Development of a Simple and Rapid ELISA of Urinary Cotinine for Epidemiological Application

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Abstract

Highly stereospecific polyclonal antibodies (anti-CN) to cotinine (CN), a major metabolite of nicotine, were prepared from rabbit antisera to CN-linked keyhole limpet hemocyanin (KLH) by removing the antibodies to KLH and to its binding regions of CN. This was achieved by using immunoadsorbents consisting of insolubilized KLH onto CNBr-activated-Sepharose 4B. A new simple and rapid enzyme-linked immunosorbent assay (ELISA) of urinary CN was developed using the anti-CN. A brief outline of the method is as follows: CN-bovine thyroglobulin complex is coated onto wells of microtiter plates (1 ng/well), and then aliquots of urine samples or standard CN solutions were added followed by appropriate dilution of the anti-CN. The bound anti-CN antibodies are quantified spectrophotometrically with horseradish peroxidase-labelled anti-rabbit IgG and 2,2'-azino-di (3-ethylbenz-thiazoline)-6-sulfonic acid. Measurement of CN concentration in urine samples can be read off on a calibration curve drawn by using standard CN solutions. The standard curve ranged from 1 ng to 4 μ g/ml with an estimated lower limit of sensitivity of 7-8 ng/ml, resulting in within/between-assay CV (coefficient of variation) of lower than 10%. The method allowed one to assay more than 40 samples in duplicate by using just one plate, and is thus easily applicable to epidemiological investigations into exposure status to tobacco smoke.

Key words: Urinary cotinine, ELISA, Exposure status to tobacco-smoke, Epidemiological application.

Introduction

Smoking enhances the risk of suffering from cancers of the lung and other organs, and from other various diseases such as ischemic heart diseases, pulmonary disorders, and gastrointestinal disorders^{1,2)}. Tobacco smoke contains more than 4000 chemicals such as nicotine, several established carcinogenic chemicals, ciliary injuring substances, CO, and various other harmful substances to health. Mainstream smoke (smoke actively inhaled) and sidestream smoke (smoke released in ambient air from cigarettes between active puffs) have similarities in composition³⁾, and in fact exposure to environmental tobacco smoke (ETS) has been linked to a number of adverse health effects in children and adults³⁾.

The majority of estimates of exposure status to tobacco

smoke in epidemiological studies have been based on questionnaire data, which is considered to be less accurate mainly because of difficulties in estimating ETS^{4,5)}. The availability of biochemical markers for tobacco smoke has recently provided researchers with a more direct measure of exposure. Cotinine (CN), a major metabolite of nicotine⁶⁾, is present in the blood, urine, and saliva of individuals who smoke or who inhale tobacco smoke produced by others. As an objective indicator of nicotine intake or confirmation of nonsmoker status, CN offers several advantages over other biochemical markers: it is a specific indicator of nicotine intake, its concentrations are not influenced by confounding factors such as diet or environment, its average biological half-life in blood is 19 hrs., and its concentration within a given individual varies by 15 to 20% over the course of a day⁷⁾. CN assay is thus a superior objective measure of exposure to nicotine^{5,8-10)}. Urine and saliva are particularly suited for epidemiological investigations, since they do not require invasive sampling techniques. CN has been measured by high performance liquid chromatography^{11,12)}, gas chromatography^{13,14)}, and radioimmunoassay^{15,16)}, however, these techniques require extensive processing of samples prior to analysis or special isotopic equipment. Although enzyme immunoassays using

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specific polyclonal and/or monoclonal antibodies have been recently developed for the measurement of CN by Langone groups¹⁷⁻¹⁹⁾ and has been gradually available in the USA, no epidemiological studies employing ELISA were found in Japan.

The present investigation was therefore undertaken to develop a rapid, simple and quantitative method to measure urinary CN with polyclonal antibodies; the method does not require strictly sterile techniques and is suitable for epidemiological application to masses of people.

Materials and Methods

1. Chemicals

Reagents used were as follows: 4'-carboxycotinine (4'-carboxy-CN) (Tokyo-Kasei Kogyo Co., Tokyo, Japan); nicotine (Maruwaka Kogyo Co., Osaka, Japan); keyhole limpet hemocyanin (KLH) and bovine thyroglobulin (TG) (SIGMA Chemical Co., MO, USA); 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCl (EDAC, Nacalai tesque, Kyoto, Japan); Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) (DIFCO Lab., MI, USA); CNBr-activated Sepharose Cl-4B (Pharmacia LKB, Uppsala, Sweden); polyethylene sorbitan monolaurate (Tween 20, Nacalai tesque, Kyoto, Japan); donkey anti-rabbit IgG antibody fractions coupled with horseradish peroxidase (HRP-IgG) (Chemicon International, Inc., Funakoshi Co., Tokyo, Japan); 2,2'-azino-di (3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) (Boehringer Mannheim, GmbH, Germany).

2. Preparation of protein coupled with CN

KLH and/or TG were incubated with 4'-carboxy-CN at a weight ratio of protein to 4'-carboxy-CN (1 : 50) by using EDAC (pH 5.0 to 6.0) at room temperature for 2 h and then at 4°C overnight. Unbound CN from CN bound KLH (CN-KLH) or TG (CN-TG) was removed by precipitating the solution with 50% saturated ammonium sulfate. Numbers of CN molecules coupled to each KLH or TG molecule were estimated spectrophotometrically by using the extinction coefficient of $A_{278\text{nm}}$ of each protein and $A_{262\text{nm}}$ of CN, and was found to be 150-200 and 50-100 molecules, respectively.

3. Production of polyclonal anti-CN-KLH antiserum

The rabbits were immunized on day 0 with 2 ml of CN-KLH (500 µg) in FCA subcutaneously. The rabbits were boosted on day 21 with gluteal and nuchal subcutaneous injections totalling 2 ml of CN-KLH (200 µg) in FIA, and on days 35, 36 and 37 with intravenous injections of 0.1, 1.0 and 1.0 ml, respectively, of CN-KLH (100 µg/ml) in physiological phosphate-buffered saline (PBS). Additional booster doses followed, consisting of 2 ml of CN-KLH (200 µg) in FIA in multiple subcutaneous injections, 4 and 7 weeks later. The rabbits were bled via their auricular vein 14 days after the last booster injection.

4. Preparation of monospecific anti-CN-antibody fraction

CNBr-activated Sepharose Cl-4B was hydrated in 1 mM HCl and transferred to 0.5 M NaCl in 0.1 M Na-carbonate buffer, pH 8.3. The activated beads were placed in reaction with KLH (10 mg/ml in the same buffered solution) overnight at 4°C with end-over-end rotation. The KLH-coupled beads were

washed successively with 0.5 M NaCl in 0.1 M acetate buffer, pH 4.0, and with 0.5 M NaCl in 0.1 M Na-carbonate buffer, pH 10.0. After blocking the unsaturated groups on the beads with 1 M glycine, the beads were washed again and packed into a small column in PBS. The rabbit anti-CN-KLH antisera were applied onto the prepared column, and the collected fraction was pooled. The same absorption procedure was repeated 2 or 3 times more until no detectable anti-KLH was found in the fraction. The finally prepared anti-KLH free fraction was kept at -80°C and used as anti-CN antibody fraction (anti-CN).

5. ELISA of urinary cotinine

After coating CN-TG (1 ng as CN) to each well of Falcon microtiter-plates (Becton Dickinson, NJ., USA), the free binding sites were blocked with 1% bovine serum albumin at 37°C for 2 h. The plates were washed 3 times with 0.05% Tween 20 in PBS. Initially, urine (100 µl) in equal amounts to standard CN solutions (100 µl, 1-4000 ng/ml as 4'-carboxy-CN) was added and then 100 µl of appropriately diluted anti-CN was subsequently added. After washing 4 times with 0.05% Tween 20 in PBS subsequent to the incubation at 37°C for 2 h, 100 µl (per well) of appropriately diluted HRP-IgG were added, and the plate was incubated at 37°C for 2 h. After washing 4 times with 0.05% Tween 20 in PBS, color was developed by adding 100 µl (per well) of 1 mM ABTS. The color development was stopped with 0.1% NaN₃ in 0.1 M citric acid, and the absorbance at 415 and 475 nm was measured on a Chromatoscanner CS-930 (Shimazu Co., Kyoto, Japan).

6. Study population

Having obtained informed consent, a total number of 301 employees (111 males: age 25 to 67 years with a mean of 29 years and 190 females: age 20 to 61 years with a mean of 36 years) from a department store were chosen. A questionnaire for each subject containing fundamental items such as age, gender, assigned work, and smoking status such as active or passive smoker was given out at a regular physical examination. After filling in the questions on the spot, the questionnaire and an aliquot of urine were collected. They were divided into 3 groups (A: 88 active smokers, B: 48 passive smokers who are not active smokers but feel they are exposed to tobacco smoke passively, and C: 165 non-smokers who feel they are not exposed to any tobacco smoke). The urine samples were stored at -80°C until assayed.

7. Statistical analysis

The coefficient of variation (CV), percentiles and the cumulative relative frequency were calculated according to the methods described by Dawson-Saunders and Trapp²⁰⁾. A statistical comparison of storage conditions of urine samples was performed by use of the Wilcoxon rank sum test²⁰⁾.

Results

1. Sensitivity and specificity

Assay sensitivity was determined by measuring the ability of known amounts of CN that can inhibit anti-CN binding to immobilized CN in the wells of microtiter plates. On the basis of this curve, a typical assay was done in triplicate (Fig. 1), only 165 ng/ml cotinine gave 50% inhibition and as little as 4-8 ng/ml could still be detected reliably (10-15% inhibition). In contrast,

Fig. 1 Inhibition of anti-CN binding to immobilized CN in wells of microtiter plates by 4'-carboxy-CN (○—○) or nicotine (□—□). The assays were done in triplicate.

the curve for nicotine (Fig. 1) shows that it is not a significant competitor with little inhibition through the range tested.

2. Influence of dilution of urinary samples on CN values

The actual assay values of five serially diluted urine samples (1/2-1/32 dilutions with distilled water) were compared with the predicted values assessed on two undiluted urine samples (CN content: 400 ng/ml and 328 ng/ml) (Fig. 2). Linearity was satisfactory over the range tested.

3. Reproducibility of CN levels within/between assays

Reproducibility of CN levels was assessed by comparing the means of replicates of 3 different urinary samples (7, 118 and 986 ng/ml of CN) and 3 standard CN solutions (5, 100 and 2000 ng/ml of 4'-carboxy-CN) taken from 10 separate assays on the same day. The within-assay CV's ranged from 4.8 to 9.7% for CN concentrations tested (Table 1). Furthermore, CN levels of 4 different urinary samples (141, 201, 255 and 432 ng/ml of CN) were separately assayed on 5 successive days, and the between-assay CV's ranged from 4.6 to 8.9% for CN concentrations tested (Table 1).

4. Influence of addition of external CN to urinary samples

4'-carboxy-CN (at concentrations of 10, 50 and 200 ng/ml) was added to urinary samples containing 153 and/or 856 ng/ml

Fig. 2 Linearity of diluted urine samples. A closed circle and a square represent the CN values of undiluted urine samples containing CN of 400 ng/ml and 328 ng/ml, respectively. Open circles and squares represent the CN values of diluted urine samples (1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32) from the respective original undiluted urine samples.

of CN, and then CN levels were assayed. Table 2 shows the actual assay values in accordance with those predicted from the calculated sum of internal and external CN in a typical assay. The differential rates between the actual values and the calculated sums were computed to be from 98.5% to 105.7%, which shows that the differences were usually less than 10% (data not shown).

5. Influence of storage of urinary samples on CN values

The CN of urine samples after storage under various conditions (5 samples for each storage condition) was measured, and the recovery ranges are shown in Table 3. Storage of urine samples at 4°C (up to 7 days), at -20°C or at -80°C for 2 months, remained stable with high reproducibility ($p > 0.05$). Although the loss of CN levels was considerable (more than 10% in some samples, $p < 0.01$) after storage at -20°C for 2 months and then at 4°C for 1-7 days, the CN levels remained relatively stable after storage at -80°C for 2 months and then at 4°C up to 7 days.

6. Relationship between the questionnaire findings on exposure to tobacco smoke and actual urine CN concentrations

A total of 301 employees of a department store was divided into 3 groups (A: 88 active smokers, B: 48 passive smokers who are not active smokers but feel they are exposed to tobacco smoke passively, and C: 165 non-smokers who feel they are not exposed to any tobacco smoke) based on their questionnaires. Urine CN concentrations were assayed by the ELISA method as described

Table 1 Reproducibility of urinary cotinine (CN) levels within/between assays.

Intra-assay ¹		Inter-assay ²	
CN, ng/ml	CV, %	CN, ng/ml	CV, %
Urinary samples		Urinary samples	
7	6.1	141	4.6
118	8.3	201	5.0
986	7.9	255	7.6
		432	8.9
Standard solution			
5	5.5		
100	4.8		
2000	9.7		

¹ Values from 10 separate assays on the same day

² Values from separate assays on 5 successive days

Table 2 Recovery of cotinine (CN) added to urinary samples.

Original CN in urine, ng/ml	CN added*, ng/ml	CN measured, ng/ml	Recovery, %
153	0	153	100.0
153	10	172	105.5
153	50	208	102.5
153	200	373	105.7
856	0	856	100.0
856	10	853	98.5
856	50	915	101.0
856	200	1050	99.4

*CN was added as 4'-carboxy-CN

Fig. 3 Relationship between the extent of the subjects' self-recognition that they are exposed to tobacco smoke and their actual urinary CN concentrations.

A: non-smokers who feel they are not exposed to any tobacco smoke (●); B: passive smokers who are not active smokers but feel they are exposed to tobacco smoke passively (■); C: active smokers (▲).

above (Fig. 3). The median, the 25, and the 75 percentile of the distribution of non-smokers, passive smokers and active smokers are 10 [2.5, 28], 95 [40, 145], 1800 [1300, 3600] ng/ml, respectively.

Discussion

In the present investigation, we developed a rapid and simple ELISA method for CN assay in quantifying the exposure status to ETS using rabbit polyclonal anti-CN antibodies. Absorption processes of the carrier protein (KLH) and the bridge antibodies which are directed against the linkage regions of KLH to CN were carried out, thereby attempting to make the antisera to CN-KLH highly specific: the anti-CN antibodies did not react with nicotine. The method was highly sensitive with a measurement range of about 4-8 to 3000 ng/ml (Fig. 1). Although the lower limit of our method seems to be less sensitive than that of the ELISA with monoclonal anti-CN¹⁷⁻¹⁹⁾, our method can be performed more rapidly because it has only 3 major steps instead 4-5 steps in the latter methods, and our method, furthermore, is deduced to be more reliable in antigen specificities because there is no use of protein A or biotin etc. Additionally, our method requires neither strict sterile special equipment nor advanced techniques which are requisite for the production of monoclonal antibodies. In addition, our method has been found to be sufficient for practical use for epidemiological investigations. In fact, the use of urine in a quantity of only 100 μ l without the step of sample dilutions for routine assay is reliable and practical. Furthermore, the method presented here has sufficiently high reproducibility and quantitateness within/between assays following the treatment of addition or dilution (Table 1, 2 and Fig.2).

In the present investigation, urine was used as test samples

Table 3 Recovery of cotinine (CN) after storage under various conditions.

Storage conditions	Recovery ranges*, %	Significance
at 4 °C, 24 h	95-101	NS
3 days	98-103	NS
5 days	96-106	NS
7 days	100-106	NS
at -20 °C, 2 mo	98-108	NS
at -80 °C, 2 mo	97-105	NS
at -20 °C(2 mo) then 4 °C(24 h)	87-96	p < 0.01
then 4 °C(3 days)	86-98	p < 0.01
then 4 °C(5 days)	84-93	p < 0.01
then 4 °C(7 days)	85-97	p < 0.01
at -80 °C(2 mo) then 4 °C(24 h)	93-102	NS
then 4 °C(3 days)	93-99	NS
then 4 °C(7 days)	98-103	NS

* CN values were assayed in 5 aliquots of the same urine samples stored under the same conditions.

NS: statistically not significant with p > 0.05

which involved no invasive techniques. This is a great advantage in epidemiology studies. CN in urine samples was found to be stable in storage at 4 °C up to 7 days, at -20 °C and -80 °C up to 2 months (Table3). The storage at -20 °C and then at 4 °C up to 7 days seems to decrease the CN levels considerably. This is most likely due to some difficulties in melting the urine homogeneously after -20 °C storage (the frozen state under this condition seemed to be unhomogenous presumably due to slow freezing and CN seemed to be absorbed on urinary insoluble materials).

The present investigation also showed considerable discrepancies between the extent of self-recognition by study subjects on exposure to ETS and their actual urinary CN concentration (Fig. 3), which is consistent with previous reports^{4,5)}. Direct measurement of ETS, e.g. CN assay in body fluids, is, therefore, of increasing importance in assessing both active and passive intake of nicotine for studies on effects of smoking on health. The method presented here allows one to assay more than 40 samples/plate in duplicate, and is thus easily applicable to epidemiological investigations into exposure status to tobacco smoke, in promoting health and in preventing diseases in the field of preventive medicine.

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