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Rapid determination of indapamide in human urine using novel low-density solvent based ultrasound assisted emulsification microextraction coupled with high performance liquid chromatography-variable wavelength detection

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Indapamide belongs to the class of thiazide-type diuretic drugs and is widely used in the treatment of hypertension and nephrolithiasis. In this work, a simple, rapid and efficient low density solvent (LDS) based ultrasound assisted emulsification microextraction (USAEME) method combined with high performance liquid chromatography-variable wavelength detection (HPLC-VWD) was investigated for the determination of a popular drug of abuse, indapamide, in human urine samples. The target compound was extracted from acidified sample solution with a few microliter amount of LDS by a USAEME method. The influence of several important experimental variables such as selection of the extraction solvent and its volume, ultrasonication time, pH and ionic strength were thoroughly examined and optimized. Under optimal conditions, the calibration was linear in concentration range from 1–100 ng mL^{-1} with a correlation coefficient of 0.9977 for the target analyte. The limit of detection based on signal to noise ratio of 3 was 0.3 ng mL^{-1} and the relative standard deviations varied from 1.2–6.6%. The proposed method provides a rapid, sensitive, low cost, easy to handle, and convenient procedure to determine indapamide in human urine samples.

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1 Introduction

3-(Aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-1-yl)benzamide (indapamide) is a diuretic and antihypertensive drug and some of its pharmacodynamic activity is related to a thiazide-type effect.[1] Clinical uses of indapamide include the treatment of nephrogenic diabetes insipidus, hypertension, hypercalciuria, glaucoma, and edema, among other diseases.[2,3] Indapamide (a popular drug of abuse) is also misused by athletes in sports for several reasons including the reduction of body weight, to reduce urinary concentration of other prohibited substances in order to avoid a positive doping test and to overcome fluid retention caused by the use of anabolic steroids.[4,5] Indapamide belongs to the group of diuretics that are banned substances in sports since 1986.[6] Moreover, to date the pharmacokinetic profiles of indapamide pills compared with conventional tablets in vivo is unknown. The analytical determination of indapamide is desirable[7] taking into account that its overdose might lead to severe hyponatraemia, with symptoms varying from nausea to seizures and coma and hypokalemia,[8,9] which could lead to fatal arrhythmia.[10] In 2007, a case report linking indapamide and hyperparathyroidism was published.[11] According to literature evidence, indapamide determination has been accomplished in various biological fluids like urine,[12,13] serum[14] and plasma.[15,16] Therefore, a simple, rapid and efficient analysis method is needed for the determination of indapamide in urine sample so as to evaluate the pharmacokinetic parameters and also to monitor its drug abuse in sports.

Most sample preparation techniques available for the extraction of indapamide in various biological matrices include liquid–liquid extraction (LLE) and solid phase extraction (SPE).[17–19] However, the disadvantage of these methods is that they are either involved in several extraction steps or yield poor separation from the biological sample's endogenous interferences, low extraction recovery and the need for a large amount of internal standard.[17,18] Ideally, sample-preparation techniques should be fast, inexpensive and compatible with a range of analytical instruments, so the current trend is towards decreasing the quantities of organic solvents, simplification and miniaturization of the sample-preparation steps.[20] To fulfill this purpose, liquid-phase microextraction (LPME) techniques like single drop microextraction (SDME), hollow-fiber LPME and dispersive liquid–liquid microextraction (DLLME) were developed.[21–25] More recently, a novel microextraction technique developed by Garcia-Jares *et al.*, named ultrasound-assisted

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emulsification microextraction (USAEME) was developed,[26] based on the emulsification of few microliters of water-immiscible extraction solvent in the aqueous sample solution by ultrasound radiation. The usage of ultrasound energy leads to enlargement of the contact surface between two immiscible liquids due to reduction of droplet size of the extractant phase, thereby facilitating mass-transfer of the analyte between the two phases. However, the main disadvantage of USAEME is that the extraction solvent must have a higher density than water in order to be sedimented by centrifugation, typically chlorinated solvents such as chlorobenzene were used, which is potentially toxic to human health and the environment. Recently, low density organic solvents were applied to substitute chlorosolvents in USAEME due to toxicity consideration.[27,28]

Hence, the goal of the present work was to investigate and develop a rapid and efficient low density solvent (LDS) based USAEME method coupled with high performance liquid chromatography-variable wavelength detection (HPLC-VWD) for the analysis of indapamide in human urine samples. It is to be noted that this is the first report of the determination of indapamide in human urine using LDS-USAEME, thus paving way as a good alternative for routine analysis of indapamide in human urine samples with advantages of simplicity, reliability, cost effectiveness and minimized matrix interferences. The effect of various experimental conditions on the extraction of indapamide are investigated and discussed in detail. The optimized procedure was successfully applied to the determination of the target analyte in human urine samples.

2 Experimental

2.1 Reagents and solutions

All chemicals used in this work were of ACS reagent grade. Indapamide (99.9%) was purchased from Fluka Chimika (Buchs, Switzerland). 1-Dodecanol (density, 0.83 g mL^{-1}) and 1-undecanol (density, 0.82 g mL^{-1}), were purchased from Merck Chemicals (Darmstadt, Germany). HPLC-grade acetonitrile (ACN), hydrochloric acid (HCl), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). Ultrapure water for all aqueous solutions was produced in the laboratory using Barnstead Nanopure water system (Barnstead, New York, USA). Stock solutions (1 mg L^{-1} of the analyte) were prepared by dissolving the analyte in methanol and stored in brown glass bottles with polymer-lined caps and kept at 2 °C. Working standard solutions were obtained daily by diluting the stock solutions.

Urine samples used for evaluation of the method were collected in glass bottles from two healthy volunteers. Obtained urine samples were diluted with ultrapure water (1 : 1) and was adjusted to pH 3 by adding 1 M HCl and filtered through 0.45 μm cellulose acetate membrane filters and stored at −20 °C prior to being used.

2.2 Instrumentation

The liquid chromatography equipment included an Agilent 1100 Series equipped HPLC system with manual injection and variable wavelength detector (VWD). A personal computer equipped with Agilent ChemStation program was used for data acquisition and processing. Extraction solvent collection and injections were carried out using a 50 μL HPLC microsyringe (SGE, Ringwood, Australia). Separation of the target analyte was accomplished using a Macherey-Nagel Nucleodur C18 HTec (5 μm, 250 mm × 4.6 mm ID) column (Düren, Germany). The mobile phase was phosphate buffer (adjusted to pH 3 with *o*-phosphoric acid) and acetonitrile (40 : 60, v/v) at a flow rate of 1 mL min^{-1}. Detection was set at 240 nm.[29] Under these chromatographic conditions, the target analyte could be baseline separated.

2.3 LDS-USAEME procedure

10 mL of spiked sample solution was placed in a 15 mL screw-cap glass test tube with conical bottom. 40 μL of 1-undecanol (as extraction solvent) was rapidly injected into the sample solution by a 0.5 mL syringe (SGE, Ringwood, Australia). The resulting mixture was subsequently immersed into an ultrasonic bath (model D80, Delta, Taiwan) for 2 min at 25 °C at ultrasound frequency and power of 43 KHz (80 W) for extraction. During this step, a turbid cloudy solution was formed in the test tube and analyte in the urine sample was extracted into the fine droplets of 1-undecanol because of the increased contact area between the extraction solvent and urine. Then the formed emulsion was centrifuged for 3 min at 4500 rpm during which the dispersed fine particles of the extraction solvent collected at the top of the conical test tube. The collected extraction solvent was measured using a 50 μL HPLC microsyringe (SGE, Ringwood, Australia), from which 10 μL was diluted with mobile phase (1 : 1) and injected into the HPLC-VWD for analysis.

3 Results and discussion

3.1 Selection of extraction solvent

The choice of an appropriate organic extraction solvent is of high importance in the USAEME process. The prime requirements for the extraction solvent include low toxicity, immiscibility in aqueous samples, low density, high extraction capability for indapamide and good chromatographic behavior. Based on the aforementioned conditions, four organic solvents including 1-octanol, 1-decane, 1-undecanol and 1-dodecanol were investigated in the current research. Fig. 1 demonstrates the peak areas obtained by using these extractants for the extraction of 100 ng mL^{-1} of the indapamide in 10 mL sample solution under USAEME conditions. Results revealed that 1-undecanol has the highest extraction efficiency for the target analyte compared to other extractants. Therefore, 1-undecanol was chosen for subsequent experiments.

3.2 Effect of extraction solvent volume

In order to investigate the effect of volume of 1-undecanol on extraction efficiency, different volumes of 1-undecanol from 30 to 70 μL were investigated. By increasing the 1-undecanol volume in the range of 30–70 μL at 10 μL intervals, the extraction efficiency gradually increased from 30 to 40 μL. Fig. 2 depicts the change trend of peak area *versus* 1-undecanol

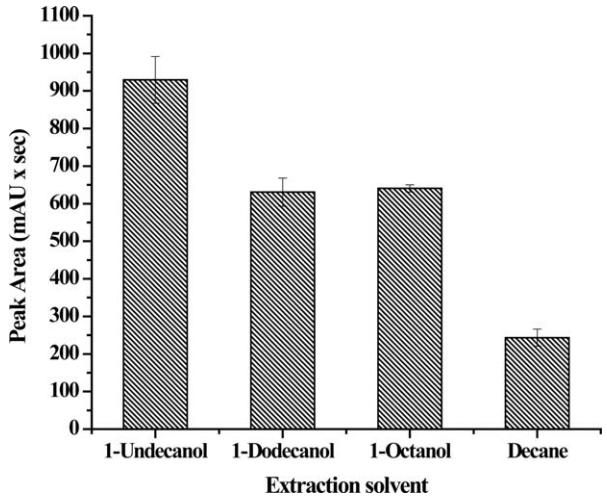

Fig. 1 Effect of extraction solvent on the extraction efficiency. Sample: 10 mL of sample solution (100 ng mL^{-1} of indapamide) at pH 3. Volume of extraction solvent: 40 μL, extraction time: 2 min, centrifugation time: 3 min at 4500 rpm, $n = 3$.

volume. It was clear that 1-undecanol showed poor extraction efficiency at low volume (30 μL). When 1-undecanol volume was increased to 40 μL, higher peak response was achieved because concentration of the analyte (indapamide) in the extraction solvent attained the maximum extraction efficiency under the proposed extraction procedure. Any further increase in 1-undecanol volume resulted in decreased extraction efficiency due to the dilution of the analyte in a higher volume of 1-undecanol.[30] Therefore, 40 μL 1-undecanol was selected as an optimal extraction solvent volume in subsequent extractions.

3.3 Effect of ultrasonication time

Ultrasonication time is one of the main factors in USAEME because ultrasonication decreases the droplet size and disperses the organic extraction solvent uniformly within the aqueous solution, resulting in large contact surface area between the organic extraction solvent and the aqueous sample, thereby resulting in very rapid transport of analyte from the aqueous phase to the organic phase. As is evident from Fig. 3, extraction efficiency increased until 2 min of ultrasonication, which was sufficient for obtaining maximum extraction efficiency and with further increase in ultrasonication time, the extraction efficiency remained almost constant probably because of attainment of equilibrium due to the complete dispersal of the extraction solvent.[31] Hence short ultrasonication time of 2 min was selected for further studies.

3.4 Effect of pH

In general, sample pH determines the existant state of analytes, thereby affecting extraction efficiency. Since analytes in neutral forms are much easier to extract than those in ionic forms, partition coefficient and extraction ability of the system are enhanced. Experimental results showed that the extraction efficiency of indapamide remains unchanged from pH 2–7 and any further increase in pH resulted in a decrease in extraction efficiency. The reason can be attributed to the fact that under alkaline pH conditions, the ionized form of the indapamide might be formed, resulting in increased aqueous solubility, thereby decreasing extraction efficiency.[32] Therefore pH 3 was used for further experiments for the better extraction with good precision for the acidified urine sample.

3.5 Effect of ionic strength

Generally, the addition of salt decreases the solubility of analytes in aqueous sample and enhances their distribution in organic phase. However, it can be seen from Fig. 4 that addition of NaCl resulted in a gradual decrease in extraction efficiency. The probable reason for the decrease in extraction efficiency could be because of decreased solubility of extraction solvent in

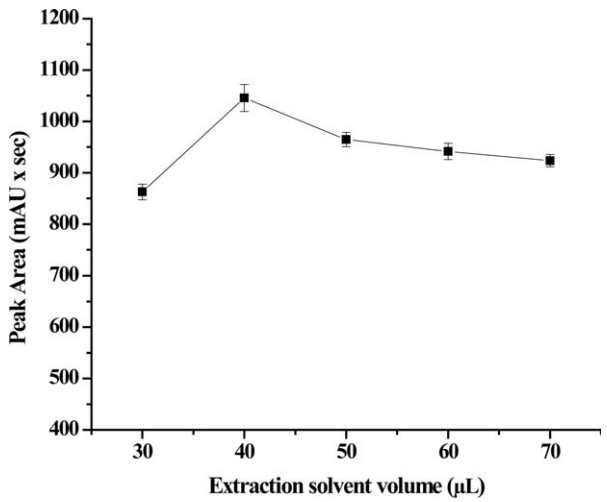

Fig. 2 Effect of volume of extraction solvent on extraction efficiency Extraction solvent: 1-undecanol, sample and extraction conditions: as in Fig. 1 except volume of extraction solvent.

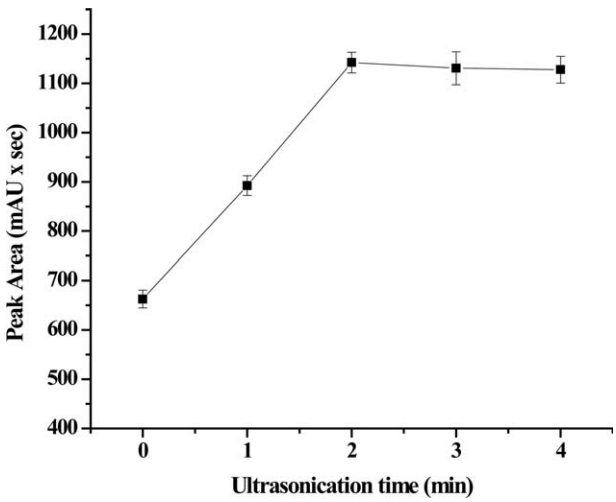

Fig. 3 Effect of extraction (ultrasonication) time on extraction efficiency sample and extraction conditions as in Fig. 2 except extraction time.

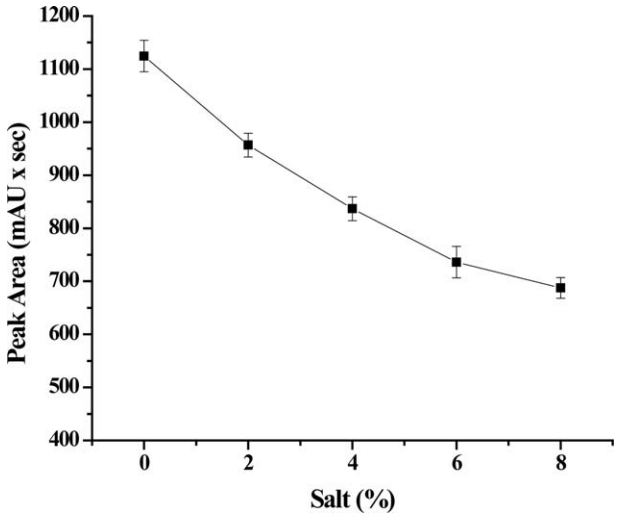

Fig. 4 Effect of salt concentration on extraction efficiency sample and extraction conditions as in Fig. 3.

aqueous sample solution in the presence of salt.[33] On the basis of the above observations; no salt was added for further extractions.

3.6 Evaluation of quantitative aspects and its application

Analytical characteristics of the proposed method such as linear range, correlation coefficient, limits of detection and precision were investigated under the optimal experimental conditions. Precision, expressed as relative standard deviation (RSD), was evaluated in terms of repeatability by performing three replicate extractions of spiked urine (100 ng mL^{-1}) and it ranged from 1.2–6.6%. For spiked urine standards, indapamide exhibited good linearity in the range of 1–100 ng mL^{-1} with a correlation coefficient (R^2) value of 0.9977. Limit of detection (LOD) was calculated as the analyte concentration equal to 3 times the standard deviation of the blank signal divided by the slope of the calibration curve and it was found to be 0.3 ng mL^{-1} and limit of quantification (LOQ) was calculated as the analyte concentration equal to 10 times the standard deviation of the blank signal divided by the slope of the calibration curve and it was found to be 1.1 ng mL^{-1}.

Validation of the proposed method was examined by spiking three analyte free urine samples with concentrations of 50, 25 and 10 ng mL^{-1} collected from three healthy adults (aged 24–30 years) and subjecting the sample to LDS-USAEME followed by HPLC-VWD analysis. Prior to LDS-USAEME, the urine samples

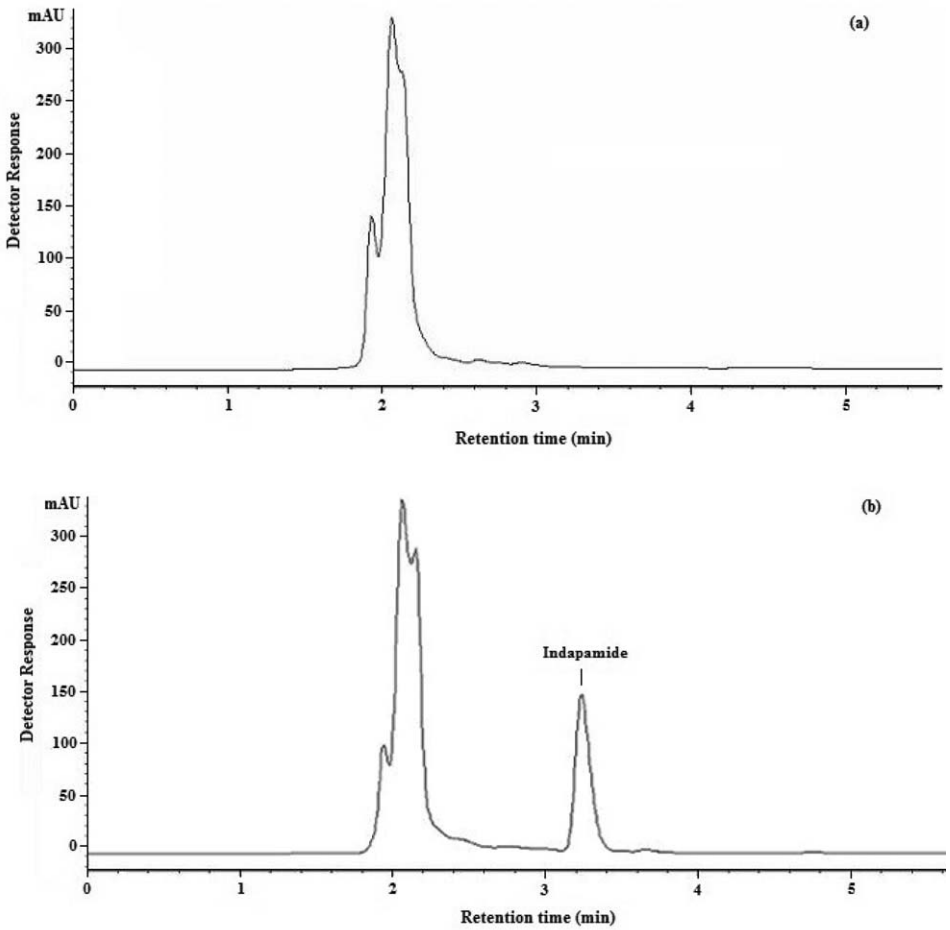

Fig. 5 Chromatogram of indapamide in (a) blank (non-spiked) urine sample and (b) spiked urine sample, by the present method. Spiked sample: 50 ng mL^{-1} of target analyte.

Table 1 Analytical results of indapamide in urine samples by the proposed method

Urine sample	Spiked concentration (ng mL^{-1})	Concentration found in real sample (ng mL^{-1})	Relative recovery (%)	RSD (%, $n = 3$)
Volunteer I	50	ND[a]	109.6	2.6
Volunteer II		ND	107.5	5.8
Volunteer I	25	ND	80	1.8
Volunteer II		ND	110.7	6.7
Volunteer I	10	ND	119	5.0
Volunteer II		ND	114	2.1

[a] Not detectable.

were adjusted to the required pH and filtered through 0.45 μm cellulose acetate membrane filters and stored at −20 °C till analysis time. Obtained urine samples were run to determine the absence of the target drug. Experimental results exhibited that no indapamide was detected in the urine samples (Fig. 5a). As can be seen in Fig. 5b, a typical chromatogram of the spiked (50 ng mL^{-1}) urine sample obtained by the LDS-USAEME-HPLC-VWD method does not have any interferences from endogenous matrix components, thereby revealing good sensitivity and good cleanup of the developed method. When the sample was spiked with 50, 25 and 10 ng mL^{-1} of indapamide and extracted (Table 1), relative recoveries obtained ranged from 80–119% with RSDs ranging from 1.8–6.7%, thereby proving that this LDS-USAEME-HPLC-VWD is a rapid, accurate and sensitive method for the analysis of indapamide in human urine.

3.7 Matrix effect

Matrix effect could change the instrumental response of the analyte when a component of urine co-elutes with the analyte of interest and causes suppression or enhancement in extraction efficiency relative to the analyte eluting in the absence of the matrix component, thereby resulting in erroneous results. In this work, the matrix effect was investigated by analyzing urine samples covering a wide range of creatinine contents (1–50 mg L^{-1}). Experimental results showed that excellent recoveries ranging from 94.6–101.7% were obtained for creatinine concentrations ranging from 1–50 mg L^{-1}, proving that creatinine adjusted urine did not have any considerable influence on extraction efficiency. Another way to reduce the impact of matrix effects is by the dilution of urine samples. In order to check the effect of urine dilution on extraction efficiency/recovery, the samples were subjected to different degrees (1, 5, 10 and 20) of dilution and it can be seen from experimental results that good enrichment factors were obtained that remained almost constant at different dilutions of the urine sample, thus proving the absence of any matrix interferences using 1-undecanol as the extraction solvent for LDS-USAEME-HPLC-VWD of indapamide. As a result of the above studies, it can be concluded that matrix influences did not affect our proposed method under optimal conditions.

3.8 Comparison of the present technique with other reported methods

Table 2 compares the figures of merit of the proposed method with other reported methods for the extraction of indapamide from various samples of interest. It can be clearly seen from the table that the volume of extraction solvent used in the proposed method is highly minimal when compared to other reported methods, and the limits of quantification (LOQs) are comparable. These merits emphasize the fact that the proposed method is highly cost effective, environment friendly and rapid.

4 Conclusion

In the present study, LDS-USAEME combined with HPLC-VWD has been successfully applied for the extraction and determination of indapamide in human urine samples. On comparison with other conventional sample preparation methods such as

Table 2 Comparison of the proposed method with other methods

Method	LOQ (ng mL^{-1})	Extraction solvent volume (mL)	Total extraction time[d] (min)	Reference
SPE-HPLC-UV	10	0.3	5	19
LLE-HPLC-AMP[a]	1	8	45	12
SPE-HPLC-AMP	1	2	2	12
LLE-UPLC[b]-UV	1	5	5.5	34
LLE-HPLC-ESI[c]-MS	0.75	5	15	14
LLE-HPLC–UV	5	4	12	29
USAEME-HPLC-VWD	1.1	0.04	5	Present method

[a] AMP – Amperometric detection. [b] UPLC – Ultra-performance liquid chromatography. [c] ESI-MS – Electrospray ionization-mass spectrometry. [d] Total extraction time refers to the overall time taken for the extraction procedure, including ultrasonication, centrifugation and vortex mixing, wherever applicable.

LLE and SPE, the proposed method offers advantages such as simplicity, minimum matrix effects, lower consumption of organic solvent, ease of operation and relatively short analysis time. Moreover, the use of less toxic low density extraction solvent facilitated the easy retrieval of the extraction solvent microdrops, thereby making the sample preparation procedure simple and environment friendly. Moreover, the linear concentration range of the proposed method covers the common drug abuse cutoff concentrations in both the initial screening test and the confirmatory test. Experimental results indicate that the present method can be used as a method of choice for the simple and efficient extraction of indapamide in human urine samples.

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