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Identification of 6-Furfuryladenine (Kinetin) in Human Urine

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In contrast to the current view of kinetin (K, N^6-furfuryladenine) as an unnatural and synthetic cytokinin, recently it has been identified in plant DNA and plant extract. Here we describe identification of K in human urine using chromatography/mass-spectrometry analysis for the first time. The amount of kinetin in urine taken from unhealthy patients lung carcinoma was established to be 0.5 ng in 20 ml and a 100-fold reduced amount in healthy subjects. Since this rare base is a potential source of structural constrains it has to be removed from DNA by enzymatic DNA-repair reactions. It seems that the presence of kinetin in human is linked to oxidative damage processes.

Key Words: **kinetin; cytokinin; animal tissues; urine analysis.**

Cytokinin is the generic name used to designate plant growth substances, which constitute a collection of naturally occurring N6 substituted adenines (1, 2). These hormones are involved in cell division, differentiation, and other physiological processes. The model of cytokinin regulation in plant cells has been proposed (3–5).

About forty years ago kinetin (K) was isolated from autoclaved herring sperm DNA and shown to act as a plant growth hormone (6). Although kinetin was originally purified from DNA, it has been assumed to be an artificial product of its rearrangement and not to occur naturally (7). Since K discovery this compound has been widely used as a cytokinin in various aspects of plant research, including applications in biotechnology and cell biology (2, 3). Some years ago, kinetin has been shown to delay the onset of many age-related characteristic that appear in normal human skin fibroblasts undergoing ageing *in vitro* (8, 9).

Recently, the presence of kinetin in an extract of the root nodules of *Casuarina equisetifolia*, a multipurpose tree produced by the inoculation of *Frankia* have been reported (10, 11). Actinomyces that make up the genus *Frankia* are distinguished by their ability to induce N-fixing root nodules on certain nonleguminous plant (10). Also palmarosa (*Cymbopogon martinii* var. *motia*) roots contain 6-furfuryladenine which increases the amount significantly after inoculation of *Glomus* species (*G. aggregatum*, *G. fasciculatum*, *G. intraradix*, and *G. mosseae*) (11).

The new findings of kinetin in DNA and the cell extracts (7) raised an obvious question about its synthesis pathway (12). Furfural has been suggested to be a putative precursor of kinetin and is formed during hydroxyl radical oxidation C5′ of deoxyribose in DNA (13–17). The presence of that aldehyde in the extracts of various cells was confirmed by its reaction with 0-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (18). Once furfural is formed in the vicinity of DNA it can efficiently react with the exocyclic amino groups of DNA components and to form the Schiff base with adenine residue, and possibly also cytosine moieties [J.B., unpublished]. This bulky substituent induces some structural constrains and should be enzymatically repaired in DNA (19). A reaction of furfural with plasmid DNA and AT-rich oligonucleotides leads to the destabilisation of DNA secondary structure. Although reaction products have not been analysed, this observation suggests the formation of a large modification of adenosine residues in DNA (20, 21). To learn more about the role of kinetin we are looking for new examples of its occurrence.

In this paper we analyse a presence of it in human urine.

MATERIALS AND METHODS

The analysis was carried out on Hewlett Packard gas chromatograph model 5980/II equipped with mass selective (GC/MS) detector model

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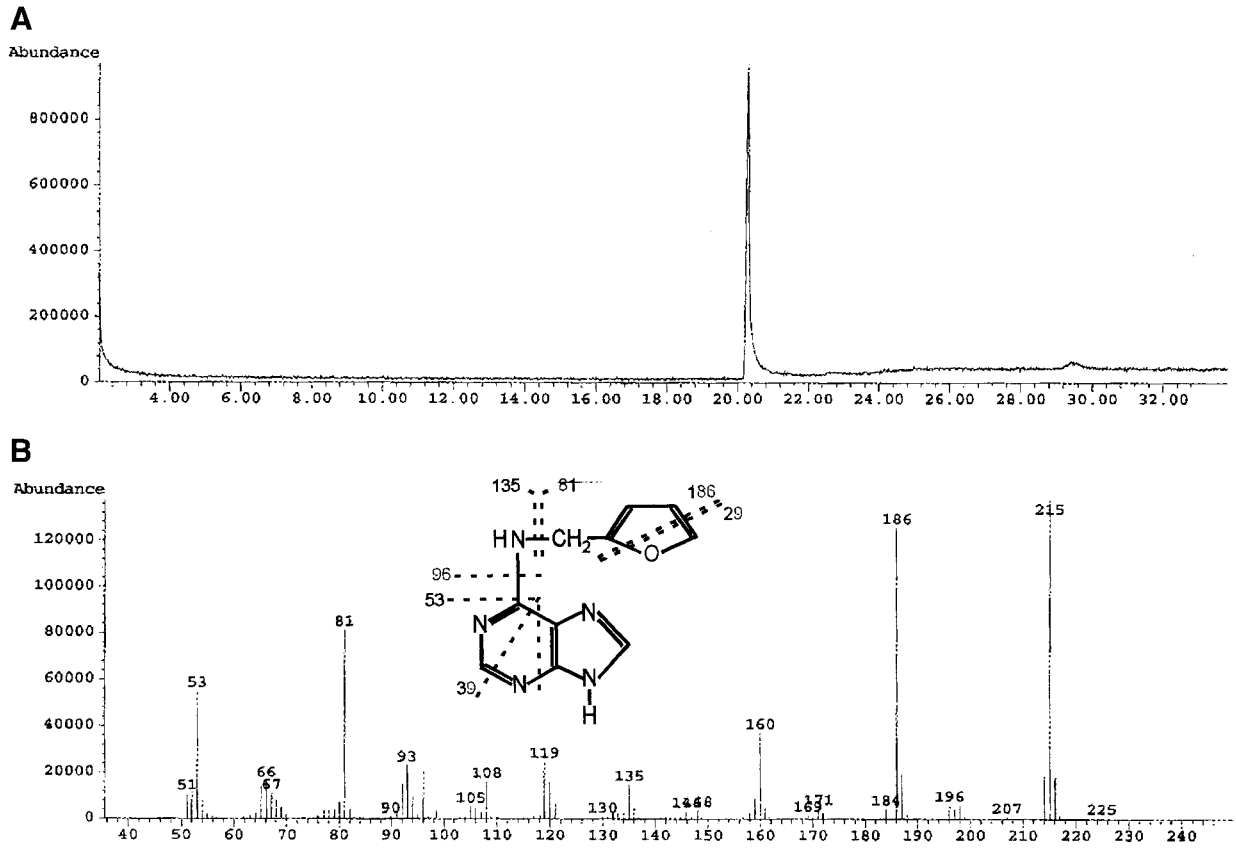

FIG. 1. Gas chromatography/mass spectra (GC/MS) analysis of kinetin. (A) The GC chromatogram shows peak with Rf 20.36, corresponding to kinetin (commercial product). (B) Electron impact spectrum of kinetin. Mass signals of 215, 186, and 81 *m/z* were used to an kinetin identification.

5071A and fused to silicon capillary column DB-5 from J&W (30 m × 0.25 mm internal diameter). Helium at flow rate 1 ml/min was used as carrier. The column temperature gradient was programmed from 140°C (held for 2 min) at 5°C/min to 300°C which was held for 5 min. The injections were made in split mode (1:10) at temperature of 250°C. For quantification of kinetin integration of two main ion signals at 215 and 186 *m/z* in mass spectrum was done. Calibration was performed for sample quantities between 100 and 300 ng. Each sample was injected twice. Single ion chromatography was used to identify mass signals characteristic to kinetin and to its quantitative analysis.

Kinetin was bought from Sigma. Human urine was collected from patients at F. Raszei Municipal Hospital in Poznan, Poland and stored at −20°C. Then 20 ml was acidified to pH 1–1.5 with HCl and incubated for two 2 h at 55°C. After adjustment of pH to 4–5.5 kinetin was absorbed on a C-18 cartridge, activated and eluted with methanol. After evaporation under nitrogen the sample was dissolved in pyridine and analysed on GC/MS.

RESULTS AND DISCUSSION

Kinetin has remarkable biological properties, which include stimulation of plant growth, retardation of leaf senescence, and modulation of the plant response to various environmental stresses (1, 2). It also delays the onset of ageing in human cell cultures (8, 9) and in insects (22, 23). Until now kinetin was believed to be an artefactual cytokinin originating from the autoclaving of herring-sperm DNA (6) or formed on DNA storage over a long period of time (7). Recently we have used high-performance liquid chromatography combined with an electrochemical detector to analyse kinetin as a natural component both of DNA and a plant cell extract (12, 24). The same type of approach showed that K is present in commercially available calf thymus DNA and in freshly extracted DNA from human cell cultures (24). The proposed mechanism for the 6-furfuryladenine formation (12) is consistent with all the available data and links the modified base with oxidative damage processes occurring in the cells for the first time. Thus, it can be recognised as a marker of DNA damage. In this paper we analysed the presence of kinetin in human urine using gas chromatography linked to mass spectrometry system.

The commercial sample of K yields a single GC peak (Fig. 1A) and gave m/e signal of 215 units and two other characteristic mass signals at m/e 186 and 81 (Fig. 1B). The m/e 186 corresponds with the fragmentation of a five-member ring sugar by releasing 29 mass units and the latter is specific to breaking the N-C bond in the side chain of 6-furfuryladenine and releasing the furfuryl group (Fig. 1B).

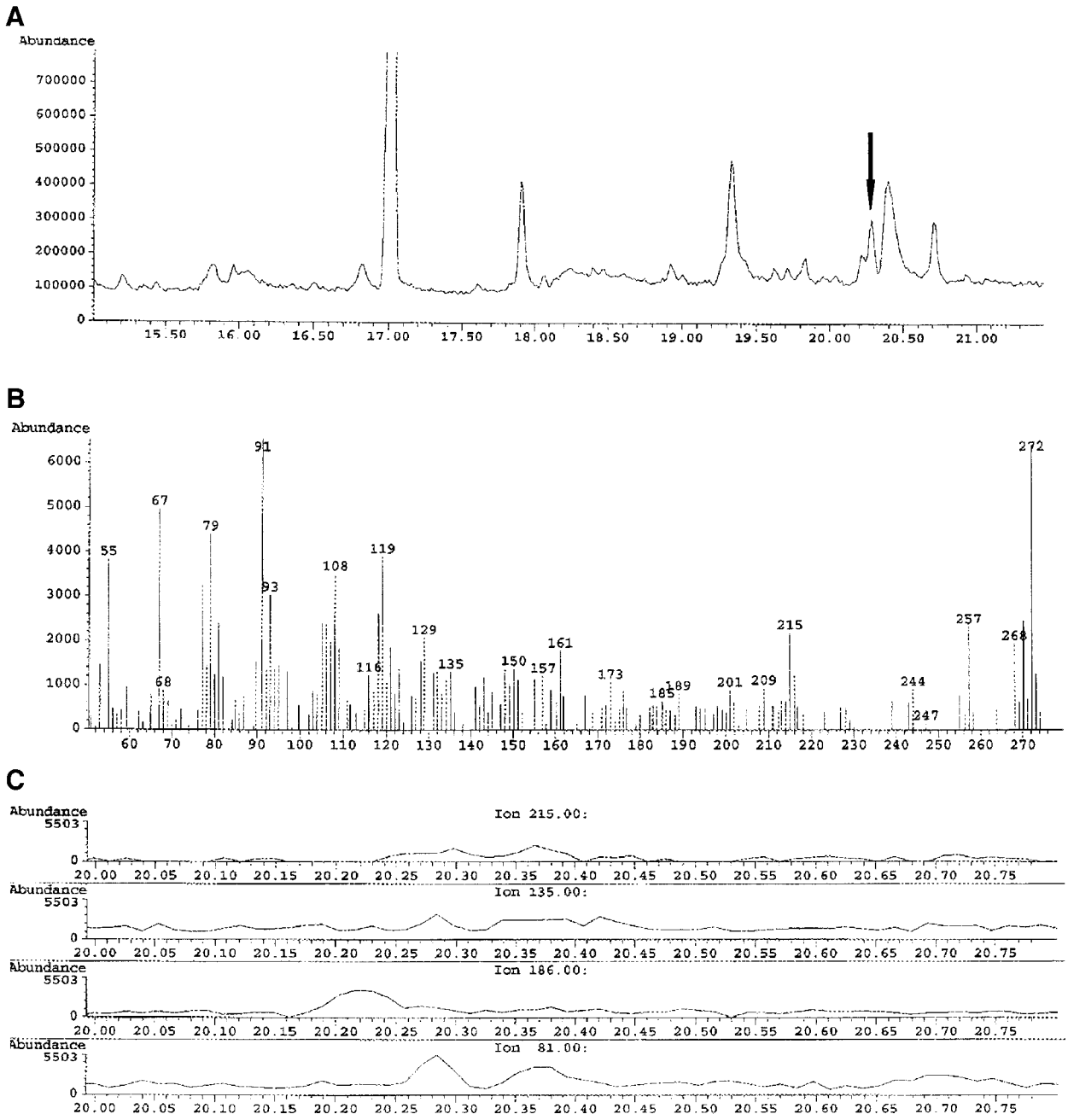

FIG. 2. GC/MS analysis of kinetin in human urine. (A) GC of human urine. The peak marked with arrow corresponds to K appeared at Rf 20.36. (B) MS of human urine shows the ms signals of 81, 135, 186, and 215 *m/z*. (C) An integration of ion signals of 215, 186, 135, 81 *m/z* derived from the compound with Rf 20.36.

The gas chromatography spectrum of human urine shows peak shifted to Rf 20.36 (Fig. 2A). Precise identification of kinetin was possible after its coinjection (Fig. 3). The mass spectrometry analysis (Fig. 2B) clearly confirmed a presence of 6-furfuryl adenine in urine. Identity of the modified DNA base was proved by appearance of a set of mass signals of 215, 186, 135, and 81 derived from the GC peak Rf 20.36. To establish a reliable method for K analysis in natural products, a quantitative relationship of the signal area and amount of K was obtained (data not shown). Integration of two peaks provided very similar data. Using the standard curve we established amount of K in urine of unhealthy patients with diagnosed cancer to be 0.5 ng in 20 ml of urine, but in healthy subjects is 10–100 times less. This analysis was done for 20 patients. Assuming 1.5 l of urine from a person per day, it means that the affected produced 38 ng (174 pmol/kg/day) of

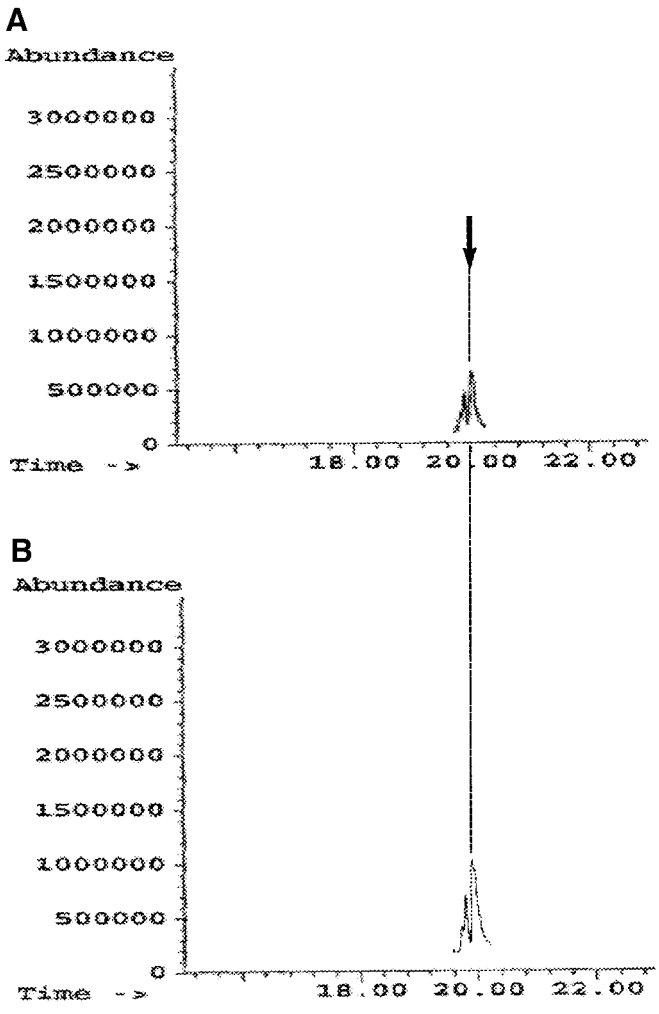

FIG. 3. Gas chromatography analysis of human urine (A) enriched with 2.8 ng kinetin (B).

K. Interestingly a total amount of 8OH-dG obtained from normal subjects was up to 300 pmol/kg/day (25). It means that the level of 8OH-dG is approximately two order of magnitude higher than kinetin. It contents in healthy patient was within the limits of detection. This is reasonable because 8OH-dG, the primary product of the nucleic acids reaction of with hydroxyl radical but kinetin is a secondary product of DNA damage.

A presence of free kinetin in coconut also suggests its enzymatic repair as we could isolate it from plant extract (12). One of repair enzymes could be a eukaryotic DNA polymerase β, which catalyses DNA synthesis during base-excision repair and also the release of 5′-terminal deoxyribose phosphate residues from incised apurinic-apyrimidic sites (12).

Finding of kinetin in urine support a view that repaired molecule can induce a synthesis of enzymes which remove modified bases from DNA and/or is involved in protection against oxygen stress (hormesis) (26). It is known that the kinetin–Cu(II) complex very efficiently catalyses O^{2-} dismutation at physiological pH (27). These properties of kinetin help us to understand why kinetin, added exogenously to various cells, has several positive effects including anti-ageing properties (28).

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