Scientific paper

Spectrophotometric Determination of 4-Acetamidophenyl N'-(Sulphanilamide) Acetate in Biological Fluids

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Abstract

A simple, accurate and low cost spectrophotometric method is proposed for the determination of the synthesized paracetamol derivative; 4-acetamidophenyl N'-(sulphanilamide) acetate (APSA) in biological fluids. The spectrophotometric method is based on a condensation reaction between the alcoholic solution of APSA and acidic solution of p-dimethylaminobenzaldeyde (DPMK) to generate a yellow colored product. The linear range for the determination of APSA was 1–10 μg mL^{-1} with molar absorptivity of 3.6877×10^4 L mol^{-1} cm^{-1} and Sandell's sensitivity of 0.001 μg cm^{-2}/0.001 absorbance unit. During the inter-day and intra-day analysis, the relative standard deviation for replicated determination of APSA was found to be less than 2.0% and accuracy was 99.20–101.60% and 99.10–101.30% in blood and urine samples, respectively. There was no interference with commonly used blood and urine sample. The developed spectrophotometric method was successfully applied to assess APSA in biological fluids.

Keywords: 4-acetamidophenyl N'-(sulphanilamide) acetate, p-Dimethylaminobenzaldehyde, spectrophotometry, biological fluids.

1. Introduction

Paracetamol is widely used as an analgesic[1] and antipyretic[2] drug. It is the member of non-steroidal anti-inflammatory drug (NSAIDs). Initial members of the class had their greatest potency as an analgesic[1] and antipyretic,[2] but newly developed members have exhibited increased potency as an analgesic and antipyretic agents[3–5] and soon agents will be available with additional activities as an anti-inflammatory,[3,6,7] antioxidant[8], immunomodulatory[9] and antimicrobial.[10] 4-acetamidophenyl N'-(sulphanilamide) acetate (APSA) is new paracetamol derivative.[5] APSA is a condensation product of paracetamol and sulfonamide moieties. APSA has been shown analgesic, antipyretic, anti-inflammatory and antibacterial properties.[5]

Several analytical techniques are available for the drug determination such as, liquid chromatography,[11] titrimetry,[12] capillary electrophoresis[13] and chemiluminescence.[14] Other, HPLC techniques,[15,16] spectrofluorimetry[17] and spectrophotometric[18,19] methods are existing for the drug determination in biological fluids. APSA is a new chemical derivative of paracetamol and no official method is available for the assay of APSA. Therefore, the establishment of sensitive and simple method for the drug determination is great importance in the area of pharmaceutical sciences.

The aim of this study to develop new simple, accurate and low cost spectrophotometric method for the determination of APSA in biological fluids. The proposed method is based on the condensation product of DMAB-APSA.

2. Experimental

2. 1. Materials

4-acetamidophenyl N'-(sulphanilamide) acetate was synthesized in our laboratory as described in the literature.[5] All chemicals used were of analytical grade. Double

distilled water was used throughout the experimental work. Blood and urine samples were obtained from Wistar albino rat at the Vidyabharti Trust College of Pharmacy, Umrakh, Bardoli, Gujarat, India.

2. 2. Instrument

UV-Visible, double beam spectrophotometer (Shimadzu-1700) equipped with a quartz cell of 1.0 cm path length was used for the absorbance measurements.

2. 3. Preparation of Stock Solutions

A stock solution of 2.0 M sulphuric acid was prepared by diluting 11.2 mL sulphuric acid up to 100 mL with double distilled water. 2.0% w/v DMAB stock solutions were prepared by dissolving 2.0 g DMAB in 100 mL of 0.1 to 2.0 M sulphuric acid solutions respectively. 1.0 mg mL^{-1} stock solution of APSA was prepared by dissolving 100.0 mg of APSA in 100 mL methanol. Blank urine sample was diluted with water in 1:100 ratio and was used during the experimental work. Standard working solutions of serum and diluted urine (100.0 μg mL^{-1}) were prepared by spiked with known volume of stock solution of APSA (100.0 μg mL^{-1}) and diluted upto 10.0 mL with methanol, respectively. Solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with suitable solvents.

2. 4. Spectrophotometric Determination of APSA

An aliquot of 0.1–1.0 mL of working solution (100.0 μg mL^{-1}) of APSA corresponding to concentration 1.0 to 10.0 μg mL^{-1} was taken in 10 mL volumetric flask, with 0.5 mL, 0.9% of DMAB solution prepared in 0.5 M sulphuric acid and diluted up to the mark with methanol. The contents of the flask were mixed well and the absorbance was measured at 446 nm against the blank reagent similarly prepared, except of APSA at reaction time 20 min and 30 °C temperature. The amount of APSA was obtained either from the calibration graph or the regression equation.

2. 5. Determination of Spiked APSA in Biological Fluids

Blank blood and dilute urine samples were spiked with convenient amounts of APSA stock solution (100.0 μg mL^{-1}). Spiked blood samples were treated with adequate volume of ethyl acetate (1:2, ratio of drug solution in methanol:ethyl acetate). The resulting mixture was shaken for 5 minute and centrifuged at 2000 rpm for 5 minute. The convenient volumes of the blood supernatants were collected in 10 mL volumetric flasks and then followed by assay method. The final concentrations of APSA in blood supernatants were 1.25, 2.50 and 3.75 μg mL^{-1} respectively. The spiked urine samples were withdrawn convenient aliquots in 10 mL volumetric flasks and then followed by the assay method. Subsequently, the concentrations of APSA in urine samples were 10, 30 and 50 μg mL^{-1} respectively. The calibration curves were prepared in spiked serum and urine samples in concentration range of 1–4 and 1–6 μg mL^{-1}, respectively. The precision and accuracy were determined during the inter-day and intra-day replicate analysis of spiked three concentrations of APSA in blood supernatants (1.25, 2.50 and 3.75 μg mL^{-1}) and urine (10, 30 and 50 μg mL^{-1}).

Stability studies of APSA in blood supernatants (1.25, 2.50 and 3.75 μg mL^{-1}) and urine (10, 30 and 50 μg mL^{-1}) were carried out including bench-top stability (at room temperature for 24 hrs) and long-term stability (–20 °C for 1 week). The results obtained for the intra-day samples (blood supernatants and urine samples) stored at different conditions were compared with those of freshly prepared intra-day samples.

3. Results and Discussion

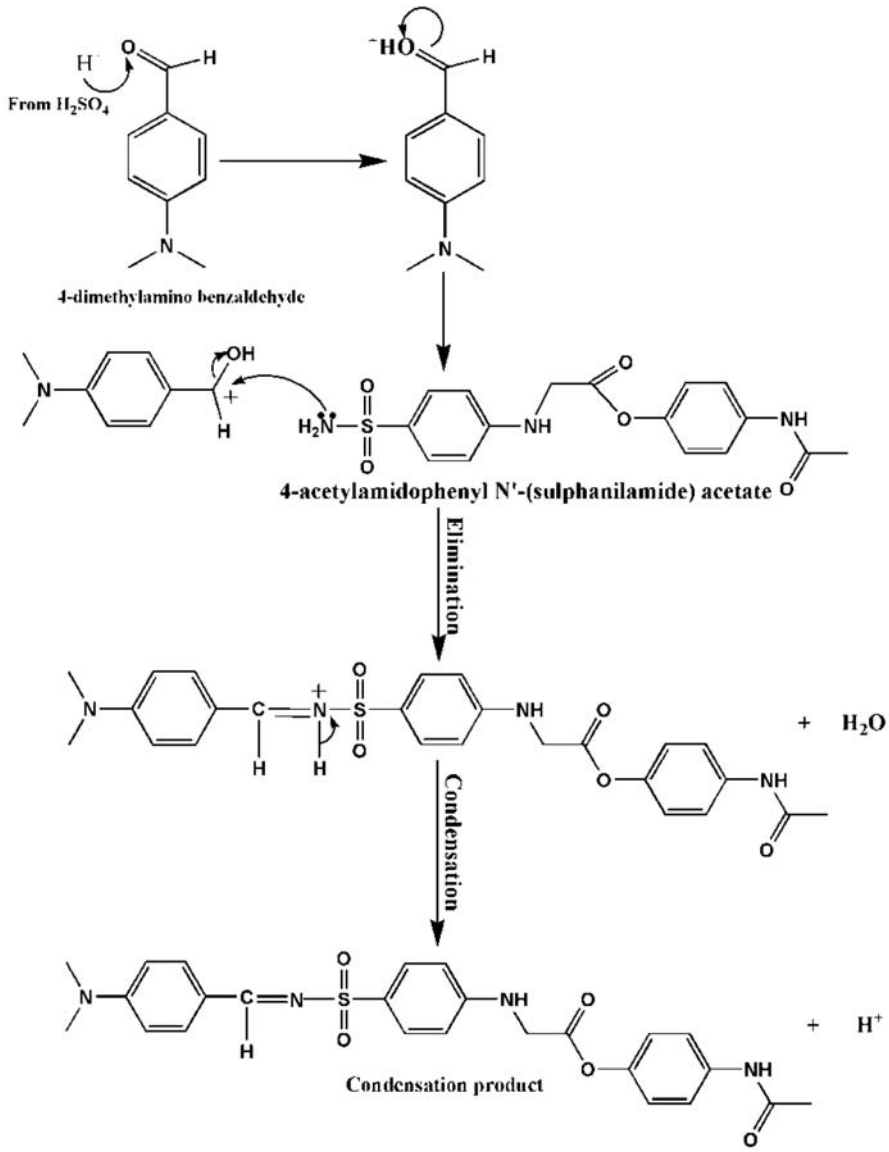
The simple, accurate and low cost spectrophotometric method for the determination of APSA in biological fluids (blood and urine) was developed using after modification in the earlier reported method.[20,21] DMAB was formed carbocation in acidic medium. The lone pair electrons of nitrogen in APSA was attacked on the carbocation of DMAB and simultaneously, water molecule was eliminated and yellow colored condensation product of DMAB-APSA was formed. The reaction between APSA and DMAB is presented in Scheme 1. The maximum wavelength was successfully determined at 446 nm.

3. 1. The Effect of Diluting Solvents

The effect of diluting solvents such as, distilled water, methanol, ethanol, propanol, butanol and acetonitrile were investigated on the absorbance of the condensation product of DMAB-APSA. In each case, after mixing of 1.0 mL of APSA (100.0 μg mL^{-1}) and 0.5 mL of DMAB solution, the resulting reaction mixture was made up to 10 mL with respective solvent. The maximum absorbance was registered on spectrophotometer against blank solvent. The

Table 1. Effect of diluting solvents.

Sr. No	Solvents	Absorbance
1.	Acetonitrile	0.029
2.	Ethanol	0.066
3.	Methanol	0.215
4.	Propanol	0.054
5.	Butanol	0.040
6.	Distilled water	0.117



Scheme 1. Formation of condensation product of DMAB-APSA.

result of the effect of diluting solvents revealed that methanol was the best solvent for the formation of yellow colored condensation product. It is presented in Table 1.

3. 2. The Effect of Reagent Concentration

The effect of DMAB concentrations on the absorbance were investigated in the concentration range of 0.1–2.0% w/v in 2.0 M sulphuric acid. The effect of sulphuric acid concentrations on the absorbance were examined in the concentration range of 0.1–2.0 M using 2.0% w/v DMAB. The effect of reagents concentration studies revealed that 0.5 M sulphuric acid and 0.9% DMAB concentration were suitable for the formation of condensation product of DMAB-APSA. Above and below these concentrations, the absorbance values were decreased. The effect of reagent concentrations is presented in Figure 1.

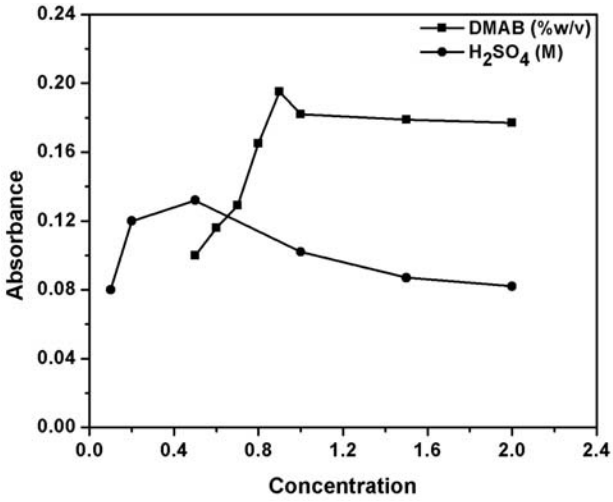


Figure 1. Effect of reagents concentrations on formation of DMAB-APSA condensation product.

3. 3. The Effect of Temperature

The effect of temperature on reaction was studied. The absorbance was measured at 10, 20, 30, 40, 50, 60, 70 and 80 °C temperatures under optimum conditions. Figure 2 showed that no significance difference was obtained at lower temperatures, while the condensation product was destroyed at higher temperature. The highest absorbance was determined at 30 °C. Therefore, 30 °C temperature was considered as an optimal temperature for the condensation reaction.

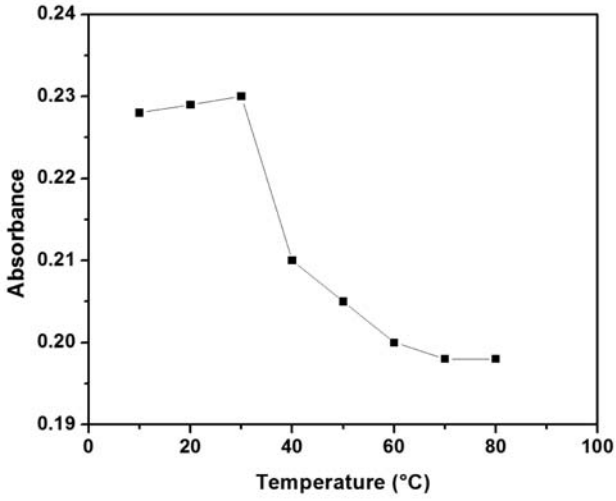


Figure 2. Optimization of reaction temperature of formation of condensation product.

3. 4. The Effect of Time

The effect of time on reaction was observed from 0 to 50 min under optimum conditions. From the figure 3, it can be seen that there was a marked increase in the color intensity within few seconds of addition of reagent and it reaches maximum in about 20 min, after it remains almost constant.

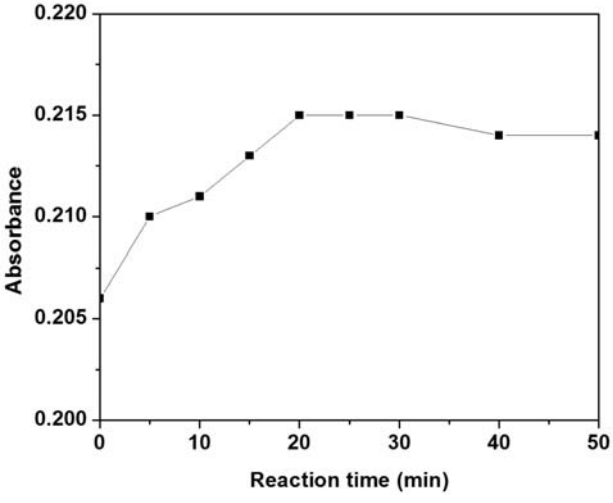


Figure 3. Optimization of reaction time for formation of condensation product.

3. 5. Stoichiometric Ratio Determination

The Job's method was used to check the stoichiometric reaction between APSA and DMAB.[22] Equimolar solutions (1 mg mL^{-1}) of the DMAB (in 0.9 M H_2SO_4) and APSA (in methanol) were used to determine the stoichiometry ratio. In seven different volumes 0, 0.25, 0.33, 0.50, 0.67, 0.75 and 1.0 mL of DMAB were taken 10 mL volumetric flasks and made up to 1.0 mL with APSA solutions. Subsequently, diluted up to 10.0 mL with methanol. The absorbance was noted at 446 nm on spectrophotometer under optimum conditions against blank reagents. The experimental result showed that APSA was combined with DMAB in 1:1 molar ratio. It is shown in Figure 4.

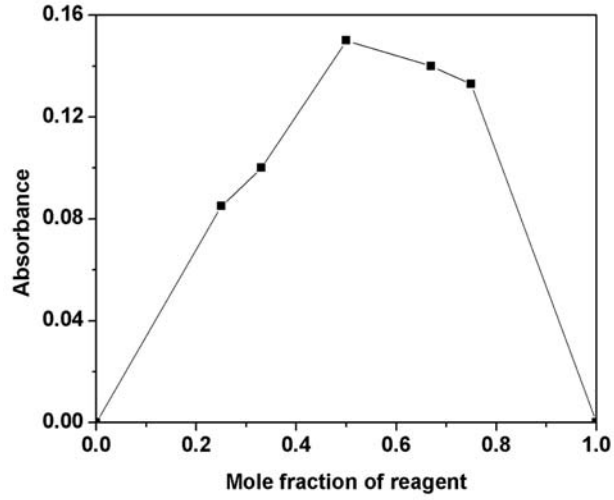


Figure 4. Stoichiometry of the condensation product of DMAB (1 mg mL^{-1}) and APSA (1 mg mL^{-1}) by Job's continous variation method.

3. 6. Spectral Characteristics and Analytical Parameters

Under the experimental conditions, the linear calibration curve was plotted in the range of 1–6 μg mL^{-1} with a significantly higher value of correlation coefficient (r) 0.9996; the representative regression equation was y = 0.1011x + 0.00167. The 95% confidence limit for the slope and intercept are 0.101 ± 0.00135 and 0.00167 ± 0.00528, respectively. The molar absorptivity was found to be 3.6877×10^4 L mol^{-1} cm^{-1} and Sandell's sensitivity of 0.001 μg cm^{-2}/0.001 absorbance unit. The validation results are presented in Table 2.

Table 2. Statistical data regarding APSA determination.

Parameters	Results
Amax	446 nm
Linear response (μg mL^{-1})	1–10
Sandell's sensitivity	0.001 μg cm^{-2}/0.001 absorbance unit
Regression equation	Y = 0.1011 X + 0.00167
Slope	0.1011
Intercept	0.00167
Correlation coefficient	0.9996
Molar absorptivity (L mol^{-1} cm^{-1})	3.6877×10^4
LOD (μg mL^{-1})	0.185
LOQ (μg mL^{-1})	0.561
95% confidence limit	Y = 0.101 X ± 0.00135 + 0.00167 ± 0.00528

3. 6. 1. The Limit of Detection (LOD) and the Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were computed from the calibration equation and using the formula,[23]

$$\text{LOD} = 3.3 \times \frac{S}{b} \qquad \text{LOQ} = 10 \times \frac{S}{b} \tag{1}$$

where, S is the standard deviation of calibration curve and b is the slope of the calibration curve. Using this formula, the LOD and LOQ of the method was found to be 0.185 and 0.561 μg mL^{-1} respectively.

3. 6. 2. Precision and Accuracy

The precision and accuracy of the method were determined as documented by the USP.[24] The precision and accuracy data are summarized in Table 3. The precision (repeatability) of the proposed method was calculated from a series of three solutions of 1.25, 2.50, 3.75 μg mL^{-1} of APSA on the same day analyses. The relative standard deviation (RSD) for three analyses was 0.86, 0.35 and 0.68% and accuracy were 98.16%, 99.92% and 100.27% respectively. The day to day precision were obtained by the repeated analyses of 1.25, 2.50 and 3.75 μg mL^{-1} APSA (three analyses) over one week. The results showed that the inter-day RSD were 0.47, 1.92 and 0.71% and accuracy were 99.52%, 98.00% and 100.08% respectively.

3. 6. 3. Determination of APSA in Biological Samples

The developed spectrophotometry method was successfully used for the determination of APSA in blood and urine samples. Spiked blood samples were deproteininzed by the mixture of methanol and ethyl acetate in 1:2 ratio, then after APSA was determined in the blood samples by the assay method. Urine sample was initially diluted with double distilled water (1:100), then after, no further treatment step was required. The LOQ and LOD were determined to be 0.24 and 0.08 μg mL^{-1} for the blood sample with acceptable linearity ranging from 1–4 μg mL^{-1}, respectively (R = 0.9998). The LOQ and LOD were examined to be 0.56 and 0.18 μg mL^{-1} for the urine sample with acceptable linearity ranging from 1–6 μg mL^{-1}, respectively (R = 0.9996).

The precision of the method based on intra-day repeatability was determined by replicating the analysis of three sets of spiked blood samples with 1.25, 2.50 and 3.75 μg mL^{-1} concentration of APSA and spiked urine samples with 10, 30 and 50 μg mL^{-1} concentration of APSA. The reproducibility (inter-day variation) of the method was obtained using the same concentration range of blood and urine as described above, but only a single determination of each concentration was made on six different days. The obtained results are shown in Table 4–5. An inter-day and intra-day analysis of APSA showed that the relative standard deviations (RSD) were found to be less than 0.6% of spiked blood samples and less than 0.9% of spiked urine samples, respectively. The accuracy of the method was determined by using the same concentrations of blood and urine samples. The recovery studies of APSA in blood and urine sample during inter-day and intra-day analysis were obtained 99.20–101.60% and 99.10–101.30%, respectively.

Table 3. Precision and accuracy of the method for determination of APSA in standard solutions.

	Drug concentration (μg mL^{-1})	Absorbance	Mean found concentration (μg mL^{-1})	Accuracy %	Precision %
	1.25	0.126	1.23	98.16 ± 0.84	0.86
Intra-day	2.50	0.254	2.50	99.92 ± 0.35	0.35
(n=3)	3.75	0.381	3.76	100.27 ± 0.68	0.68
	1.25	0.127	1.24	99.52 ± 0.47	0.47
Inter-day	2.50	0.249	2.45	98.00 ± 1.88	1.92
(n=3)	3.75	0.381	3.75	100.08 ± 0.71	0.71

Table 4. Precision and accuracy of the method for determination of APSA in blood samples.

	Spiked drug concentration in blood sample (μg mL^{-1})	Absorbance	Mean found concentration (μg mL^{-1})	Accuracy %	Precision %
	1.25	0.146	1.27	101.60 ± 0.34	0.33
Intra-day	2.50	0.261	2.48	99.20 ± 0.55	0.55
(n=3)	3.75	0.385	3.78	100.80 ± 0.48	0.48
	1.25	0.143	1.24	99.20 ± 0.27	0.27
Inter-day	2.50	0.265	2.52	100.80 ± 0.68	0.67
(n=3)	3.75	0.381	3.73	99.47 ± 0.71	0.71

Table 5. Precision and accuracy of the method for determination of APSA in urine samples.

	Spiked drug concentration in urine sample (μg mL^{-1})	Absorbance	Mean found concentration (μg mL^{-1})	Accuracy %	Precision %
	10.0	1.002	10.03	100.30 ± 0.14	0.14
Intra-day	15.0	1.485	14.87	99.10 ± 0.35	0.35
(n=3)	20.0	2.019	20.22	101.10 ± 0.18	0.18
	10.0	1.012	10.13	101.30 ± 0.87	0.86
Inter-day	15.0	1.150	15.12	100.80 ± 0.48	0.48
(n=3)	20.0	1.996	19.98	99.90 ± 0.31	0.31

Table 6. Stability of APSA in blood supernatants and urine (n=3).

	Spiked drug concentration (μg mL^{-1})	Accuracy %	Bench-top stability	Long-term Stability
	1.25	101.60 ± 0.34	99.83 ± 1.57	100.15 ± 2.00
blood	2.50	99.20 ± 0.55	100.41 ± 2.74	101.42 ± 1.17
(n=3)	3.75	100.80 ± 0.48	102.40 ± 1.48	98.91 ± 0.82
	10.0	100.30 ± 0.14	99.70 ± 1.05	98.50 ± 1.65
Urine	15.0	99.10 ± 0.35	98.94 ± 0.59	102.36 ± 0.47
(n=3)	20.0	101.10 ± 0.18	100.47 ± 1.21	99.53 ± 1.23

The stability of APSA in the rat blood and urine samples was assessed by typical storage and handling conditions (Table 6). After bench-top, the changes in the ratios of APSA to those of the fresh intra-day samples were negligible (99.83–102.40% for blood supernatants, 98.94–100.47 for urine). In the case of long-term stability, the recovery ratios were comparable to those of the fresh intra-day sample (98.91–101.42% for blood supernatants, 98.50–102.36% for urine).

3. 7. Effect of Interfering Coexisting Components

Under the same experimental condition of APSA, the effect of interfering species was evaluated by addition of various concentrations of interfering species to a fixed amount of APSA (10 μg mL^{-1}). The tolerance of the various interferents (e.g. Ca^{+2}, Na^{+}, K^{+}, Mg^{+2}, Zn^{+2}, Fe^{+2}, L-alanine, Glycine, Tyrosine, glucose, uric acid, paracetamol and L-ascorbic acid) are shown in Tabel 7. The results of Table 7 revealed that the most interfering species in

Table 7. Effect of common interferents on the determination of 10 μg mL^{-1} APSA.

Coexisting substance	Ratio of Coexisting substance to APSA	% relative error
Ca^{+2} (Cl^{-})	1:75	3.7
Na^{+} (Cl^{-})	1:150	3.2
K^{+} (Cl^{-})	1:75	1.0
Mg^{+2} (Cl^{-})	1:0.4	4.2
Zn^{+2} (Cl^{-})	1:0.4	–2.1
Fe^{+2} (Cl^{-})	1:0.4	–1.4
L-Alanine	1:75	3.7
Glycine	1:25	2.4
Tyrosine	1:25	1.1
Uric acid	1:0.025	–1.7
Paracetamol	1:1	1.4
L-ascorbic acid	1:1	2.5

small amounts were found to small effects on the determination of APSA under permission of ±10% relative error. In practice, interference free determination of APSA is possible, when the dilution of urine samples up to 1000-fold, deproteinization of blood samples and by applying the standard addition method. Hence, the selectivity was achieved by the proposed method is good and it is possible to determine the APSA.

4. Conclusion

The present work is concerned with the determination of APSA in biological fluids. The proposed spectrophotometric is considered to be simple, convenient and cost effective and it is also accurate and sensitive for determination of APSA in biological fluids. The proposed method can be used as stability-indicating method and can determine APSA in biological samples without interference from coexisting components. Moreover, the adopted method is inexpensive and do not required sophisticated techniques or instruments. It can be considered useful and promising for developing routine quality control analysis of APSA in biological samples. The proposed method is valid for application in laboratories lacking of liquid chromatography instruments (HPLC, GC).

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Povzetek

Za določanje sintetiziranega derivata paracetamola, 4-acetamidofenil *N*'-(sulfanilamid) acetata (APSA), v bioloških tekočinah predlagamo preprosto, točno in poceni spektrofotometrično metodo. Spektrofotometrična metoda je osnovana na reakciji kondenzacije med APSA v alkoholni raztopini in *p*-dimetilaminobenzaldehidom (DMAB) v kisli raztopini, pri kateri nastane rumeno obarvan produkt. Linearno območje za določevanje APSA je bilo 1–10 μg mL^{-1} z molarno absorptivnostjo 3,6877 × 10^4 L mol^{-1} cm^{-1} in Sandellova občutljivost 0,001 μg cm^{-2}/ 0,001 enote absorbance. Pri določitvi »inter-day« in »intra-day« ponovljivosti je bil relativni standardni odklon za APSA pod 2,0%; točnost je bila 99,20–101,60 % za vzorce krvi in 99,10–101,30 % za vzorce urina. Običajno prisotne spojine v vzorcih krvi in urina niso interferirale. Razvito spektrofotometrično metodo smo uspešno uporabili za določitev APSA v bioloških tekočinah.