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# Modest Simultaneous Determination of Acetylsalicylic Acid and Salicylic Acid in Plasma by High Performance Liquid Chromatography UV Detection

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## Abstract

Objective: Acetylsalicylic acid (ASA) is one of the drugs used in antiplatelet therapy. ASA is rapidly hydrolyzed to salicylic acid (SA) and has low levels in plasma. Analysis of ASA and SA in plasma has been done using LC-MS/MS, UHPLC, and HPLC with hydrolysis and fluorescence detector. Compared to those methods, HPLC equipped with UV detector is less expensive. The method using HPLC with UV detector also has been done but not sensitive enough according to the LLOQ. Therefore a sensitive and selective analysis method needs to be developed. This study aims to develop an analytical method of ASA and SA in human plasma using HPLC with UV detector. Method: The method used in this study is Reversed Phase - High Performance Liquid Chromatography using $C_{18}$ column (Waters, Reliant™ 5 µm; 250 x 4.6 mm) with UV-Vis detector and was detected at wavelength of 230 nm. This method was developed using furosemide as internal standard (IS). Human plasma containing ASA and SA was extracted after protein precipitaion method using 15% perchloric acid with a liquid-liquid extraction method using ethyl acetate. Result: The method obtained was linear ($r \geq 0.99$) at concentration range of 0.05-1.5 µg/mL for ASA and concentration range of 0.2 – 5.0 µg/mL for SA. The validation result of ASA and SA analytical method fulfilled the validation requirement of EMEA Bioanalytical Guideline in the year 2011. Therefore the method provides modest, sensitive, and selective measurements of ASA and SA concentrations.

**Keywords**: *Acetylsalicylic acid, Salicylic acid, Furosemide, HPLC, Validation, Human plasma.*

## Introduction

Acetylsalicylic acid (ASA) has an antiplatelet effect [1] by irreversible inhibition of cyclooxygenase (COX)-1, thus blocking the formation of thromboxane $A_2$, which is a potential vasoconstrictor and platelet aggregation trigger [2]. Recently, ASA is used in low dosages for long-term application to prevent heart attack, stroke, and blood clot process [3]. ASA has low levels in plasma and a short half-life of approximately 15 to 20 minutes due to easy hydrolysis to SA [4].

In pharmacokinetic studies, in addition to ASA as parent compound, SA is also analysed as its metabolite. Analysis of ASA and SA in human plasma has been carried out by some researchers employing LC-MS/MS, [4-8] UHPLC, [9] and HPLC with hydrolysis and fluorescence detector [10]. Although these techniques were quite sensitive with lowest LLOQ values of ASA and SA up to 1 and 80 ng/ml, respectively [4].However, these methods need an IS that is difficult to obtain and utilize complex instruments. The present method using HPLC with UV detector also has been described [11-14]. HPLC equipped with UV detector is less expensive compared to LC-MS/MS, UHPLC, and HPLC with a fluorescence detector. The obtained LLOQ value derived from one of the methods using HPLC with UV detector was 0.1 µg/mL [11].

According to EMEA 2011; the LLOQ should not be higher than 5% of the $C_{max}$ [15]. The previous LLOQ [11-14] was not sensitive enough when referred to ASA's $C_{max}$ value that is around 1.01 µg/mL.12 therefore, the sensitive and selective method to analyse ASA and SA in the plasma using HPLC with UV detector is needed. This study aimed to attain an improved and validated method for analysing ASA and SA simultaneously by

employing HPLC with UV detector and using furosemide as IS. Furosemide was used as IS because it is easy to be acquired.

## Materials and Methods

Acetylsalicylic acid standard was purchased from Novacyl-France (Saint Fons, France), salicylic acid standard was purchased from BPOM (Jakarta, Indonesia), and furosemide was purchased from Ipca Laboratories Ltd. (Mumbai, India). HPLC grade acetonitril, potassium dihydrogen phosphate, perchloric acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). Aquabidest was purchase from PT. Ikapharmindo Putramas (Jakarta, Indonesia). Human plasma was purchased from Indonesian Red Cross (Jakarta, Indonesia).

### Intrumentation

The HPLC system used was LC-20AD series (Shimadzu, Tokyo, Japan), equipped with pump DGU-20A$_5$, UV-Vis detector SPD-20A, column oven CTO-10 AS vp, autosampler SIL-20A (Shimadzu, Tokyo, Japan) and Reliant™ C18 (250 x 4.5 mm; 5 µm) column (Waters, Massachusetts, United States). The mobile phase used was acetonitrile-20 mM phosphate buffer pH 2.5 (35:65 %v/v) with a flow rate of 1 mL/min. The injection volume was 20 µL and the UV detector was operated at 230 nm.

### Preparation of Standard Stock Solution, Calibration Standard, and Quality Control Samples

Stock solution of ASA and SA was prepared at 1.0 mg/mL in acetonitrile. From these stock solutions, working standard solutions of ASA and SA were prepared in acetonitrile. The stock solution of furosemide as IS was prepared at 1.0 mg/mL in methanol. A working concentration of the internal standard (10 µg/mL) solution was prepared in methanol.

All solutions were stored at 4°C and brought to room temperature before use. Calibration standard samples of ASA (0.05, 0.08, 0.1, 0.3, 0.6, 1.0, 1.2, 1.5 µg/mL) and SA (0.2, 0.6, 1.2, 1.8, 2.6, 3.4, 4.2, 5.0 µg/mL) were obtained by spiking an appropriate volume of the variously diluted stock solutions to human plasma. Quality control (QC) samples of ASA were prepared at concentration of 0.15 µg/mL, 0.725 µg/mL, and 1.125 µg/mL for low, medium, and high quality control (QCL, QCM, and QCH), respectively. The QC samples of SA were prepared at concentration of 0.60 µg/mL, 2.40 µg/mL, and 3.75 µg/mL for low, medium, and high quality control (QCL, QCM, and QCH), respectively. All aliquot plasma were stored at freezer -20°C and brought to room temperature before use.

### Sample Preparation

A 250 µL plasma was added of 20 µL IS solution (10 µg/mL) and 25 µL 15% perchloric acid. After vortexing, a 500 µL of ethyl acetate was addded as extraction solvent. The mixture was vortex-mixed for 3 min and centrifuged at 12000 rpm for 3 min. The supernatant phase was transferred into evaporation tube and evaporated to dryness under nitrogen at 50°C. The residue was reconstituted with mobile phase and vortex-mixed for 15 s. A 10 µL aliquot was injected into the HPLC system for analysis.

### Method Validation

The method validation assessment includes selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision, recovery, carry over, dilution integrity, and stability of the analytes in biological matrix in accordance with the international guideline for bioanalytical method validation [15-16].

### Selectivity

Selectivity test was carried out by exercising plasma taken from six different individual sources. The measurements were done by observing interferences on each plasma, where the acceptable limit is less than 20% of analyte's LLOQ and 5% of the internal standard [15].

### Linearity

Calibration curve was constructed in the range of 0.05 µg/mL to 1.5 µg/mL for ASA and 0.2 µg/mL to 5.0 µg/mL for SA. This curve consisted of blank plasma (plasma without analyte and internal standard), zero (plasma without analyte but with internal standard), and seven concentration points. Linearity was obtained by applying the linear regression equation and regression coefficient calculation. Moreover % diff calculation with conditions was not exceeding ± 15% for all concentration levels except LLOQ, which was not over than 20% [15].

### Lower Limit of Quantification

LLOQ was obtained by analysing ASA and SA in low concentration. The analyte response should be quantified reliably, with an acceptable accuracy and precision (less than 20% for each criterion) [15].

### Accuracy and Precision

Intra-and Interday accuracy and precision test were performed using five replicas of four concentrations (LLOQ, QCL, QCM, and QCH) within three consecutive days. Acceptance requirements were %diff and %KV is not more than 15% in QC samples and not more than 20% in LLOQ [15].

### Recovery

Recovery was calculated at three QC levels (QCL, QCM, and QCH) by comparing the spiked plasma sample area with spiked post-extraction samples. The value of %KV obtained is not more than 15% [16].

### Carry Over

Carry over was performed by blank injection after injecting high concentration samples or ULOQ. The acceptability requirements of this parameter were that the response of the analyte is less than 20% of LLOQ and 5% for internal standard [15].

### Dilution Integrity

Dilution integrity was carried out by diluting the standard stock solution with plasma to obtain a concentration above ULOQ, which is 2x the concentration of QCH. Afterward it was diluted with plasma to reach a concentration of ½ and ¼ times from initial concentration. Accuracy and precision were observed with acceptability requirements not exceeding 15% [15].

### Stability

Stability test of the stock solutions of ASA, SA and IS was carried out at room temperature for 0, 6 and 24 hours for short-term stability and at storage temperature (-4°C) for 0, 7, 14, and 25 days for long-term stability. Tests were performed twice for each stock solution with the %diff value not exceeding ± 2%. Sample stability test was done by analyzing QCL and QCH in short-term storage (0, 3, and 6 hours at room temperature) and long-term storage (0, 7, 14, and 25 days at -80°C).

In addition, the stability of the sample was also done by analyzing QCL and QCH after three freeze-thaw cycles and the stability of autosampler (0 and 24 hours of autosampler storage). Tests were carried out in triplicate in each concentration with %diff and %KV values not exceeding ± 15% [15].

## Results

### Method Development

### Optimization of Chromatographic Conditions

This study began with optimization of chromatographic conditions consisting of optimization of mobile phase combination, mobile phase composition, pH of the mobile phase, flow rate, column temperature and internal standard. Based on the obtained chromatogram, a combination of acetonitrile-20 mM phosphate buffer pH 2.5 (35:65) produced a better separation therefore this mobile phase was selected for further analysis.

The flow rate of 1.0 mL/min was selected as optimum one because it had better resolution and the temperature of 35 °C was selected as the optimum because it produced faster retention time and it could prolong the column age with lower pressure. The internal standard variation used in this study besides benzoic acid was paracetamol and furosemide because these two compounds have physicochemical properties similar to analytes. Based on the chromatogram, the retention time of paracetamol does not differ much from benzoic acid, whereas at that retention time there was considerable interference in the plasma. Therefore, furosemide was chosen as the internal standard for this study.

### System Suitability Test

After acquiring optimum analysis conditions, then a system suitability test was carried out before conducting an analysis to ensure that the conditions used were in accordance with the optimum conditions. The system suitability test was carried out in five replications and the coefficient of variation in the peak area ratio and the retention time of the analyte and internal standard were observed. The coefficient of variation requirement (% KV) is <2%. The results obtained from each system suitability test was CV value <2% with the analysis time of each injection of 14 minute.

## Optimization of Sample Preparation

Optimization was conducted by testing the protein deposition method, liquid-liquid extraction, and the combination of those two mentioned methods. In the protein deposition extraction method, it used extraction solutions with variations of acetonitrile and methanol. The liquid-liquid extraction method applied extraction solution with variations of diethyl ether, ethyl acetate, and n-hexane.

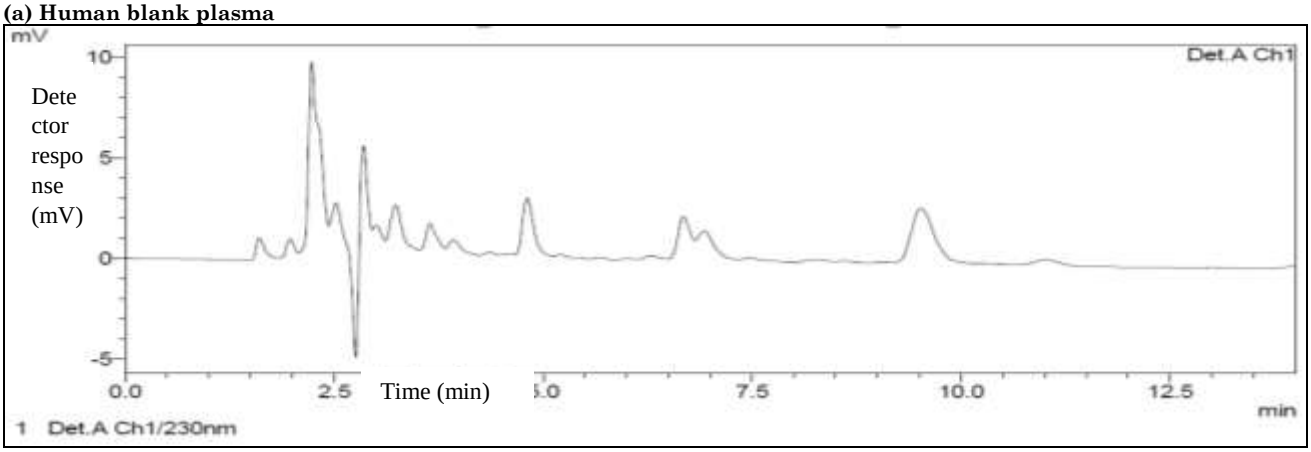
While, the protein deposition method with a combination of liquid-liquid extraction was carried out with two variations of protein precipitation, acetonitrile, and 15% perchloric acid, with diethyl ether and ethyl acetate as extraction solvents. Based on the obtained results, the protein deposition method with a combination of liquid-liquid extraction was chosen because it produced the largest area with the smallest interference.

## Method Validation

The validation result of ASA and SA analytical method fulfilled the validation requirement of EMEA Bioanalytical Guideline in the year 2011 and FDA in the year 2001 consisting of selectivity, LLOQ, linearity, accuracy and precision, recovery, dilution integrity, carry-over, and stability.

The chromatogram resulted from the analysis of blank plasma sample and spiked LLOQ of ASA, SA, and IS was shown in Figure 1. The calibration curve was linear and acceptable. The data acquired from the inter-day calibration curve for ASA and SA was shown in Table 1. LLOQ value was 0.05 µg/mL for ASA and 0.2 µg/mL for SA (Table 2). Accuracy and precision data for intra- and inter-day plasma samples for ASA and SA are shown in Table 3. Finally, the results of the stability test of ASA and SA in human plasma were shown in Table 4.

**(a) Human blank plasma**




**(b) Human plasma spiked with analyte at LLOQ**

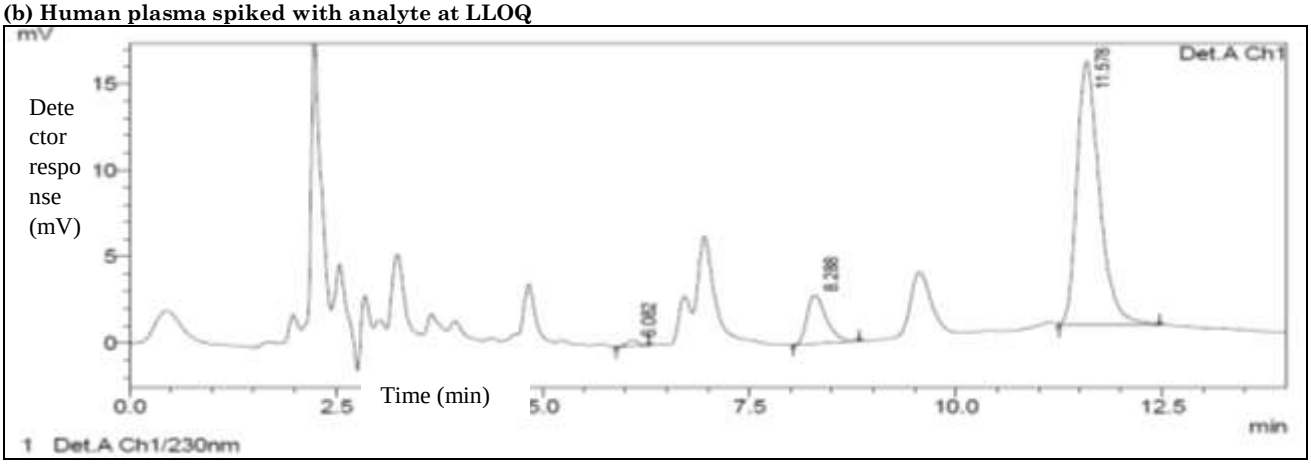



**Figure 1: Representative chromatograms of ASA, DA, and IS in (a) human blank plasma; (b) human plasma spiked with analyte at LLOQ**

**Table 1: System suitability test**

| Parameter | Mean±SD (CV, %) (n=5) | | |
|---|---|---|---|
| | ASA | SA | IS |
| tR (min) | 6.064±0.003 (0.06) | 8.116±0.008 (0.10) | 11.529±0.007 (0.06) |
| Area | 182612±779 (0.43) | 515831±2086 (0.40) | 453282±3710 (0.82) |
| Tf | 1.346 ± 0.002 (0.17) | 1.656 ± 0.005 (0.29) | 1.247 ± 0.009 (0.74) |
| N | 5135±6.405 (0.12) | 4850±8.679 (0.18) | 5539±13.507 (0.24) |

**Table 2: Inter-day calibration curve of ASA (up) and SA (down)**

| Replica inter-day | r | Slope (b) | Intercept (a) |
|---|---|---|---|
| 1 | 0.9971 | 0.3937 | 0.0158 |
| 2 | 0.9974 | 0.3506 | 0.0129 |
| 3 | 0.9980 | 0.3797 | 0.0059 |
| Mean | 0.9975 | 0.3747 | 0.0115 |
| **Replica inter-day** | **r** | **Slope (b)** | **Intercept (a)** |
| 1 | 0.9966 | 0.5938 | -0.0049 |
| 2 | 0.9983 | 0.6167 | -0.0697 |
| 3 | 0.9997 | 0.3797 | 0.0238 |
| Mean | 0.9982 | 0.5301 | -0.0169 |

**Table 3: The accuracy and precision from LLOQ of ASA (up) and SA (down)**

| LLOQ conc. (µg/mL) | Measured conc. (µg/mL) | Accuracy (%diff) | Precision (%CV) |
|---|---|---|---|
| 0.05 | 0.0425 | -15.09 | 2.45 |
| | 0.0417 | -16.60 | |
| | 0.0433 | -13.41 | |
| | 0.0410 | -17.99 | |
| | 0.0408 | -18.34 | |
| **LLOQ conc. (µg/mL)** | **Measured conc. (µg/mL)** | **Accuracy (%diff)** | **Precision (%CV)** |
| 0.20 | 0.1962 | -1.88 | 0.89 |
| | 0.1961 | -1.97 | |
| | 0.1985 | -0.77 | |
| | 0.1941 | -2.94 | |
| | 0.1944 | -2.81 | |

**Table 4: The intra- and inter-day accuracy and precision of ASA (up) and SA (down)**

| Nominal conc. (µg/mL) | Intra-day | | Inter-day | |
|---|---|---|---|---|
| | Mean accuracy (% diff) | Precision (%CV) | Mean accuracy (% diff) | Precision (%CV) |
| 0.05 | -17.00 to 3.46 | 9.59 | -18.78 to 6.85 | 9.11 |
| 0.15 | -2.76 to 10.54 | 4.76 | -5.39 to 10.54 | 5.17 |
| 0.725 | 1.59 to 13.47 | 4.68 | 0.27 to 13.57 | 3.81 |
| 1.125 | 12.51 to 14.69 | 0.75 | -0.11 to 14.72 | 3.70 |
| **Nominal conc. (µg/mL)** | **Intra-day** | | **Inter-day** | |
| | **Mean accuracy (% diff)** | **Precision (%CV)** | **Mean accuracy (% diff)** | **Precision (%CV)** |
| 0.20 | -14.61 to 9.56 | 9.39 | -14,61 to 18.09 | 9.44 |
| 0.60 | -14.65 to -7.31 | 3.19 | -14.65 to -7.31 | 2.60 |
| 2.40 | -14.29 to -11.88 | 1.40 | -14.29 to 1.93 | 5.46 |
| 3.75 | -7.59 to 5.70 | 5.31 | -14.31 to 8.82 | 8.68 |

**Table 5: The stability test results of ASA and SA in human plasma**

| Stability experiments | Stable to- | |
|---|---|---|
| | ASA | SA |
| Short-term storage (25°C) | 3 h | 3 h |
| Long-term storage (freezer -80°C) | 25 days | 25 days |
| Autosampler | 24 h | 24 h |
| Freeze-thaw | 3 cycles | 3 cycles |

## Discussion

### Optimization of Mobile Phase Combination

The mobile phase combination was tested using isocratic elution mode with a combination of acetonitrile - phosphate buffer 20 mM pH 2.5 (45:55) and 0.1% acetonitrile - formic acid (40:60). The internal standard used in this optimization of chromatographic conditions was benzoic acid. Based on the obtained chromatogram, a combination of acetonitrile - 20 mM phosphate buffer pH 2.5 produced a better separation therefore this mobile phase was selected for further analysis.

### Optimization of Mobile Phase Composition

The composition of mobile phase used was acetonitrile – phosphate buffer 20 mM (28:72), (35:65), and (45:55). Based on the obtained chromatogram, the higher the concentration of acetonitrile, the quicker the retention time will be yet its resolution was getting smaller. Hence, the mobile phase composition of acetonitrile – phosphate buffer 20 mM (35:65) was chosen since it resulted in a passable resolution and retention time was fast enough.

### Optimization of pH of the Mobile Phase

The pH variation used in this study was 2.5; 3.0; 3.5. This was considered because, at pH 2.5, all three analytes tended to be in the form of unionized molecules. At the pH of 3.0,

only two peaks appeared from all three analytes due to similar polarity of ASA and SA. Whereas, at pH 3.5, the peaks of ASA and SA overlapped within each other as they were eluted contiguously. This happened due to SA tends to be ionized and polar. Therefore, the condition at pH 2.5 was chosen as optimum pH for the mobile phase since it had good resolution.

### Optimization of Flow Rate

Variation of the flow rate used in this study was 0.8; 1.0; and 1.2 mL/min. The faster the flow rate is, the faster the retention time of the analyte becomes however the resulted area will be smaller and the column pressure increases. Thus, the flow rate of 1.0 mL/min was selected as optimum one since it had the sizable area with better resolution.

### Optimization of Column Temperature

The variation of column temperature used was 25. 30 and 35°C.Based on the obtained data, higher column temperature causes faster retention time and the column pressure is on the wane due to the decreasing of the mobile phase's viscosity used. The decreased viscosity could reduce the column pressure [15]. Consequently, the temperature of 35 °C was selected as the optimum because it produced faster retention time and it could prolong the column age with lower pressure.

### Selectivity

The chromatogram resulted from the analysis of blank plasma sample and spiked LLOQ of ASA, SA, and IS were not found interfering peaks from the endogenous component at the retention time of ASA, SA, and IS.

### Linearity

The calibration curve of seven concentration point in the range of 0.05 – 1.5 µg/mL and 0.2 -5.0 µg/mL for ASA and SA respectively was linear and acceptable. As of the obtained linear regression equation, it displayed the correlation coefficient (r) of > 0.99 for ASA and SA as well as the %diff value of the calculated concentrations was acceptable.

### Lower Limit of Quantification

The LLOQ was 0.05 µg/mL for ASA and 0.2 µg/mL for SA, with %CV and %diff of back calculated concentrations of LLOQ was acceptable.

### Accuracy and Precision

Accuracy and precision data for intra- and inter-day plasma samples for ASA and SA showed that accuracy and precision value were suitable with required criteria.

### Recovery

The mean extraction recoveries for ASA at three concentrations of QCL, QCM, and QCH were 87.76%, 108.19%, and 106.92%, with %CV values were 6.23%, 0.92%, and 7.92%, respectively. The mean extraction recoveries for SA at three concentrations of QCL, QCM, and QCH were 100.88%, 89.34%, and 87.55%, with %CV values were 5.18%, 2.01%, and 4.76%, respectively. While for the IS at concentration of 1000 µg/mL was 87,47% with %CV value was 3.31%.

### Carry Over

The carry-over test of ASA and SA met the required values where they obtained %carry-over in the range of 7.86% up to 11.66% for ASA and from 4.76% to 8.40% for SA.

### Dilution Integrity

The results of the dilution integrity test of ASA and SA was also suitable because it encountered the requirements of accuracy and precision with a value of the obtained %diff and %KV were not exceed ± 15% at each dilution concentration.

### Stability

Stock solutions of ASA and SA in acetonitrile and IS in methanol were stable at room temperature storage for 24 hours, while at -4 °C, ASA and SA were stable for 14 days, while IS was for 25 days. The results of the stability test indicated that ASA and SA were quite stable during storage conditions and sample preparation.

## Conclusion

The present method provides a modest, sensitive, and selective measurement of ASA and SA of realistic concentrations in human plasma. The LLOQ obtained was 0.05 µg/mL for ASA and 0.2 µg/mL for SA using protein precipitation in combination with liquid-liquid extraction for the sample preparation. The IS used was furosemide, which is cheaper and easy to be found. In conclusion, an alternative method for simultaneous

determination of ASA and SA in human plasma using HPLC with UV detection was succesfully developed and validated.

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