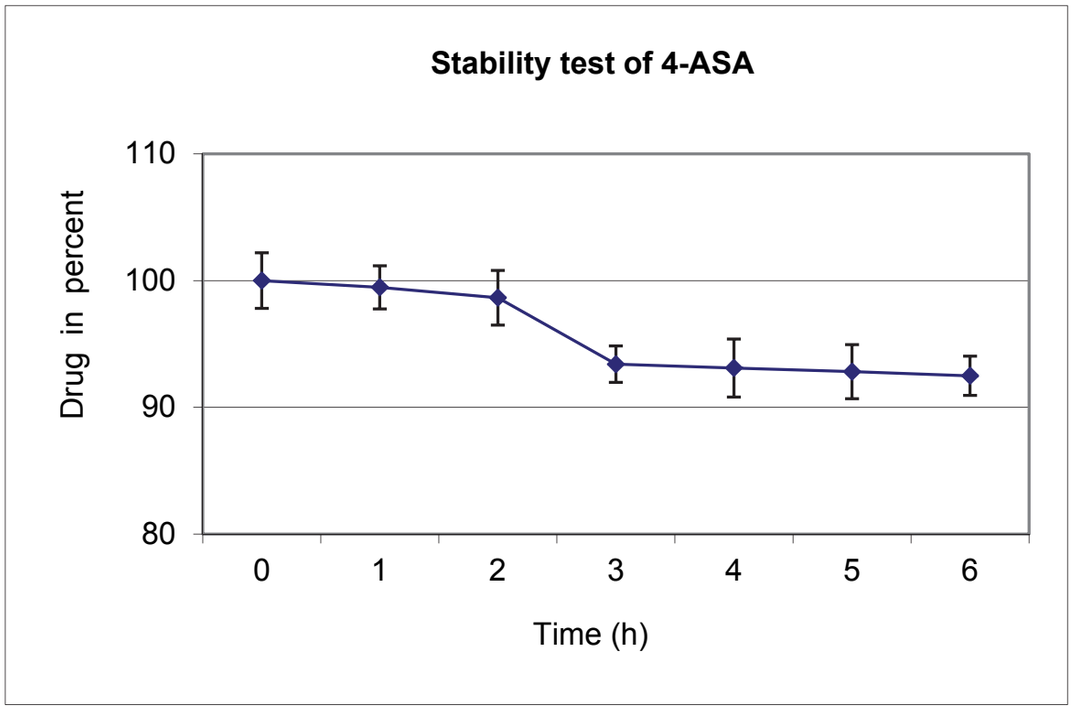

Fig. 5. Study of 4ASA stability during 6h. Bars represent standard deviations of the mean (n=3)

The determination of 4ASAconcentration following the degradation, was a positive point on the specificity of the drug.
It would mean that the first breackdowned products did not absorb at 300nm. However new peaks at lower retention time (<3min) were detected on seven day-old samples at 300nm.The later consideration deals with the specificity of the method and its ability to separate degradation products for pure drug.

4.3 Application to biological samples

The validated analytical method was applied to male Sprague-Dawley rats.
Compared to the results given by working standard solutions, good peak shape and acceptable sensitivity were obtained with the same mobile phase (acetonitrile /water 30:70) from diluted plasma samples containing 4ASA. Good precision (CV=3.39%) and reproducibility were also observed during 4ASA quantification in plasma samples (Table **4**).

	Area	%
Mean	8849,978	100,7479
RSD	152,8166	2,718627

Table 4. Evaluation of the Intra assay precision (n=8)

The accuracy of these values was confirmed by back calculating the concentration of the calibrations standards.
The retention time was from 4.3 to 5.2 min and did not interfer with other compounds from plasma. This period was closed to that obtained with those obtained with aqueous solution (4.3-4.8min). The concentration range (10-100µg/ml) in plasma is shorter than the aqueous medium concentration (1 to 125µg/ml). The regression equation was Y=88.72 x-88.38 and coefficient of correlation (r^2) was 0,999.With regard to the partition test, plasma level of 4ASA reached a maximum of 101.95% (Figure 6).

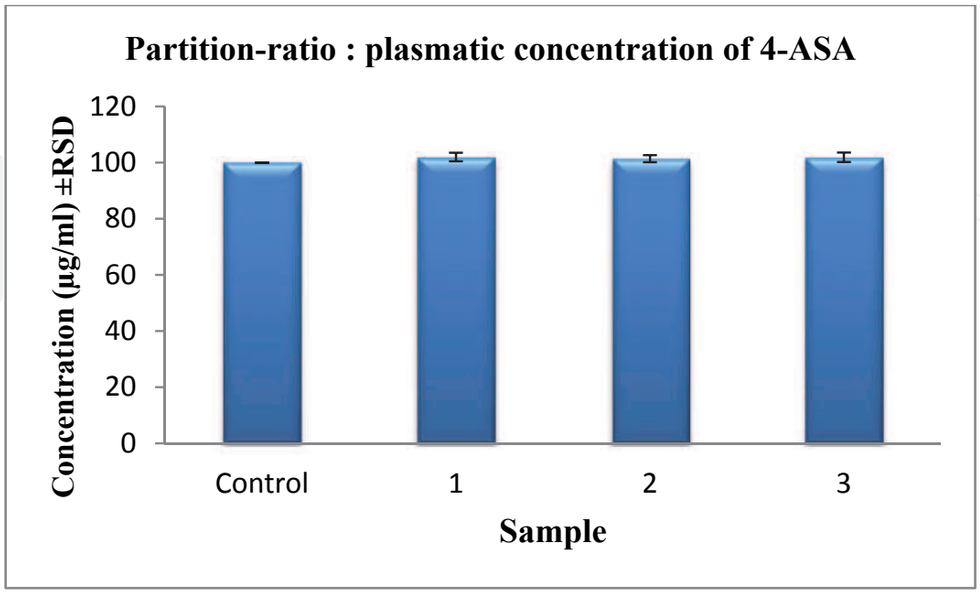

Fig. 6. Plasmatic concentration of 4ASA after partition-ratio.
Bars represent standard deviations (n=5)

The average recovery of assay was 93.54%, 100.89% and 100.23% for low, medium and high concentrations of 4ASA in plasma respectively (Table **5**).

Concentration (µg/ml)	RSD (%)	
Expected	Measured	
25	23,39	1,74
50	51,45	3,28
100	100,3	1,65

Table 5. Responses in recovery tests at low, mean and high value. Mean ±RSD

Stability tests were investigated using the procedure described above. The results indicated that 4ASA was stable in plasma at -4°C for 24h (Figure **7**).

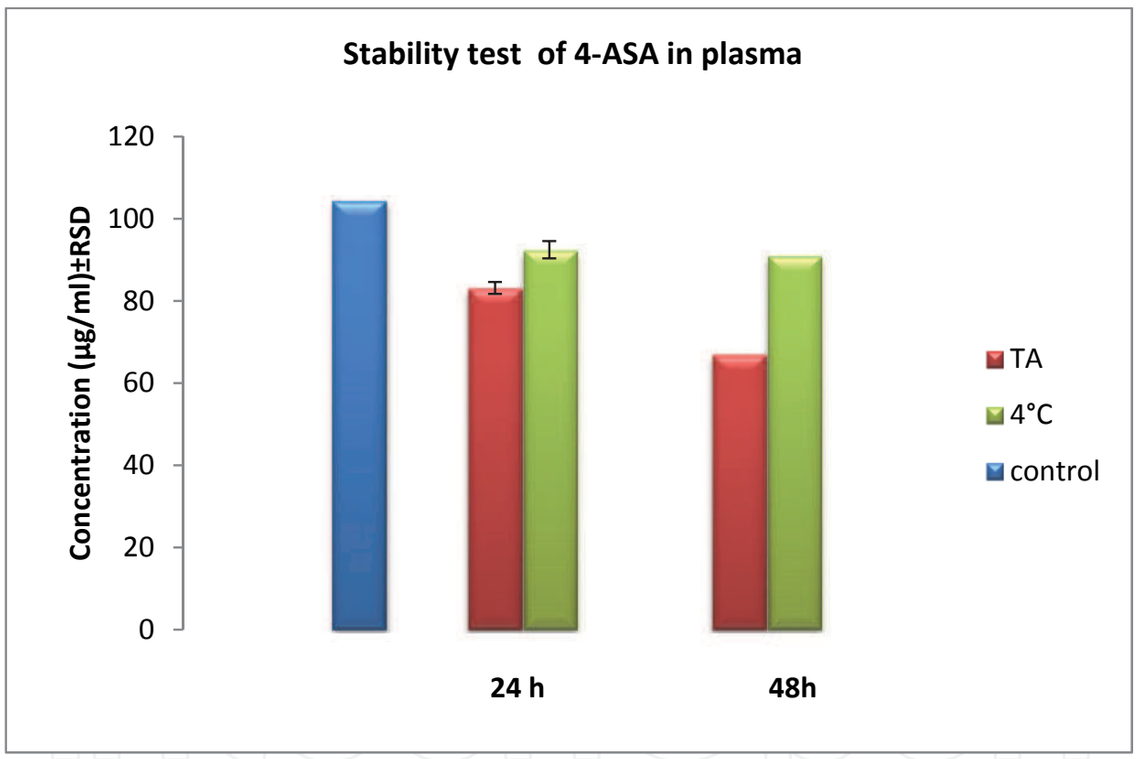

Fig. 7. Stability test of 4ASA in plasma after 48h at room temperature (RT) and at 4°C.
Bars represent standard deviations of the mean (n=5).

Since 4ASA was degraded in human plasma in 24 hours at room temperature and would be useful for immediate-release solid oral dosage forms. The profile of bioavailability test curve was characteristic of a dosage form with extended release. Because the plasma content of 4ASA was almost constant, it could mean that equilibrium of the balance absorption/ degradation was maintained (Figure **8**).
We notice that stability of the analyte during sample collection was investigated using the procedure described above. In connection with bioavailability, these results were unexpected because the concentration remains relatively constant.

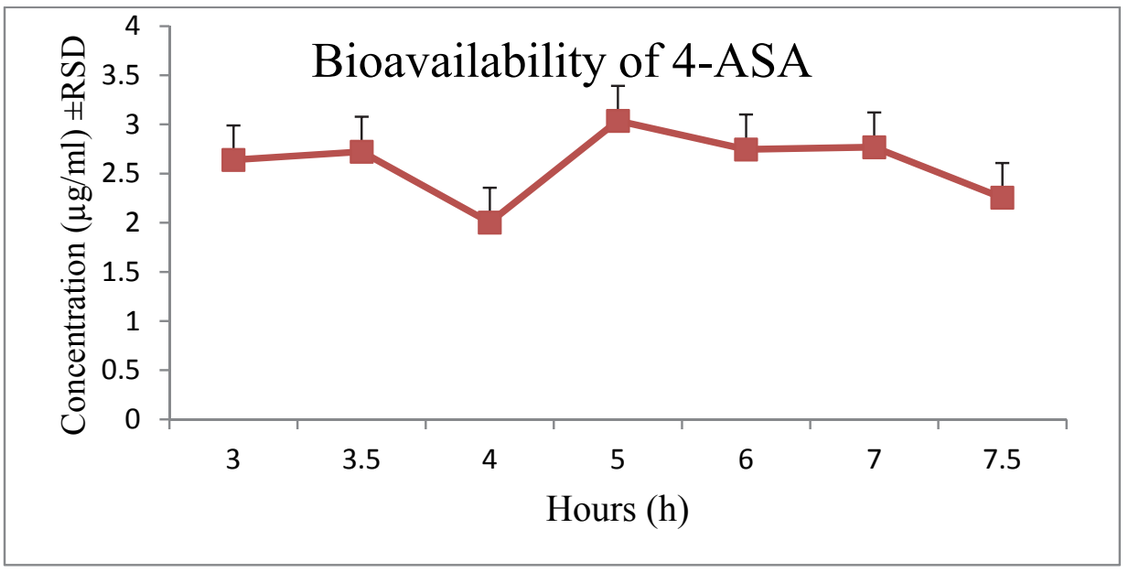

Fig. 8. Plasma concentration-time profile after i.v. administration of 25 mg/kg 4- ASA after 7.5h. Bars represent standard deviations of the mean (n=4).

5. Conclusion

In pharmaceutical analysis, often a method validation is required in order to meet the strict regulations, set by the regulatory authorities**.** This method was then developed and validated for the determination of 4ASA. Thus specificity, precision, accuracy, linearity, recovery and the limits were acceptable with the execution run time for control studies. It would be very useful for release profile study in simulated intestinal medium of the analyte in question. These results indicated that the RP-HPLC method was specific for the determination of 4ASA in plasma, under the chromatographic conditions employed.
Through this method the retention time was reduced considerably compared to previous results obtained by Gennaro *et al* [13]. With these present results it is possible to save time and solvents used to constitute the mobile phase. In addition, studies in plasma indicated that this method is suitable for pharmacokinetic studies.

6. Acknowledgements

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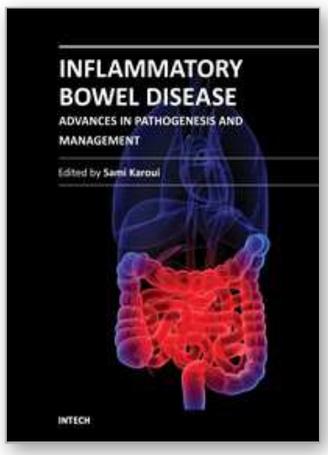

Inflammatory Bowel Disease - Advances in Pathogenesis and Management

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This book is dedicated to inflammatory bowel disease, and the authors discuss the advances in the pathogenesis of inflammatory bowel disease, as well as several new parameters involved in the etiopathogeny of Crohn's disease and ulcerative colitis, such as intestinal barrier dysfunction and the roles of TH 17 cells and IL 17 in the immune response in inflammatory bowel disease. The book also focuses on several relevant clinical points, such as pregnancy during inflammatory bowel disease and the health-related quality of life as an end point of the different treatments of the diseases. Finally, advances in management of patients with inflammatory bowel disease are discussed, especially in a complete review of the recent literature.

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