

Identification of Adenosine-3', 5'-Cyclic Monophosphate Purified From Leukaemic Human Urine

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Abstract

Human urinary Adenosine-3',5'-cyclic monophosphate (cAMP) was studied in 90 normal healthy volunteers (49 males and 41 females) aged between (11 months -55 years), and 86 leukemia patients (48 males and 38 females) of four types (25 ALL, 28 AML, 14 CLL, 19 CML) aged between (11 months - 65 years). The study includes the following:-

Extraction and purification of urinary cAMP from the interfering nucleotides, proteins, phosphates and pyrophosphates, by using Zinc sulphate –Barium hydroxide precipitation.

The extracted cAMP was purified by using Dowax 50W-H⁺ hydrogen form column chromatography (1x5 cm). Identification of the purified cAMP, this was achieved by applying the following techniques:

a- U.V analysis: - Scanning the samples and standard cAMP at (220-300 nm) in acidic, basic and neutral pH by using (Helios alpha type) spectrophotometer. Shifts in the wave length were found in acidic and basic mediums comparing with the neutral pH for all of standard, normal and leukemia cAMP.

b- TLC analysis: - Using Silica gel G60 (20 X 20 cm) TLC plates with solvent system (n-butanol: glacial acetic acid: water, (2:5:3 V/V). The R_f value for standard was 0.44 cm and for purified normal and leukemia cAMP were found to be closer to the standard value (0.43 and 0.45 cm respectively).

c- HPLC analysis: - Using HPLC 10AVP consist of two deliver pumps, with methanol: water 50:50 V/V as mobile phase and flow rate of 1ml/min, the separation was performed on reversed phase (250 X 4.6 mm column). The eluted chromatogram was monitored by U.V-VIS 10AV-SPD detector The retention time of urinary cAMP obtained from normal healthy and leukemic patients was 3.02 min which was close enough to the standard retention time.

The results of the study showed that the level of purified normal and leukemia cAMP are of the values 15.87 ± 2.4 μ mole/24 hours and 20.13 ± 0.881 μ mole/24 hours respectively.

Keywords : cAMP, Human urine.

Introduction

The second messenger concept of hormone action was proposed by Sutherland et al. [1] who described the hormone as the first messenger and cAMP as an intracellular second messenger.

The intercellular response to a large number of extra cellular signals was found to be due to the change in the cAMP level, as in the situation with adrenaline secreted from the adrenal glands in to the blood stream of mammals.

This occurs in response to a number of stimuli, such as fear, shock and stress, and leads to glycogen breakdown to glucose in the liver for energy supply.

The interaction between the hormone and its receptor, adenylate cyclase activity is affected in such a way as to cause increased or decreased production of intercellular cAMP [2].

It is the altered level of intracellular cAMP that then changes the metabolic behavior of the cell so that it exhibits the characteristic response to the hormone.

The increased level of cAMP leads to a variety of responses. This type of response is dependent on the nature of the target cell, for example, stimulation by catecholamine of liver [3], muscle [4] and adipose tissue [5], causes the activation of glycogen phosphorylase and lipolysis in these tissues. On the other hand, the increased concentration of cAMP by catecholamine stimulation in smooth muscle led to the muscle relaxation [6].

Changes in the intracellular concentration of cAMP indirectly bring about alteration in the activity of many different enzymes.

Increased cAMP in cells can diffuse to extracellular fluid. Hormones that increase hepatic (glycogen) or renal (parathyroid hormone) cAMP bring about an increased level of the cyclic nucleotide in blood and urine, respectively. Although this may be useful for diagnostic purposes, extracellular cAMP has little or no biologic activity in mammals [7].

Several studies have demonstrated that determination of cAMP in 24 hour urine specimens might be useful for the clinical investigation of endocrine disorders, especially of the parathyroid [8-13] and thyroid gland [14,15].

The evaluation of urinary cAMP provides an index of parathyroid activity, which is useful for clinical discrimination between patients with PTH and non-PTH-mediated hypercalcaemia [16,17].

An increased urinary cAMP has been found in hypercalcaemic patients that secreted a PTH-like peptide (ectopic PTH) [10,19]. It has been stated that, when patients with malignant lesions and hypercalcaemia have normal urinary cAMP output, the pathogenesis is not PTH-related. In these cases hypercalcaemia is probably due to prostaglandins or other osteolytic factors [20,21].

Plasma and urine cAMP levels are generally unchanged in normocalcemic cancer patients; however, increased or decreased cyclic nucleotide levels have also been reported [22-24].

On the other hand, increased cGMP levels and reduced cAMP / cGMP molar ratios in plasma and urine seem to be a more constant feature of cancer patients, with a relatively low frequency of false-negative values in the properly controlled studies [22,25].

Experimental

Specimen collection

Twenty four hours urine samples of the human leukemia patients and normal healthy control were collected. Eighty six samples of the leukemia patients were used throughout the project and they were admitted to the center cancer hospital of Sulaimania. These samples include 48 males and 38 females, aged between 0.11-65 years with all four types of leukemia (25 ALL, 28 AML, 14 CLL, 19 CML). These samples were under treatment for variety of periods.

Urine samples of 90 normal healthy volunteers, 49 males and 41 females, aged between 0.11-55 years in the Sulaimania city were used.

This selection was used in order to determine the cAMP concentration in different age groups in normal and leukemia patients and comparing the normal and leukemia patient of the same age.

Each person was provided with a clean reagent bottle containing chloroform to inhibit bacterial growth. Urine samples collected were brought to the laboratory, its volume was measured, then 10 ml of the sample was pipette from the bottle and then centrifuged at 3000 r.p.m for 10 minutes to remove any impurity or crystalline forms in the urine samples.

The cAMP concentration in the supernatant was assayed in the same day and the remain sample was stored in the refrigerator.

Isolation of cAMP

The procedure adopted consisted of the following steps:-

1-Elimination of impurities and crystalline forms:

This was carried out by taking 10 ml of the urine sample to test tube and then centrifuged at 3000 r.p.m for 10 minutes.

2- Precipitation of proteins, phosphates, pyrophosphates and all other nucleotides:

A protocol of Brooker [26] was adopted so that cAMP was isolated in the supernatant. The procedure was used by taking 3 ml of centrifuged urine sample to the test tube and then the addition of 0.4 ml of 5% ZnSO₄ (5gm of Zinc sulphate dissolved in 100ml distilled water) was followed, then 0.4 ml of 0.3N Ba(OH)₂ (4.725gm of dissolved in 100ml distilled water) was added. Mixed well and then centrifuged for 10 minutes at the 3000 r.p.m, this would precipitate protein, phosphates , pyrophosphates and all nucleotides except cAMP [26-28].

3- Column chromatography purification:

The technique was used to purify the extracted cAMP; a volume of 3 ml of the supernatant (containing cAMP) from step 2 was loaded on a Dowex 50W hydrogen form of the dimension (1 X 5 cm) and eluted with distilled water at flow rate of 3ml/min. The eluted fractions of 3 ml were collected [29] (fig-1).

4- After the collection of 15 fractions, it was found that fraction No.2 contains the highest activity of the purified cAMP as shown in the figure (1).

Same results were obtained in which fraction No.2 represents the activity of cAMP purified from normal and leukemia urine.

Identification

Ultraviolet spectrophotometry:-

cAMP has a strong characteristic U.V spectrum with a maximum λ at 258 nm at PH 7. Regular use of this property was made in identification and quantification.

To prepare standard solution, approximately 1 milligram of cAMP was dissolved in 25 ml distilled water, the PH was adjusted to 7, where 3 ml of cuvette has been used as a sample container and after the appropriate dilution, scanned the absorbance using (Helios alpha type) ultraviolet spectrophotometer [30].

Absorption at neutral pH: -

After the neutralization of the standard solution, 3ml of the standard cAMP solution was scanned by spectrophotometer between 220 – 300 nm. A maximum absorbance at 258 nm was obtained.

The same protocol was used for normal and leukemia urinary extracts of cAMP. In which fraction No.2 contained separated and purified cAMP and reflected a sharp peaks at $\lambda=258$ nm [31.] (fig-2).

Absorption at acidic and basic pH: -

Three ml of standard cAMP solution and same volumes of extracted cAMP from normal and leukemic urine were acidified at pH=2 by the addition of 1N HCl. These fractions were scanned using spectrophotometer between 220 – 300 nm. Maximum absorbances were obtained at the wave length of 256 nm.

Same protocol was used but the fractions were made basic at pH=12 using 1N NaOH and scanned by using spectrophotometer between the 220 – 300 nm. Maximum absorbances were obtained at wave length of 260 nm [31,32].

TLC Analysis:

TLC plate:-

A uniform thin layer Silica gel G60 was used as the stationary phase in the project to separate cAMP. Silica gel G60 was mounted at the thickness of 300 μ m on the glass plate and the area of (20 X 20 cm).

The Silica gel G60 plate was kept at 100 C for 60 minutes for reactivation before the application of samples.

Sample Preparation:-

The extracted cAMP (fraction 2) was evaporated to dryness under vacuum at 55C, after dryness cooled and then mixed with 20 micro liter distilled water [33].

Sample Application:-

A Micropipette was used for applying spotting 2 (cm) from the edge of the plate. Typical spot sizes were 5 μ L. Drying of the spots was achieved by using an air blower; more samples were then applied if necessary.

Plate Development:-

Development of the Plate was achieved in a glass tank in which the inside wall has been covered by filter-paper (Whatman No. 1) in order to ensure equilibration after saturation of the atmosphere within the tank by the solvent vapor. Developing solvent was added to a depth of about 1.5 cm of the tank. The tank top was covered to reach the equilibrium within 60 minutes and the plate was then placed vertically in the tank.

Spot Detection:-

Ultraviolet light absorbing spots were visualized by spraying with 0.01% ethanolic solution of 2, 7-dichlorofluoresceine; this was used for nucleotides, cyclic nucleotides which appeared as dark areas on a fluorescent background.

Several solvent systems were used, but n-butanol: glacial acetic acid: distilled water; 2:5:3 v/v was the major system used as previously suggested [34, 35]. The R_f values for the standard and sample are shown in table (1).

HPLC Analysis:-

HPLC 10AVP consist of two deliver pumps, 10 AVP controller, the injection was reodyne 7125 USA, the eluted chromatogram was monitored by UV-VIS 10 AV-SPD Detector. The separation was performed on a reversed phase (250 x 4.6 mm h.d). Column 5 μ m particle size, type DB-C-18. Mobile Phase: methanol: water 50/50 v/v. Flow rate: 1ml / min. Temperature: 25 C. Detection: UV set at 260 nm.

Firstly 5 μ L of mobile phase without standard and samples was injected to compare with another chart.

The standard was tested by taking 1ml of standard and added to 1ml of mobile phase. After mixing in vortex, 5 μ L was injected into HPLC and by the detector recording the chromatogram chart and measuring the retention time.

For the normal and leukemia patient samples repeating the same method as shown above for the standard, after recording the result measuring the retention time and for the identification of the standard and the samples comparing the retention time of them [33, 36].

Results and Discussion

Identification of cAMP Using U.V Analysis:-

It is known that cyclic adenosine 3', 5'- nucleotides contains hetero nitrogenous bases, and the basic principle of UV analysis for such type of compounds depends on the presence of such nitrogenous bases [37,38].

As cAMP and other derivatives contain an adenosine base which is a conjugated ring system, these compounds have a strange characteristic UV spectrum with a maximum wave length of 258 nm at PH 7.0 [38]. Regular use of this property was made in identification and quantification.

Fraction 2 was identified as cAMP. Absorption spectra in the UV region (230-320nm) are shown in fig (2). The sample which contains fraction 2 and the standard were found to have maximum wave length of 256,258 and 260 nm at pH 2, 7 and 11 respectively (fig-3).

These results were similar to those previously described [32] in which cAMP fractions were purified from different tissues.

The UV spectrum of the standard cAMP was compared with the spectra of extracted cAMP of normal and leukemic patient.

Figures (2and3) illustrate the close similarity between the standard and extracted cAMP samples and confirm that fraction 2 contains the highest activity of cAMP. This is in consistence with the findings of Krstulovic et al [36].

The close similarity between those spectra shown in fig (3-1) tends to confirm the identity and the purity of standard and urine extracted cAMP. No interferences were seen from other naturally occurring constituents in the gradients elution separation of the cAMP extract. The analysis does not require any pre-concentration and its sensitivity is of considerable importance in analyzing limited amounts of samples for the exceedingly low concentration of this compound.

The alternative isocratic elution mode optimized for the selective analysis of cAMP, offers a rapid and reliable method for the determination of this important compound in the biological samples. The method of analysis employed in this study lend themselves easily to several simple identification methods, which are necessary if the identity of the components of complex mixtures of biological compounds is to be determined valuably and unambiguously.

This method coupled with on appropriate sample preparation can be used for biological samples of different origin.

Identification of cAMP using TLC analysis:-

Thin layer chromatography analysis has been routinely used for the analysis and identification of cAMP and its derivative, in which the R_f values were for the comparison between standard and samples.

As shown from table (1) the R_f value for standard cAMP is 0.44 cm. The same results were obtained when applying TLC identification technique on the purified peaks of cAMP for both normal and leukemic patient (0.43 and 0.45 cm respectively). The chances that chromatographic peak is a compound other than cAMP seem remote. Bases or nucleosides would be eliminated by the other extractions. The Zinc-Barium precipitation, the Dowex-50W column and by thin layer chromatography were used for this purpose. Similar results were reported by Kuo et al [82] and Brooker [33].

Identification of cAMP using HPLC technique:-

High performance liquid chromatography is the most versatile and widely used type of elution chromatography, the technique is used by chemists to separate and determine a variety of organic, inorganic and biological materials [40].

Figure (4) shows the HPLC chromatogram of standard, extracted normal and leukemic patient urinary cAMP at the retention times of 3.02 min respectively. The values of retention time confirm the successful extraction of both normal and leukemic patient urinary cAMP as they are the same as that of the standard.

The retention times obtained in this study differed from that previously reported by Newton [32], Brooker [33] and Krstulovic [36], whose findings also show different retention times. These variations can be explained by differences in the type of HPLC model, length of column, types of mobile phase and its flow rates, and the type of packed column material.

Data obtained from the three techniques used for the analysis and identification of cAMP provide strong evidence for the similarity between the standard and extracted urinary cAMP, despite slight differences between them, which we believe may be due to differences in purity, sensitivity of the instruments and methods used.

The results of this study showed that the level of the purified normal and leukaemic cAMP are of the values 15.87 ± 2.4 $\mu\text{mole}/24$ hour and 20.13 ± 0.881 $\mu\text{mole}/24$ hour respectively.

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Table(1): TLC- analysis for standard CAMP and normal and Leukaemic Urinary CAMP.

Parameter	Rf Value
St. CAMP	0.44
UN. CAMP	0.43
UL. CAMP	0.45

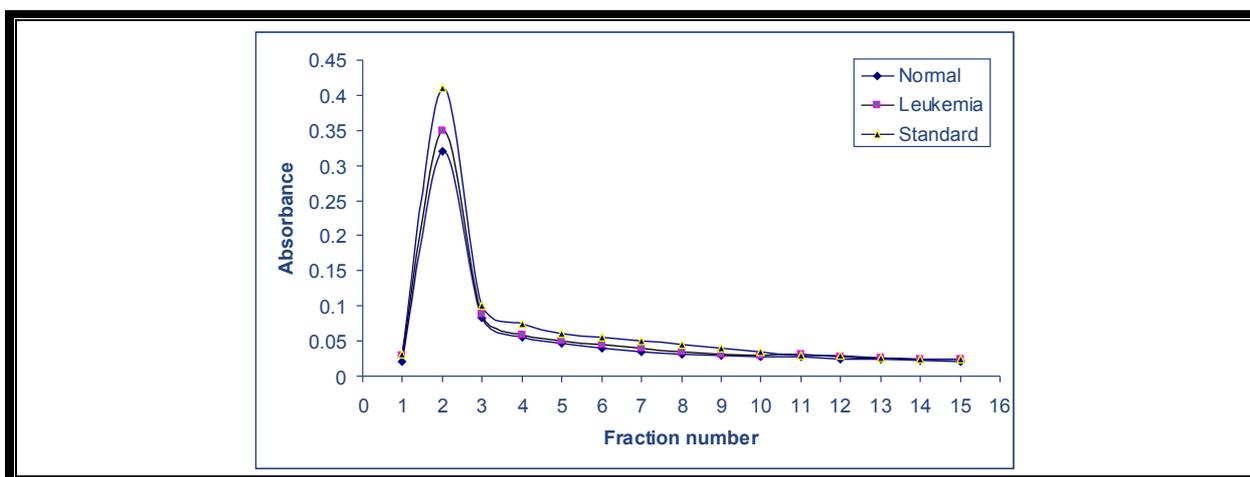


Fig .(1) : Elution profile of Standard cAMP and normal and leukaemic urinary cAMP using Dowex 50W column chromatography

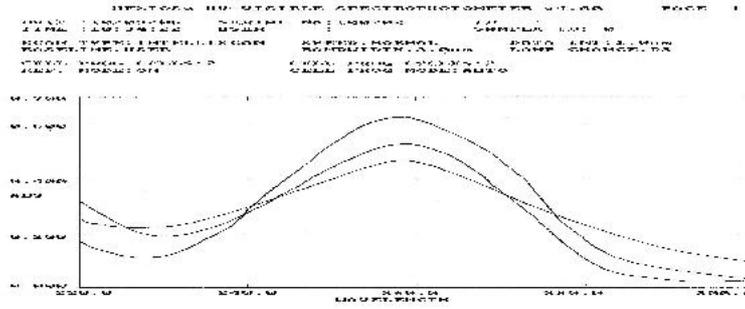


Fig. (2) : UV – analysis of the standard cAMP and the purified cAMP (fraction 2) from normal and leukaemic urine using neutral P^H.

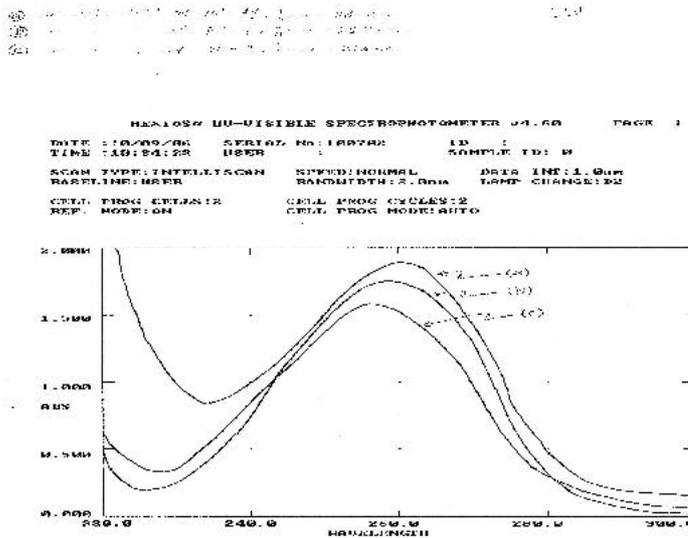


Fig. (3) : UV – analysis of the purified leukaemic cAMP (fraction 2) at basic (a), neutral (b), and acidic P^H (c).

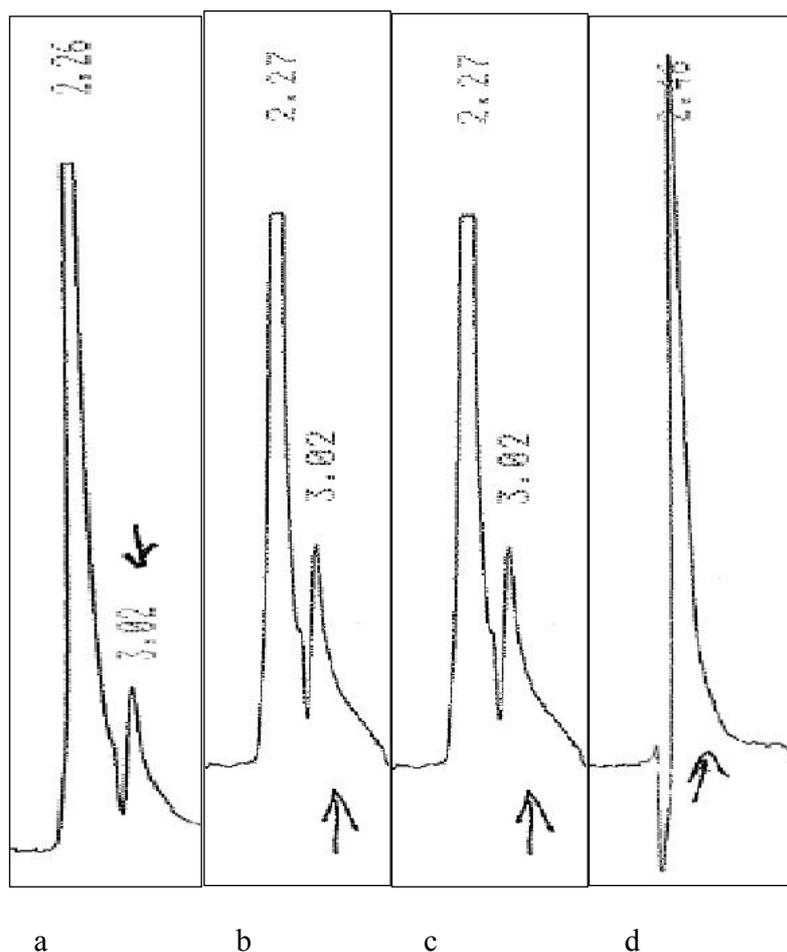


Fig . (4) : HPLC – analysis of standard cAMP and normal and leukaemic urinary cAMP. Analysis reflects same retention times of all (3.02).

- a. Standard cAMP.
- b. Purified cAMP from normal urine.
- c. Purified cAMP from leukaemic urine.
- d. Mobile phase (blank) MeOH / H₂O 50:50 v/v.

تشخيص الادينوسين الاحادي الفوسفات الحلقي المنقى من ادرار المصابين بمرض ابيضاض الدم

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الخلاصة

درست صفات الادينوسين الاحادي الفوسفات الحلقي (cAMP) المعزول من ادرار الاشخاص الطبيعيين (49 ذكرا و 41 أنثى) تتراوح اعمارهم ما بين 11 شهر والى 55 عام وقورنت القيم الناتجة مع مثيلاتها في ادرار الاشخاص المصابين بمرض ابيضاض الدم (48 ذكرا و 38 أنثى) ولنماذج متنوعة من المرض (25 حالة ALL، 28 حالة AML، 14 حالة CLL، 19 حالة CML) تراوحت اعمارهم ما بين 11 شهرا والى 65 عاما. تضمنت الدراسة الخطوات الاتية :

عزل و تنقية الادينوسين الاحادي الفوسفات الحلقي من متداخلاته من النيوكليوتيدات ، و البروتينات ، و الفوسفات ، و الفوسفات الثنائية باتباع أسلوب ترسيبها باستخدام مزيج الزنك - هيدروكسيد الباريوم ثم حمل الراشح المعزول على عمود تجزئة من نوع Dowex 50W-H⁺ ذي الابعاد 5 x1 سم. شخصت الاجزاء الناضحة والمحتوية على cAMP باتباع طرائق التحليل الاتية:

أ . تحليل طيف الاشعة فوق الحمراء : اجري مسح الاجزاء الناضحة لكل من الادرار المنقى الطبيعي والمرضي بطول موجي تراوح ما بين 200-300 nm وفي الاوساط المعتدل والحامضي والقاعدي وقورنت نتائج ذلك مع مثيلاتها من المادة القياسية. لوحظ ان هناك حيودا في الامتصاصية متشابهة لكل في الوسط الحامضي والقاعدي عما عليه في الوسط المعتدل.

ب . تحليل كروماتوغرافية الطبقة الرقيقة: استخدمت الواح السليكا G60 وسطا للفصل مع نظام مذيب مكون من مزيج البيوتانول وحمض الخليك الثلجي والماء بنسب 2 : 5 : 3 V/V على التوالي. حسبت قيم Rf لكل من القياسي و الطبيعي والمرضي ، اذ كانت قيمها متشابهة مع بعضها.

ج : تحليل HPLC : استخدم جهاز HPLC 10A VP المكون من مضختان بأعتقاد مزيج الميثانول والماء طورا فاصلا وبنسب 50:50 V/V وبمعدل جريان 1مل لكل دقيقة وبأبعاد عمود تجزئة مساوي الى 250X4.6mm توبعت الاجزاء الناضحة بوساطة جهاز المطياف من نوع UV-Visible 10AV – SPD detector حسب زمن الارتداد لمادة cAMP في الادرار الطبيعي والمرضي و قورنت مع مثيلاتها القياسي ، اذ كانت متساوية (3.02).

اثبتت الدراسة من ان مستويات مادة cAMP المنقاة من الانرار الطبيعي والمرضي هي 2.4 ± 15.87 و 20.13 $0.88 \pm$ مايكرومول\ 24 ساعة على التوالي.