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# Simple and Rapid Determination of Diuretics by Luminescent Method

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## ABSTRACT

Diuretics are drugs widely used in treatment of heart failure and hypertension and as doping agents in sports. Wrong prescription and excessive abuse can lead to negative side effects. Despite the effectiveness of methods usually used for the determination of diuretics (gas or liquid chromatography, capillary electrophoresis), they do not always provide necessary sensitivity. Moreover, sample preparation increases time of analysis. A rapid and sensitive luminescent method for determination of 5 diuretics (amiloride, bendroflumethiazide, bumetanide, furosemide, triamterene) in aqueous solutions and amiloride and triamterene in human urine is described. Intrinsic luminescent properties of protolytic forms of diuretics were studied in order to provide highly sensitive analysis. Investigation of interfering influence of diuretics was carried out to provide selective determination of triamterene, bumetanide and furosemide in aqueous mixtures of diuretics. Influence of urine at luminescent properties of diuretics was studied. The possibility of determination of triamterene and amiloride in human urine as individual substances and in mixture was proved. Simple and rapid technique for their determination in human urine was elaborated. The techniques elaborated for determination of triamterene in presence of other diuretics and furosemide in presence of commensurate amount of bumetanide allow enhancing specifity of analysis. Sufficient selectivity and sensitivity were reached in determination of amiloride and triamterene in human urine. The reduction of time of analysis due to avoiding sample preparation merits the techniques proposed.

**Keywords:** Furosemide; Bumetanide; Amiloride; Triamterene; Thiazides; Separation; Luminescence; Human Urine

## 1. Introduction

Diuretics are drugs that increase the amount of urine produced and enhance the excretion of electrolytes and water with urine as a result of disruption of ion transport in the kidney [1]. They are widely used in clinical practice in the treatment of hypertension and in different kinds of edema as well as for the correction of acid-base balance, in the treatment of intoxication and some injuries. Despite their effectiveness, diuretics may cause serious problems in case of wrong administration and excessive abuse [2]. Thus, it is of primary importance to control the intake of diuretics in order to avoid negative side effects.

Diuretics are prohibited for samples taken out of and in competition according to World Anti-Doping Agency (WADA) [3]. In sports, athletes misuse diuretics for several reasons: to reduce body weight in order to qualify for a lower weight, to reduce the urinary concentration of other prohibited substances to avoid a positive doping result and to overcome fluid retention caused by the use of anabolic steroids. Diuretics need to be detected at or a lower level than the minimum required performance limit (MRPL) −250 ng·mL$^{-1}$ [4]. The factors mentioned demand to carry out rapid, multicomponent, accurate and selective analysis for the determination of pharmaceuticals in urine.

The widely used methods for the determination of diuretics in urine are liquid and gas chromatography with mass-spectroscopic detection. These methods provide necessary selectivity and expressivity [5-13]. The main disadvantage of the methods is insufficient selectivity towards the urine components. Necessary selectivity is achieved by preliminary liquid-liquid or solid phase extraction. However, it leads to an increase in analysis time.

Other highly sensitive method of analysis of biological

molecules is luminescent method. Isopotential fluorimetry and fluorimetry in combination with partialleast squares multivariative calibration for simultaneous determination of amiloride and triamterene in human urine are described in [14,15]. Although these methods are characterized by high specifity and rapidity, high dilution of urine and low recoveries of diuretics lead to low sensitivity. Besides, the interfering influence of other diuretics was not studied.

Sensitive method of determination of furosemide based on its luminescent properties was proposed by Ioannou *et al.* [16], but the selectivity of method was not reached. Determination of amiloride and furosemide in urine involves separation of the substances on nylon membrane [17]. The method of the luminescent determination of triamterene and its metabolite in urine demands preliminary separation on octadecyl (C18) discs that consist of glass fibber embedded with C18 bonded silica, providing a hydrophobic surface for retaining nonpolar compounds, and the method of determination of triamterene in pharmaceuticals—separation on MP1 (a mixed phase of nonpolar and strong cation which involves both reversed phase and cation exchange) [18]. Literature data concerning luminescent determination of other diuretics have not been found yet.

The luminescent properties of some representatives of different classes of diuretics (loop, thiazide and potassium sparing) in aqueous solutions and in human urine with the aim to develop method of determination of furosemide, bumetanide, chlorthiazide, hydrochlorothiazide, bendroflumethiazide, triamterene and amiloride were studied in present work.

## 2. Materials and Methods

### 2.1. Instruments

Luminescence spectrometer LS55 (Perkin Elmer, USA) equipped with a xenon impulse lamp source and 1.0 cm cell, spectrophotometer UV-VIS Unico UV-2800, pH-meter with glass electrode, centrifuge OPN-3Y4.2 (Russia), evaporator in dry nitrogen Liebisch (Germany), analytical balance KERN ABS (Germany), pipettes of adjustable volume Eppendorf (Germany) 2 - 20 μL, 20 - 200 μL, 100 - 1000 μL.

### 2.2. Chemicals and Reagents

Stock standard solutions were prepared for 7 diuretic drugs (amiloride, bendroflumethiazide, bumetanide, chlorthiazide, furosemide, hydrochlorothiazide, triamterene) (Merck, Germany) by dissolving 1.0 mg of the diuretic in 10 mL of methanol (Sigma, USA, qualification "HPLC grade") to obtain concentration of 0.1 $mg \cdot mL^{-1}$. The methanol stock solutions of diuretics were stored in a tightly closed container in a cool and dry place. Working solutions were obtained by taking an appropriate volume of standard solution, evaporating the methanol in a nitrogen stream and diluting it with water. Standard aqueous solution was freshly prepared daily.

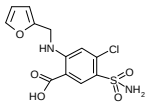
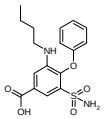
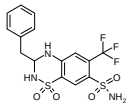
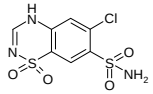
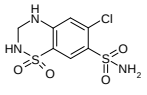
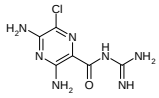
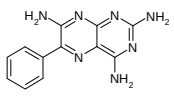
The pH was regulated by adding of fixed volume of acid or alkali. Hydrochloric acid and sodium hydroxide (both Merck, Germany) were prepared by dilution of the initial concentrated solution.

Urine samples were collected from 5 volunteers who did not consume banned substances. Urine samples were stored in polypropylene bottles at a temperature of −20˚C. Before conducting the experiment samples were defreezed and centrifuged for 10 min at 3000 rpm. Urine samples were diluted 10 times and the pH value was measured. Spiked urine samples were prepared by adding an aliquot of diuretic and appropriate volume of hydrochloric acid to negative urine samples after sample preparation to reach pH 2.0 or 4.0.

## 3. Results and Discussion

The diuretics selected for investigation can be classified as strong acids (bumetanide and furosemide), weak acids (thiazide derivatives) and basic compounds (triamterene and amiloride) (**Table 1**). Considering this fact and that

**Table 1. The characteristics of selected diuretics [1].**

| Name | Structure | $pK_a$ | |
|---|---|---|---|
| | | $pK_{a1}$ | $pK_{a2}$ |
| Furosemide |  | 3.9 | - |
| Bumetanide |  | 1.4 | 3.7 |
| Bendroflumethiazide |  | 8.5 | - |
| Chlorthiazide |  | 6.7 | 9.5 |
| Hydrochlorthiazide |  | 7.9 | 9.2 |
| Amiloride |  | 8.7 | - |
| Triamterene |  | 6.2 | - |

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pH of urine of healthy person is 5.5 - 7.0, it was necessary to study luminescent properties of their different protolytic forms.

It is seen that at pH of urine of healthy person furosemide and bumetanide exist as anions in solutions: furosemide as monoanionic and bumetanide as dianionic. The spectra of excitation and emission of molecular and anionic forms of furosemide and bumetanide are shown in **Figures 1** and **2**. It is seen that luminescent properties of protolytic forms of both diuretics differ appreciably. The intrinsic luminescence of furosemide at pH > 5 significantly decreases due to the destruction of rigidity of molecule which can be explained by breach of intermolecular hydrogen bonds with carboxylic groups of other furosemide molecules [17].

In contrast to furosemide the highest intensity of luminescence is peculiar to dianionic form of bumetanide, which can be explained by simultaneous deprotonation of the benzoic acid and of the anilinium ion with consequent formation of stable structure [19]. Dianionic form of bumetanide exhibits intensive excitation band at 325 nm with emission at 420 nm. So, at pH of urine bumetanide is characterized with high luminescence intensity while furosemide demonstrates low intensity that is the evidence of possibility to determine amount of furosemide.

In acidic medium (pH 2) furosemide demonstrates strong intrinsic luminescence with maxima $\lambda_{ex}/\lambda_{em}$ 270/410 nm in contrast to bumetanide. These properties are served as the basis for the determination of furosemide in presence of commensurate amount of bumetanide after acidifying urine sample till pH 2.

Bendroflumethiazide and hydrochlorothiazide at pH of urine exist in molecular form, while in case of chlorothiazide at pH 5.5 - 7.0 molecular and monoanionic forms coexist in solution. The spectra of excitation and emission of bendroflumethiazide, hydrochlorothiazide and chlorothiazide are shown in **Figures 3** and **4**. The highest luminescence intensity of bendroflumethiazide is peculiar to its molecular form ($\lambda_{ex}/\lambda_{em}$ 270/400 nm) which exists at pH 5.5 - 7.0. Anionic form is characterized by negligible emission. Luminescent properties of protolytic forms of hydrochlorothiazide and chlorothiazide do not differ much. Maxima of excitation and emission for protolytic forms of both diuretics coincide which can be explained by their identical molecular structures. Luminescent intensity of chlorothiazide is 5 times more than of hydrochlorothiazide. However, considerable overlapping of excitation and emission spectra makes their simultaneous determination impossible. At the same time, determination of bendroflumethiazide in presence of commensurate amount of hydrochlorothiazide is possible due to low luminescent intensity of the latter.

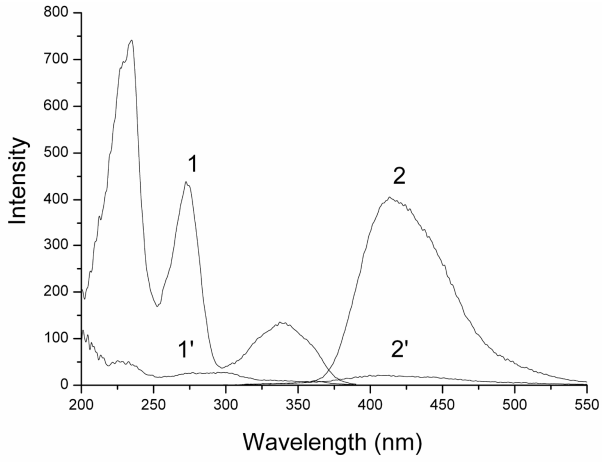


**Figure 1. Excitation and emission spectra of molecular (1, 2) and monoanionic (1', 2') forms of furosemide. pH = 2.0 (1, 2), pH = 6.0 (1', 2'), $\lambda_{ex}$ = 270 nm, $\lambda_{em}$ = 410 nm.**

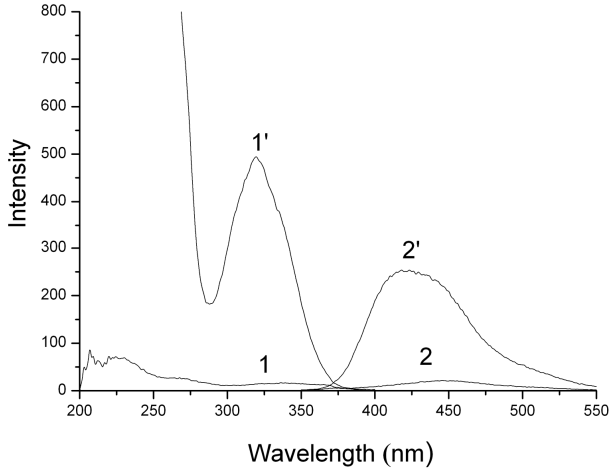


**Figure 2. Excitation and emission spectra of monoanionic (1, 2) and dianionic (1', 2') forms of bumetanide. pH = 2.0 (1, 2), pH = 6.0 (1', 2'), $\lambda_{ex}$ = 325 nm, $\lambda_{em}$ = 420 nm.**

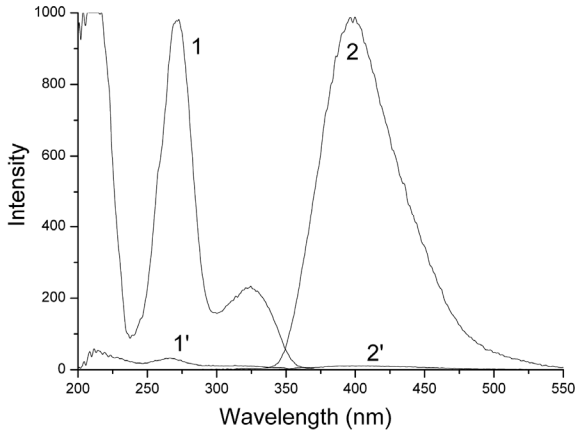


**Figure 3. Excitation and emission spectra of molecular (1, 2) and anionic (1', 2') forms of bendroflumethiazide. pH = 6.0 (1, 2), pH = 10.0 (1', 2'), $\lambda_{ex}$ = 270 nm, $\lambda_{em}$ = 400 nm.**

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At pH of urine amiloride is present in solution in protonated form, and triamterene in protonated and molecular forms. Molecular forms of amiloride and triamterene are characterized with intensive luminescence (**Figures 5** and **6**). Protonated forms which predominate at pH of urine have less intensive luminescence. At pH 4.0 insignificant bathochromic shift for excitation ($\Delta\lambda_{ex}$ = 6 nm) and emission maxima ($\Delta\lambda_{em}$ = 4 nm) is observed. This fact can be explained by protonation of aminogroupes [18].

Determination of amiloride in mixture with triamterene is impossible due to high luminescent intensity of the latter. However, triamterene can be determined in presence of any amount of amiloride. Elaborated method of determination of triamterene as individual substance in aqueous solution without pre-concentration demonstrates

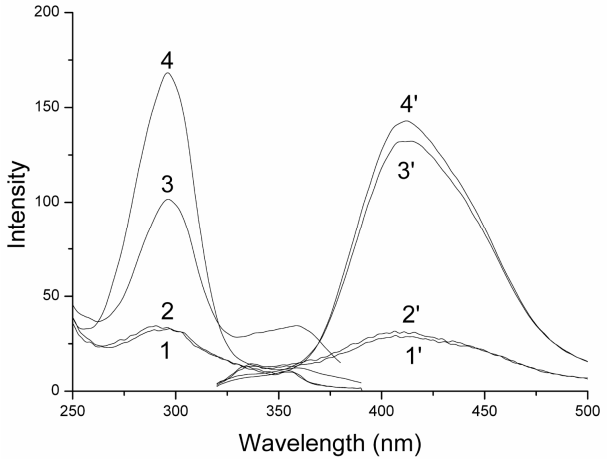


**Figure 4. Excitation and emission spectra of molecular (1, 1') and anionic (2, 2') forms of hydrochlorothiazide and of molecular (3, 3') and anionic (4, 4') forms of chlorothiazide. pH = 6.0 (1, 3), pH = 10.0 (2, 4), $\lambda_{ex}$ = 295 nm, $\lambda_{em}$ = 405 nm.**

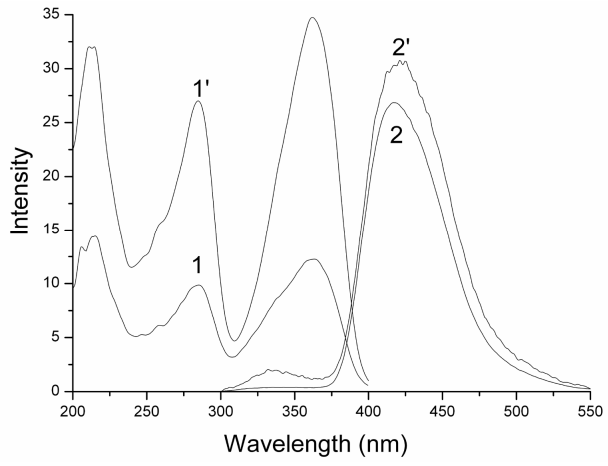


**Figure 5. Excitation and emission spectra of protonated (1, 2) and molecular (1', 2') forms of amiloride. pH = 6.0 (1, 2), pH = 10.0 (1', 2'), $\lambda_{ex}$ = 285 nm, $\lambda_{em}$ = 420 nm.**

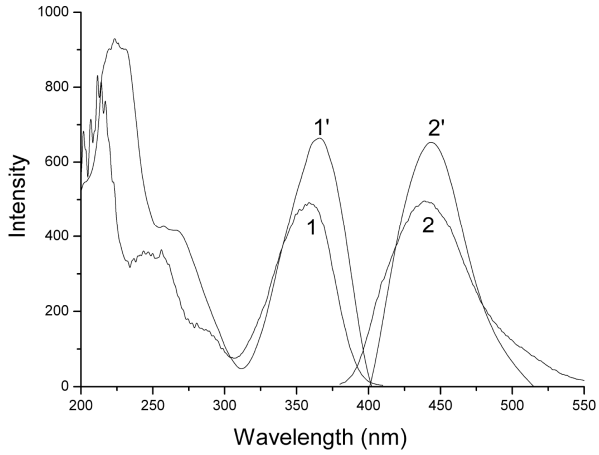


**Figure 6. Excitation and emission spectra of protonated (1, 2) and molecular (1', 2') forms of triamterene. pH = 6.0 (1, 2), pH = 10.0 (1', 2'), $\lambda_{ex}$ = 360 nm, $\lambda_{em}$ = 440 nm.**

the results competitive with that obtained in [18]. The same LOD and linearity range were achieved without solid-phase extraction which decreased time of analysis and simplified the method of determination.

The excitation and emission intensities as function of diuretic concentration under optimal conditions were studied. Limit of Detection was calculated as relation of threefold standard deviation of noise signal to coefficient of instrumental sensitivity. The results are shown in **Table 2**.

## 3.1. Interfering Influence

The highest sensitivity is inherent for the methods of determination of furosemide and triamterene as it can be seen in **Table 2**. At pH of urine all diuretics except furosemide show luminescent properties. Furosemide at pH > 5 does not interfere with determination of other diuretics but all diuretics except bumetanide interfere with its determination. Furosemide and bumetanide can be determined in one sample by adjusting pH. So, at pH 2.0 bumetanide molecule does not emit light which allows determination of furosemide. On the contrary, at pH 6.0 bumetanide is characterized with high luminescence intensity, and furosemide molecule does not emit light. The possibility of determination of one diuretic in presence of other in commensurate amount with sufficient sensitivity was proved. Results are shown in **Table 3**.

Due to overlapping of spectra of all diuretics and to high emission intensity triamterene at optimal conditions and at pH of urine impedes determination of studied diuretics, but diuretics in concentration lower or equal to concentration of triamterene do not impede its determination. Thus, for determination of other diuretics in presence of triamterene its preliminary separation is indispensable.

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**Table 2. Some analytical figures of merit of determination of diuretics by intrinsic luminescence in aqueous solutions. $R^2$ = 0.998 - 0.999.**

| Diuretic | pH | $\lambda_{em}^{max}$ ($\lambda_{ex}^{max}$), nm | $I = (a \pm \Delta a) + (b \pm \Delta b)$ C, μg·mL$^{-1}$ | | | LOD, μg·L$^{-1}$ | Linearity range, μg·L$^{-1}$ |
|---|---|---|---|---|---|---|---|
| | | | $I$ | $a \pm \Delta a$ | $b \pm \Delta b$ | | |
| Furosemide | 2.0 | 410 (270) | $I_{ex}$ | 9 ± 4 | 1988 ± 19 | 6.0 | 10 - 2000 |
| | | | $I_{em}$ | 14 ± 6 | 1980 ± 26 | 9.0 | |
| Bumetanide | 6.0 | 420 (325) | $I_{ex}$ | 3 ± 4 | 392 ± 4 | 30 | 50 - 5000 |
| | | | $I_{em}$ | 1 ± 2 | 395 ± 2 | 20 | |
| Bendroflumethiazide | 6.0 | 400 (270) | $I_{ex}$ | 16 ± 5 | 988 ± 13 | 20 | 10 - 5000 |
| | | | $I_{em}$ | 17 ± 6 | 992 ± 15 | 20 | |
| Amiloride | 6.0 | 420 (285) | $I_{ex}$ | 12 ± 7 | 489 ± 7 | 40 | 50 - 5000 |
| | | | $I_{em}$ | 16 ± 6 | 413 ± 6 | 40 | |
| Triamterene | 4.0 | 440 (360) | $I_{ex}$ | 1 ± 1 | 3805 ± 26 | 0.8 | 1 - 1000 |
| | | | $I_{em}$ | 2 ± 1 | 3802 ± 3 | 0.8 | |

**Table 3. Determination of furosemide (Fur) and bumetanide (Bum) in aqueous solutions.**

| pH | Added, μg·L$^{-1}$ | | Found, μg·L$^{-1}$ | | Recovery, % | | RSD, % | |
|---|---|---|---|---|---|---|---|---|
| | *Fur* | *Bum* | *Fur* | *Bum* | *Fur* | *Bum* | *Fur* | *Bum* |
| 2.0 | 0 | 200 | 0 | 2.8 | 0 | 1.4 | - | 8.8 |
| | 200 | 0 | 198.6 | 0 | 99.3 | 0 | 3.7 | - |
| | 200 | 200 | 203 | 0 | 101.5 | 0 | 6.9 | - |
| 6.0 | 0 | 200 | 0 | 197 | 0 | 98.5 | - | 4.0 |
| | 200 | 0 | 5.1 | 0 | 2.6 | 0 | 8.9 | - |
| | 200 | 200 | 0 | 205 | 0 | 102.5 | - | 3.8 |

## 3.2. Urine Analysis

Determination of diuretics in urine is complicated due to the influence of matrix which contains a variety of organic substances. Most of these substances exhibit high absorbance in the ultraviolet region [20] and have strong background luminescence that interferes with the direct determination of diuretics. As a result, the urine must be diluted and the fluorescence intensity should be measured at the maximum of the highest excitation wavelength where the urine exhibits low absorbance.

Dilution in 10 times and measuring of intensity signal versus blank urine was proved to be effective in order to avoid an interfering influence of matrix and distortion of the spectra. As it was established earlier the highest luminescence intensity was inherent to molecular forms of triamterene and amiloride (pH > 8.0 and 9.5, respectively), but alkalization of urine till pH 9.5 - 10.0 leads to appearance of turbidity. Thus, to avoid additional step in sample preparation including filtration determination of these diuretics was realized at pH 4.0 at which both diuretics exist in protonated forms.

As it can be seen from **Figure 7** triamterene in urine is characterized by high intensive excitation and emission with maxima at 370 and 440 nm, respectively.

Triamterene as individual substance can be determined in urine by either excitation or emission intensity with LOD obtained 5 and 6 μg·L$^{-1}$, respectively. Considering the dilution of urine in 10 times, LOD is higher than that in aqueous solutions but is still enough to detect triamterene with necessary sensitivity. The linearity range is

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20 - 500 μg·L$^{-1}$.

Amiloride does not exhibit luminescence in urine (**Figure 8**) which can be explained by binding with urine components such as derivatives of kynurenine, xanthurenic and folic acids [20]. As individual substance it can be determined by excitation spectra with LOD 30 μg·L$^{-1}$ and linearity range 200 - 2000 μg·L$^{-1}$. The presence of triamterene makes determination of amiloride by excitation spectra impossible since their maxima coincide. Overlapping leads to the increase of total signal intensity. However, determination of amiloride in presence of any amount of triamterene is still manageable by spectrophotometry, by intrinsic absorbance at 360 nm (**Figure 9**), in area where other diuretics in urine do not absorb. The linearity range is 50 - 1000 μg·L$^{-1}$ with LOD 10 μg·L$^{-1}$ obtained. Determination of triamterene in presence of any amount of amiloride is possible by its emission spectrum at wavelength 440 nm.

High recoveries of amiloride and triamterene from human urine and low relative standard deviations (**Table 4**) are the evidence of accuracy and reproducibility of the method given. Retaining of necessary specifity on reduction the analysis time compared with that proposed in [18] owing to avoiding solid-phase extraction is important achievement of our work.

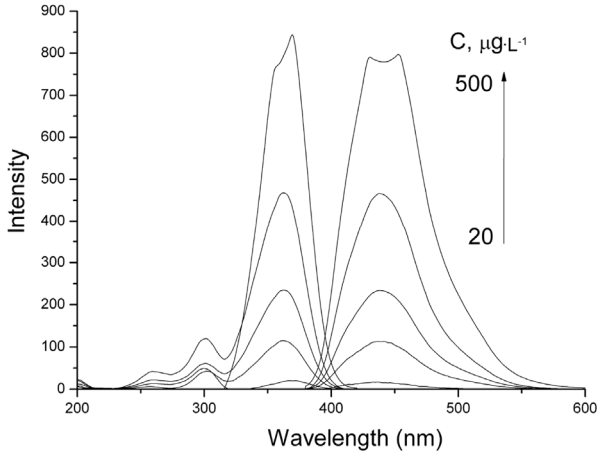


**Figure 7. Excitation and emission spectra of triamterene in urine. C = 20 - 500 μg·L$^{-1}$. pH = 4.0. $\lambda_{ex}$ = 360 nm, $\lambda_{em}$ = 440 nm.**

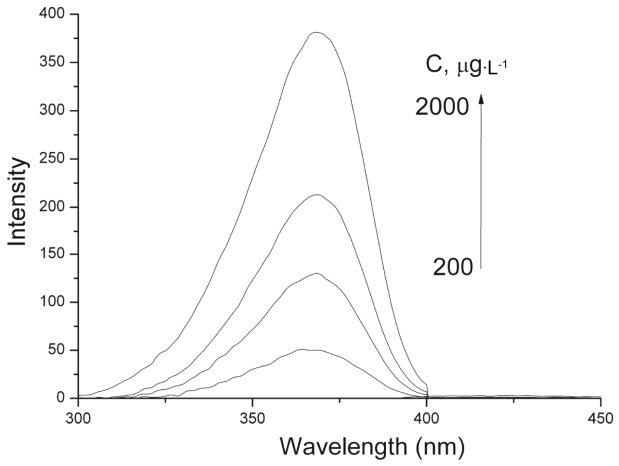


**Figure 8. Excitation spectrum of amiloride in urine C = 200 - 2000 μg·L$^{-1}$. pH = 4.0. $\lambda_{ex}$ = 285 nm, $\lambda_{em}$ = 420 nm.**

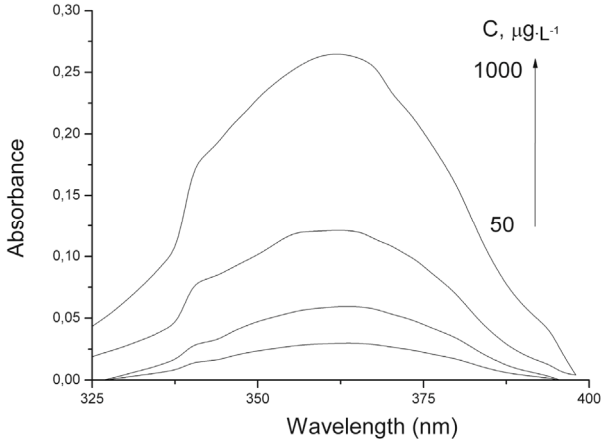


**Figure 9. Spectrum of absorbance of amiloride in urine. C = 50 - 1000 μg·L$^{-1}$. pH = 4.0. $\lambda$ = 360 nm.**

Bumetanide, furosemide and bendroflumethiazide were proved to exhibit high luminescence under optimal conditions in aqueous solutions. The influence of complex urine matrix leads to disappearance of luminescent properties of these diuretics at optimal conditions. Luminescence quenching probably can be explained by their binding with components of urine, supposedly with aminoacids [20]. So, determination of bumetanide, furosemide and bendroflumethiazide assume preliminary sample preparation of urine.

## 4. Conclusions

Intrinsic luminescent properties of different protolytic forms of furosemide, bumetanide, bendroflumethiazide, amiloride and triamterene are taken as a principle of sensitive determination of the diuretics in aqueous solutions. The simplicity and rapidity of method make it a useful tool in determination.

Techniques elaborated for determination of triamterene in presence of other diuretics and furosemide in presence of commensurate amount of bumetanide allow enhancing specifity of analysis. Other studied diuretics can not be determined in presence of triamterene which requires its preliminary separation.

Dilution of urine and measuring the signal versus blank solution allowed avoiding matrix influence in case of triamterene and amiloride analysis and elaborating simple, selective and sensitive method for their determination. Other diuretics suppose to be binded with components of urine resulting in luminescence quenching

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**Table 4. Analytical recoveries of amiloride and triamterene from human urine.**

| Amiloride | | | | | | | Triamterene | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Added, $\mu g \cdot L^{-1}$ | Found*a, $\mu g \cdot L^{-1}$ | Recovery, % | RSD, % | Found*b, $\mu g \cdot L^{-1}$ | Recovery, % | RSD, % | Added, $\mu g \cdot L^{-1}$ | Found*, $\mu g \cdot L^{-1}$ | Recovery, % | RSD, % |
| 225 | 227 | 100.9 | 8.7 | 228 | 101.3 | 5.7 | 22.5 | 22.7 | 100.9 | 6.2 |
| 400 | 397 | 99.2 | 6.2 | 402 | 100.5 | 6.4 | 90 | 88.6 | 98.4 | 5.9 |
| 1000 | 998 | 99.8 | 6.4 | 1000 | 100.0 | 6.5 | 250 | 252 | 100.8 | 5.8 |
| 1500 | 1508 | 100.5 | 5.3 | 1510 | 100.6 | 5.5 | 400 | 396 | 99.0 | 7.6 |

*Average of 3 measurements. [a]Quantity found by luminescent method. [b]Quantity found by spectrophometry.

which demands preliminary sample preparation of urine.

Triamterene in urine can be determined in presence of studied diuretics by emission intensity with high sensitivity. Simple method of amiloride determination in human urine was proposed. It does not include long sample preparation but provides high specifity of analysis with sufficient sensitivity. The reduction of time of analysis due to avoiding sample preparation merits the techniques proposed.

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