Determination of Urinary Total Hydroxyproline by HPLC with UV and EC Detectors

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Summary: Hydroxyproline is found in high concentrations in connective tissue proteins. It is remarkably useful to detect it because variation in urine levels of hydroxyproline is associated with various diseases. High performance liquid chromatography methods, in which ultraviolet and electrochemical detectors were used, were developed and validated for the determination of hydroxyproline in urine. Both methods included acid hydrolysis and derivation. The most appropriate time, temperature and pH were identified to optimize derivatization processes.
Limits of detection were calculated as 1.57 μg/mL, 0.9 μg/mL and limits of quantification were 4.76 μg/mL, 2.73 μg/mL for UVD and ECD methods, respectively. Precision and accuracy of the methods were obtained ≤10.9 % (RSD), ≤11.5% (RE). The recoveries were found 108±6% and 102±7% for UVD and ECD methods, respectively.

A strong, positive and linear correlation was found between hydroxyproline values obtained from UVD and ECD methods ($p<0.01$, $r=+0.98$). After optimization and validation of these methods, OHP levels in urine samples which were obtained from non-smokers and smokers were analyzed and, observed OHP/creatinine levels were compared. Results of both methods showed that total urinary OHP values of smokers were significantly higher than non-smokers ($p<0.05$).

Keywords: Hydroxyproline, Tobacco smoke, Urine, HPLC, Ultraviolet detection, Electrochemical detection.

Introduction

Proline and hydroxyproline (OHP) are present in various amounts in certain structural proteins, such as high in collagen and low in elastin [1]. OHP is a secondary amino acid and, approximately 50% of proline is hydroxylated to OHP, which provides H-bonding sites and is necessary for the protein triple helical structure in connective tissue [2]. OHP ratio of proline in lung collagen is higher than other tissues [3]. For this reason, there is considerable interest in OHP levels as a marker of various pathological conditions related to collagen degradation in lungs [4]. Degradation can be related to a pulmonary damaging effect of NO_2, oxidative stress, hypoxia and above all derived from tobacco smoke [5, 6].

OHP is released during the breakdown of collagen in connective tissues [3] and is not recycled to form new collagen [7-9]. It is present in plasma in -free, -peptide and -protein form [10, 11]. Most of the free OHP is reabsorbed by the tubules while OHP containing peptides and proteins pass through the kidney, so approximately 90% of urinary OHP is peptide bound [12]. Peptide and protein bound form of OHP became free OHP with acid hydrolysis and this allows determining total free urinary OHP.

Many methods have been proposed to determine OHP values in human urine. These include colorimetric methods [13], enzyme immunoassay [14], electrogenerated chemiluminescence [1], micellar electrokinetic capillary electrophoresis [15], gas chromatography mass spectrometry [16], gas chromatography flame ionization detection [17] and high performance liquid chromatography (HPLC) [18, 19].

The aim of this study was to develop sensitive and reproducible methods for quantifying OHP in urine and to determine whether there was a relation between smoking and hydroxyproline levels and to verify this by two methods.

Experimental

Chemicals

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole and cysteic acid were obtained from Fluka (Neu-

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Ulm, Germany) and Merck (Darmstadt, Germany), respectively. Triethylamine was purchased from Merck (Hohenbrunn, Germany). Trans 4-hydroxy-L-proline was obtained from Sigma Aldrich (St. Louis, ABD) and N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) was obtained from Aldrich (Steinheim, Germany). Acetone, sodium dihydrogen phosphate dihydrate, acetonitrile, hydrochloric acid, methanol (Merck, Darmstadt, Germany) was used as reagent and solvent. The mobile phase was filtered through a 0.45 μm pore-size filter (Alltech, Deerfield, USA). Specimens were filtered with a 0.45 μm pore size syringe filter (Sartorious, Goettingen, Germany)

Reagents and solutions

Ultraviolet Detection Method

A stock solution of hydroxyproline was dissolved in HCl (20 mmol L^{-1}) and dilutions were prepared in water and stored at -18˚C. 5 mol L^{-1} sodium hydrogen carbonate solution was prepared every week (pH 9.5). Cysteic acid (2 mg mL^{-1}) was diluted to 40 μg mL^{-1} with the sodium hydrogen carbonate solution immediately, before the sample preparation. 10 mg mL^{-1} FDNDEA, used as derivatization reagent, was prepared weekly in acetonitrile.

Electrochemical Detection Method

A stock solution and standards of hydroxyproline were prepared in 25% methanol and stored at -18˚C. A solution of sodium hydrogen phosphate (0.5 mol L^{-1}, pH 12.5) was prepared every week and 10 μg mL^{-1}NBD-Cl, used as derivatization reagent was prepared weekly in absolute methanol.

Instrumentation

The separation was performed by Agilent 1100 (Hewlett-Packard, Santa Clara, CA, USA) high-performance liquid chromatography (HPLC) system. The analyses were carried out with a system of isocratic pump (G1310A) (Santa Clara, CA, USA), a Waters Nova-Pak C18 column with dimensions (60 Å, 4 μm) 3.9 x 150 mm (Milford, MA, USA) and manual injector (G 1328A, Rheodyne) with a loop volume of 20 μL. Column suitable for use with a buffer at pH 2-8 range. Quantification of UVD and ECD methods were performed by G1314A (Agilent, Waldbronn, Germany) and Coulochem II (ESA) (Rochester, New York), respectively.

Chromatographic Conditions

The mobile phase of ultraviolet detection (UVD) method was a mixture of 80% acetate buffer, which was fixed by mixing 1 L water and 3 mL glacial acetic acid with 10 mL triethylamine, then acetate buffer was adjusted to pH 4.3 and 20% acetonitrile. The flow rate was 1.3 mL min^{-1} and the UVD was set at 360 nm. The mobile phase of electrochemical (ECD) method was a mixture (80:20, v/v) of 50 mM NaH_2PO_4 and methanol, and this mixture was adjusted to pH 3.4 with phosphoric acid. The rate of flow was set to 0.8 mL min^{-1} and the analytical cells were set at 350+ and 640+ mV for detector 1 and 2, respectively. All mobile phases were prepared at room temperature, than filtered 0.45 μm filter. Optimization, validation and urine samples were analyzed at ambient temperature.

Methods

Both of the methods include acid hydrolysis and derivatization step.

Ultraviolet Detection Method

It was required to add to each glass tube 50 μL urine samples, 50 μL water, 100 μL HCl and mix well for hydrolysis. These samples were incubated at 100˚C for 16 h and then dried under nitrogen. The residue was gently dissolved in 200 μL acetone and dried under nitrogen again. The final residue was redissolved in 250 μL of cysteic acid solution. 50 μL, taken from this solution, was added to 50 μL FDNDA solution, derivatized at 100˚C for 20 min and then dried. After the residue was dissolved in 500 µL of mobile phase. Injection volume of this solution into HPLC was 20 μL.

Electrochemical Detection Method

It was required to add to each glass tube 50 μL urine samples, 50 μL 12 M HCl and to mix well. These samples were incubated at 115˚C for 20 hour and the samples were dried under nitrogen. The last residue was dissolved in 200 μL 25% methanol and to each solution was added, 200 μL derivatization buffers and 50 μL NBD-Cl. This blend was incubated for 10 min at 80˚C for derivatization, process was stopped by adding 50 μl 1 M HCl and cooling immediately. 20 μL of final solution was injected into HPLC.

Creatinine detection method

The urine sample was diluted 200 times, acidified to pH 2.2 and analyzed by C18 column and 235 nm ultraviolet detectors. As the mobile phase; a 0.05 M phosphate buffer having a pH value of 7.4

was used. The flow rate of the mobile phase was set at 1 mL/min for HPLC.

Method Validation

The method for each sample was verified in accordance with the following criteria in accordance with the International Conference on the Adaptation of Technical Requirements for the Determination of Drugs for Human Use [20]. Validation processes were performed for UVD and ECD methods, separately.

Linearity

After the chromatographic conditions were established, spiked human urine standards were prepared from the known concentrations of analytes. Their concentrations ranged from 5-100 μg mL^{-1}. For these concentrations, five individual repeats were injected and linearity was obtained with the correlation coefficients (r^2) They are 0.9998 and 0.9995, UVD and ECD methods, respectively.

Accuracy and Precision

In order to determine inter-day accuracy and precision of the assay, spiked samples containing known hydroxyproline concentrations (5, 25, 100 μg mL^{-1}) were analyzed on five separate days. In order to determine intra-day accuracy and precision, spiked samples containing known hydroxyproline concentrations (5, 25, 100 μg mL^{-1}) were analyzed five times.

Sensitivity

Sensitivity of the method was evaluated by determining LOD and LOQ. They were determined based on the standard deviation of the response and the slope of the calibration curve. The LOQ and LOD were repeated ten times for confirmation.

Recovery

The recoveries were determined in urine samples at the concentrations of 25-100 μg mL^{-1} (n=5).

Sample Collection

The study was permitted by the Local Ethics Committee of Ankara University in 25/07/2011 (Decision number 34-739). Subjects were 24 non-smoking and 24 smoking volunteers from Kırıkkale State Hospital healthcare personnel in Kırıkkale-Turkey. First morning urine samples; which are widely used for creatinine detection in medical diagnosis and scientific studies [21, 22, 23], were collected in amber glass tubes and as mentioned in the method section. Creatinine in urine was determined for the ratio of hydroxyproline to creatinine. All Statistical Analysis were performed by using Statistical Package for the Social Sciences (SPSS) 15.0 for MS Windows.

Results and Discussion

All urine samples were hydrolyzed overnight, evaporated to dryness and derivatized.

Optimization of Derivatization

The most critical step of the methods was derivatization. Hence, the most appropriate time, temperature and pH of the derivatization were investigated in order to maximize the amount of the FDNDEA-OHP and NBD-OHP. The reaction of OHP with FDNDEA and NBD-Cl are shown in Fig. 1 and Fig. 2.

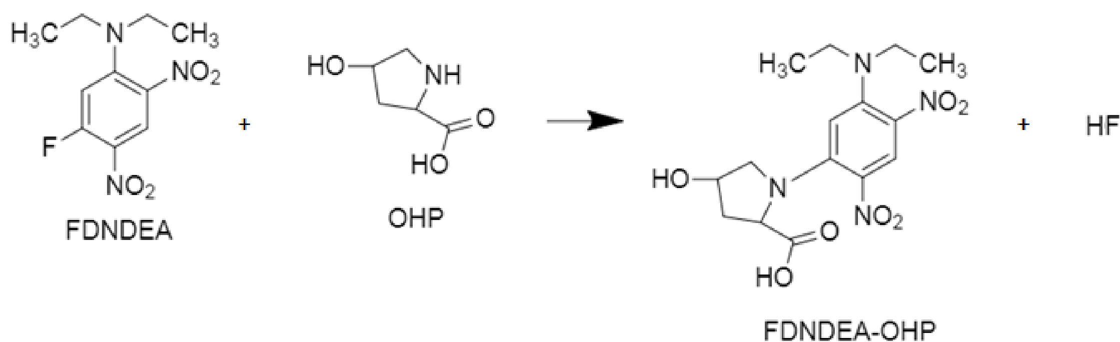

Fig. 1: Derivatization reaction of OHP with N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA).

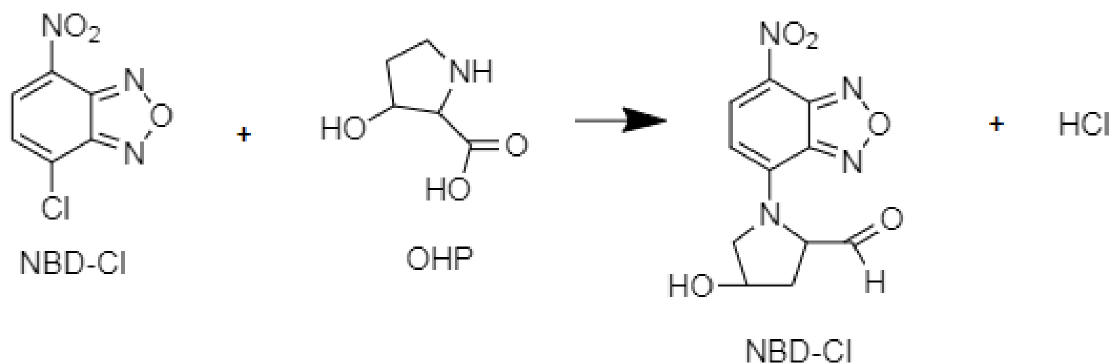

Fig. 2: Derivatization reaction of OHP with 4-Chloro-7-nitrobenzofurazan (NBD-Cl).

Five aliquots of hydroxyproline solutions were derivatized in triplicate at different pH (range 8.9-9.7), time (range 10-30 min) and temperature (range 80-110°C) values for UVD method. As shown in Fig.3, 5(a), 6(a) the best reaction yields, in terms of peak areas were obtained at pH of 9.5, time of 20 min. and temperature of 90 °C.

Minimum five aliquots of hydroxyproline solutions were derivatized in triplicate at different pH (range 9.0-13.0), time (range 5-20 min) and temperature (range 50-100°C) values for ECD method. As shown in Fig. 4, 5(b), 6(b) the best reaction yield, in terms of peak areas were obtained at pH of 12.5, time of 10 min and 80°C. Derivatization did not occur at pH<9. Covering these details, in Table 1, the pH, time and temperature values at which the best reaction efficiency is obtained in derivatization are given for both UV and EC detectors.

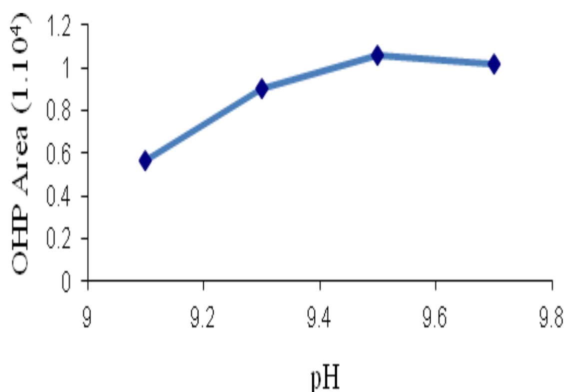

Fig. 3: UVD method derivatization yields for OHP at various pH values.

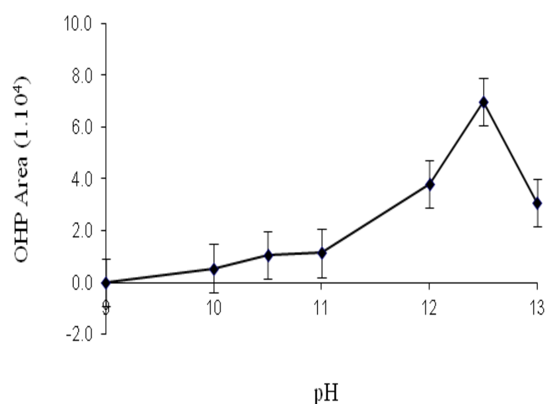

Fig. 4: ECD method derivatization yield for OHP at various pH values.

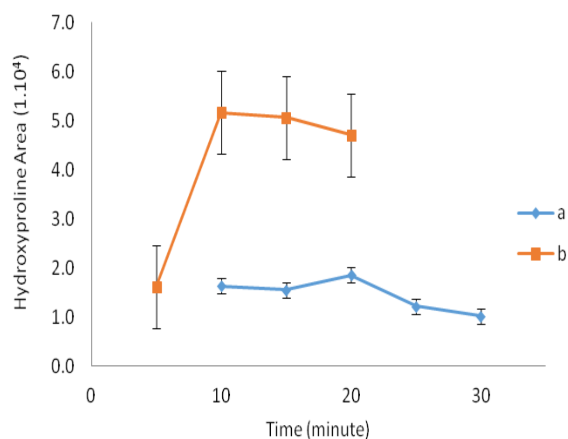

Fig. 5(a): UVD method derivatization yields for OHP at various time. (b) ECD method derivatization yield for OHP at various time values.

Table-1: The pH, time and temperature values at which the best reaction efficiency was obtained in derivatization.

Detector	pH	Time (min.)	Temperature (°C)
UV	9.5	20	90
EC	12.5	10	80

Chromatographic Separation

Typical sequences of chromatograms are shown in Fig.7 and Fig.8 which were obtained from UV and EC detectors, respectively. Fig.7 demonstrates a typical separation of FDNDEA derivatives of OHP (25 μg mL^{-1}). The retention times of the analytes were 2.6 min for OHP and 4.8 min for cysteic acid (40 μg mL^{-1}). Fig.8 demonstrates a typical separation of NBD derivatives of OHP (25 μg mL^{-1}). The retention times of OHP, eluted as separate symmetrical peak, were 3.2 min.

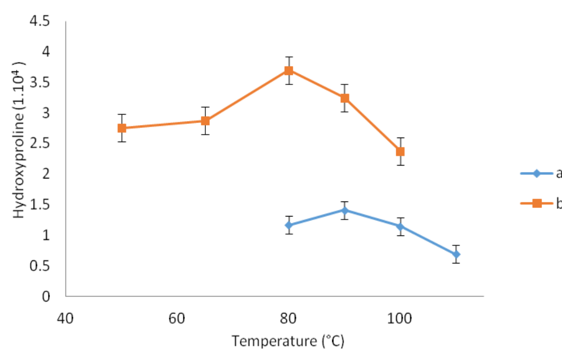

Fig. 6.(a): UVD method derivatization yields for OHP at various temperature values. (b) ECD method derivatization yield for OHP at various temperature values.

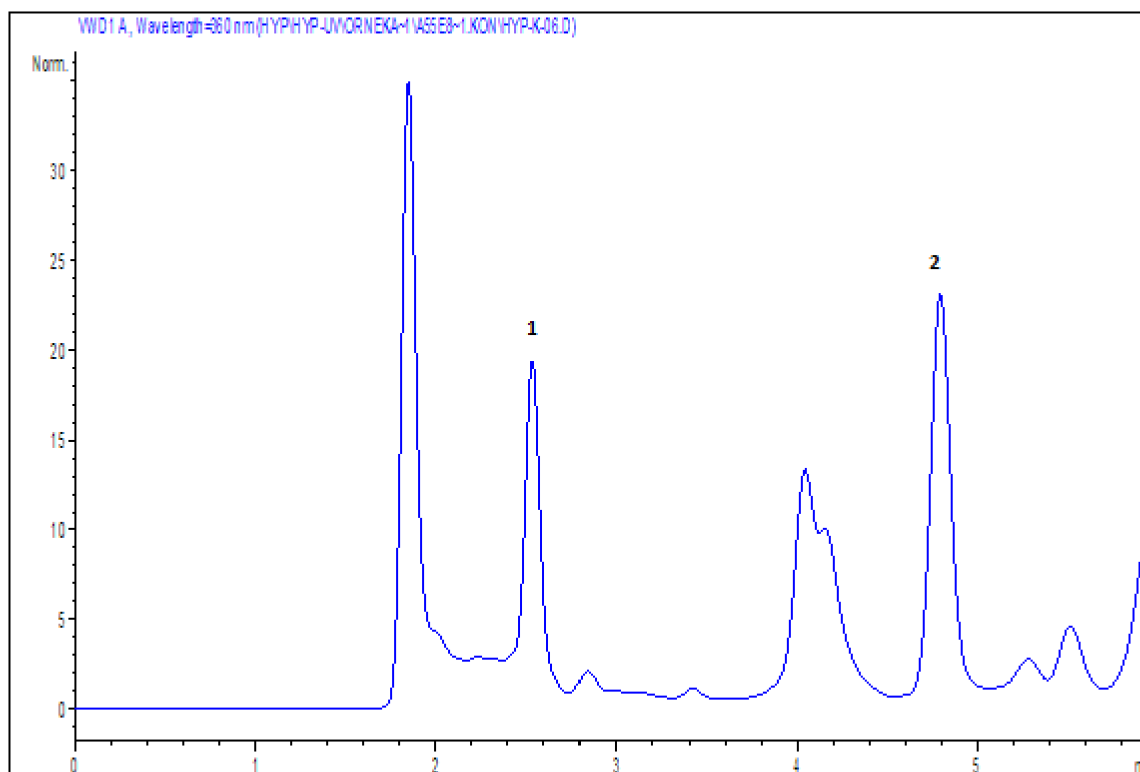

Fig. 7: Separation of FDNDEA-OHP (25 μg mL^{-1}) and cysteic acid in spiked samples with HPLC-UV method. Peak 1,ISTD; Peak 2, FDNDEA-OHP.

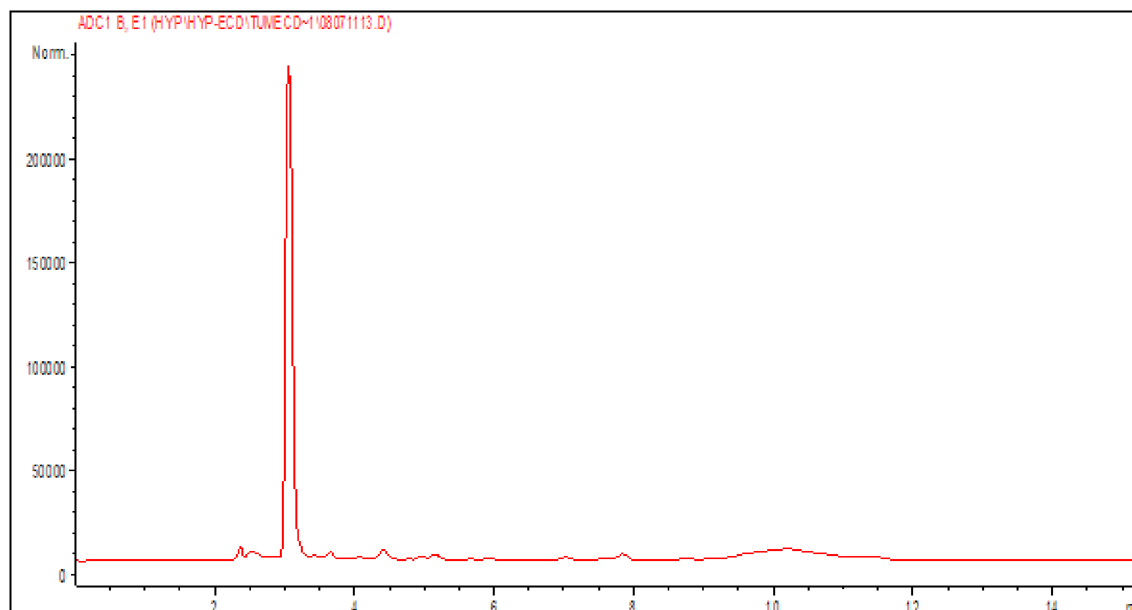

Fig. 8: Separation of NBD-OHP (25 μg mL^{-1}) in spiked samples with HPLC-EC method. Peak, NBD-OHP.

Table-2: LOD and LOQ values for the determination of hydroxyproline.

Method	LOD	LOQ
HPLC-UV detector	1.57 μg/mL	4.76 μg/mL
HPLC-EC detector	0.9 μg/mL	2.73 μg/mL
HPLC-a diode-array detector [18]	2 μg/mL	
HPLC-a diode-array detector [19]	75 fmol/μL	

Table-3: Intra-day assay precision and accuracy for the determination of hydroxyproline.

Detector	Expected Concentration (μg mL^{-1})	Observed Concentration (Mean±SD) μg mL^{-1}	Precision (%)	Accuracy (%)
	5	5.6±0.3	5.4	11.0
UV	25	26.0±1.0	3.8	6.6
	100	101.0±4.0	4.0	0.7
	5	5.5±0.6	10.9	10.8
EC	25	25.0±3.0	12.0	4.2
	100	108.0±4.0	3.7	7.3

Table-4: Inter-day assay precision and accuracy for the determination of hydroxyproline.

Detector	Expected Concentration (μg mL^{-1})	Observed Concentration (Mean±SD) μg mL^{-1}	Precision (RSD%)	Accuracy (RE%)
	5	5.5±0.2	3.6	11.5
UV	25	26.8±1.1	4.1	5.5
	100	103±6.0	5.8	1.1
	5	5.8±0.2	3.4	10.9
EC	25	26.4±1.4	5.3	1.7
	100	105.0±6.0	5.7	8.2

Analytical Variables

The analytical methods were validated to demonstrate the linearity, precision, accuracy, sensitivity and recovery. Validation data showed that the relationships were linear in the range of 5–100 μg mL^{-1}, for UVD (r^2=0.9998) and ECD (r^2=0.9995) methods. Precision (relative standard deviation %, RSD%) was determined with five individual replicates at three different concentrations (n=5) for each method. Accuracy (random error %, RE%) was also determined for the three concentrations of analytes. Table-3 and Table-4 show the RSD values of intra-day and inter-day precision and accuracy. Limit of detection (LOD) and limit of quantitation (LOQ) values were calculated. LOD were 1.57 μg mL^{-1}, 0.9 μg mL^{-1} and LOQ were 4.76 μg mL^{-1}, 2.73 μg mL^{-1} for UVD and ECD methods, respectively. The concentration of hydroxyproline in the blend of 20 samples of urine was measured as blank and free

OHP. The recoveries were carried out by adding 100 μg mL^{-1} of standard hydroxyproline to urine pool formed by mixing 20 urine samples. Then, the samples of urine, in which determined concentration was added, was then measured. The recoveries were calculated 108±6 (5.8%) and 102±7 (7.0%) for UVD and ECD methods, respectively.

Comparison of Samples

Creatinine is important in assessing kidney function and showing glomerular filtration rate. Since the direct measurement of the glomerular filtration rate is very difficult, it is indirectly calculated by means of the clearance calculation, which means the rate of cleaning of the various substances. An ideal substance to be used for clearance measurement; freely present in the circulation, freely filtered from the glomerular basement membrane, not secreted through the nephron and not reabsorbed, endogenously produced at a constant speed and easily measurable. The most commonly used method is creatinine clearance. For this reason, we calculated that creatinine by finding the ratio of creatinine [OHP] / [creatinine] in each urine sample.

Urine samples of 24 non-smoker and 24 volunteers smoker were quantified by the described methods. Values were expressed as the ratio of OHP/creatinine. The concentration of OHP/creatinine for non-smokers in urine ranged from 0.51-2.58 (UVD) and 0.62-2.63 (ECD). The concentrations of OHP/creatinine for smokers in the urine ranged from 0.57-3.65 (UVD) and 0.68-3.72 (ECD). The mean concentration ratios of non-smokers' OHP in urine were obtained 1.33±0.56 μg mL^{-1} and 1.33±0.59 μg mL^{-1} for UVD and ECD methods. The mean concentration ratios of smokers' OHP in urine were obtained 1.79±0.88 μg mL^{-1} and 1.77±0.85 μg mL^{-1} for UVD and ECD methods, respectively. Urine creatinine levels were not significantly different between smokers and nonsmokers. There was a positive, strong and linear correlation between urinary OHP values of smokers and non-smokers ($r=+0.98$, $p<0.05$). However, the ratio of OHP/creatinine was significantly lower in the non-smokers' group ($p<0.05$). Thus, the analysis of student t test provided evidence for significant differences ($p<0.05$) between the groups of non-smokers and smokers. Smokers were categorized according to the number of cigarettes smoked per day. Groups included low smokers (less than 10 cigarettes per day), moderate smokers (10–20 cigarettes per day) and heavy smokers (over 20 cigarettes per a day), and were 11, 6 and 7 people, respectively.

The statistical analysis of the results from the three groups showed that the values recorded for the heavy smokers were significantly different from the low smokers and moderate smokers groups ($p<0.01$).

In this study, we have used the determination methods described by Paroni et al. 1992 [18] and Welch et al. 1993 [19]. These methods were modified and optimized. There was no sample pre-treatment before derivatization, other than hydrolysis. OHP in urine was separated and detected from an injection volume of 200 μL urine for both of the methods. The chromatographic run takes 6 minute from injection to injection and this short time provides considerable advantages in routine analysis. In our study, LOD was 1.8 μg mL^{-1} (UVD) and 0.9 μg mL^{-1} (ECD), which was lower than obtained from previous studies [18, 19]. LOD and LOQ values in our study and literature were shown in Table-2. Table-3 and 4 include precision and accuracy data for the methods at low, medium and high concentrations. Intra-assay precision of both methods proved to be less than 10.9% and accuracy less than 11.48% in the linear range of the assay. When compared with other methods, these methods have the advantage of having higher sensitivity, lower cost, no interference and use of a less complicated system. Other studies report the mean of OHP excretion in urine as 26±0.98 μg mL^{-1} [18], 33±6.45 μg mL^{-1} [24] and 38 μg mL^{-1} [19]. In our study, the mean of OHP for non smokers and smokers were estimated as 36.8±13.6 μg mL^{-1} and 45.5±19.0 μg mL^{-1} and, respectively.

This study was carried out using urine samples taken from groups of non-smokers (n=24) and smokers (n=24). Groups were established by taking into consideration the gender, age and health of participants. We found no significant gender related difference, as already reported by other authors [18, 25]. The average age of non-smokers was between 25- 40 (Median 29) and that of smokers was 24-39 (Median 31.5). Health was considered because diseases with lung injury (for example, lung fibrosis, emphysema, lung cancer) have high levels of urinary OHP. Collagen is a major constituent of the lungs. Hydroxyproline is secondary and predominant amino acid present in collagen which is released with breakdown of collagen and not recycled to form new collagen. Published results show that urinary hydroxyproline is a biochemical marker which indicates the breakdown of lung by smoking [5].

The hydroxyproline in the urine can be a biomarker of many different biochemical events and diseases. A study showed that hydroxyproline (OHP)

is a sign of collagen degradation by high performance liquid chromatography [26]. Hydroxyproline has been considered as a biomarker of other diseases. 4-hydroxyproline (OHP) in urine have been suggested as disease biomarkers for bone turnover and osteoporosis [27]. One of the purpose of our study was also to compare the amounts of hydroxyproline in the urine of non-smokers and smokers. Our results, supported by our methods, showed that the ratio of OHP/creatinine was significantly lower in the group of non-smokers than in the group of smokers ($p<0.05$). There are many factors affecting the ratio and amount of hydroxyproline in the urine.

The urinary OHP level can be affected by many metabolic ways. One of these metabolic ways is related to oxygen. Oxygen is required for hydroxylation of proline to hydroxyproline [28]. Tissue oxygen has been reduced by smoking so this total hydroxyproline level may change. Hydrogen cyanide also inhibits oxygen delivery at the cellular level. Thirty minutes after smoking, there is an average decrease in oxygen tension. Collagen accumulation in wounds can be limited by this tissue oxygen tension [29, 30]. Furthermore, smoking induces oxidative stress and this promotes a faster catabolism of ascorbic acid (vitamin C). By the decrease of ascorbic acid, the enzymatic hydroxylation of the proline was effected. Ascorbic acid behaves like a cofactor of this reaction [31, 32]. It is clear that oxidative stress is also related to OHP level. Additionally, cadmium, which is exposed with cigarette, has been found to depress the procollagen production in fibroblasts [29]. These reasons demonstrate that urinary OHP level is effected by smoking. In our opinion the effect of smoking on lung collagen could be demonstrated by measuring hydroxyproline level in urine.

Changes in the ratio of hydroxyproline/creatinine could be caused by changes in the concentration of the urinary hydroxyproline or creatinine. Some investigators report that when smokers' creatinine serum levels were compared to non-smokers, low urinary creatinine concentrations could be observed in smokers [5, 33]. However, in addition to studied and given creatinine measurements, the association between urinary creatinine concentration and smoking was also studied in our study, and no statistical difference for urinary creatinine was found between smokers and non smokers ($p>0.05$). Also, high amounts of urinary OHP from bone and skin degradation can conceal the degraded hydroxyproline originating from the lungs. There are a large number of factors that influences OHP level in urine. The level of hydroxyproline in urine can be affected by diet, disease and degradation of collagen in other tissues [7-9]. For these reasons, it is difficult to determine the source of the urinary OHP. However, in this study the known difference between two groups was smoking. Thus, the others factors that might affect urinary hydroxyproline levels were accepted as the same and the change in measured urinary hydroxyproline was associated with smoking. In a study conducted in 2002, OHP levels were approximately doubled in the smoke-only group compared with the control group [34].

Derivatizing agents FDNDEA and NBD-Cl allow components to be detected by ultraviolet and electrochemical detectors. FDNDEA is a pre-column derivatizing agent used for analysis of amino acids [35, 36]. The advantages of FDNDEA as a derivatization agent are that it is connected by a simple reaction step, it forms a stable structure, and the step of disconnection after attachment is not necessary. In this technique, UV detection at 360 nm removes the fundamental problems that may occur when working in shorter wave lengths in degradation systems. In addition, it has been reported that the derivative is stable and insensitive to light [35]. The other derivatizing agent NBD chloride is useful for detecting small quantities of amines and amino acids. These derivatives have been reported to be stable and insensitive to light [37]. Verification trials yielded satisfactory results indicating reliable methods for determining total urine OHP.

Our studies were originally focused on determining the hydroxyproline concentration in relation to smoking by two different HPLC detection techniques; ultraviolet (UVD) and electrochemical detectors (ECD). Main differences of UVD method and ECD method are derivatizing agents, derivatizing temperature and detection. In the UVD method; we used FDNDEA as the derivatizing agent which reacted at 100˚C, and detection was made at 360 nm. In the ECD method; we used NBD-Cl as the derivatizing agent which reacted at 115˚C, and detection was made at 350+ and 640+ mV. We have investigated and compared the urinary OHP excretion in smokers (n=24) and non-smokers (n=24). This study is the first study which includes sample preparation for hydroxyproline concentration and analyzing by two different methods.

Conclusions

We have developed high throughput methods for simultaneous quantification of urinary hydroxyproline. The methods were rapid, sensitive, accurate and simple. A strong, positive and linear

correlation was found between these methods ($p<0.01$, $r=+0.98$). Our results supported by these methods showed that the ratio of OHP/creatinine were significantly lower in the group of non-smokers ($p<0.05$). When low, moderate and heavy smokers' urinary OHP levels were compared with each other, the results showed that heavy smokers had different levels compared to those from the low and moderate smokers groups ($p<0.01$).

This result support the idea that hydroxyproline may be a biomarker that can be used as a lung destruction product due to smoking. These simple sensitive, cheap, fast and highly reproducible UV and ECD methods, which were developed and validated, have the potential to become a new clinical routine method for evaluating collagen degradation in lungs with smoking.

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Compliance with Ethical Standards: Conflict of interest

Author Betül İŞİNER KAYA declares that she has no conflict of interest. Author Emrah DURAL declares that he has no conflict of interest. Author Erdal KENDÜZLER declares that he has no conflict of interest. Author Tülin SÖYLEMEZOĞLU declares that she has no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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