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Diagnostic methods for identifying aristolochic acid in biological samples: Narrative Review.

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Abstract

Introduction: Aristolochic acid (AA) is derived from the *Aristolochia* family and has been widely used as herbal medicine for treating various diseases. However, the International Agency for Research on Cancer and the European Medicines Agency (EMA) have classified aristolochic acid as a Group 1 carcinogen due to its nephrotoxicity and carcinogenic properties, thereby restricting its use.

Objective of the review: This article is a narrative review whose objective is to carry out a systematic review of the diagnostic methods for detecting aristolochic acid in the body. The goal is to establish a more precise diagnosis regarding the pathologies induced by its consumption.

Important points: During the biotransformation of AA, AA-DNA adducts are formed, which are highly mutagenic and inhibit DNA replication. These adducts are recognized as specific biomarkers. Among the methods for identifying aristolochic acid are: next-generation sequencing (WGS), dual spectroscopic strategy, UPLC-MS/MS, terminal transferase-dependent PCR, and parametric magnetic resonance.

Conclusion: These methods offer experimental approaches that are relevant to the scientific community. However, executing these methods continues to be challenging due to the resources needed .

Keywords:

Aristolochic acid, nephropathy, carcinoma, diagnostic methods.

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Aristolochic acid (AA) is a member of the nitrophenanthrene family of carboxylic acids that is abundant in plants of the Aristolochia family [1]. Its use dates back thousands of years, with the most essential record being in the 1990s, since it was used as an herbal medicine for weight loss, arthritis, inflammation, and gout. However, a considerable percentage of people who consume it develop kidney damage [2]. In the United States, the International Agency for Research on Cancer and the European Agency for the Evaluation of Medicines established aristolochic acid as a group 1 carcinogen because of its nephrotoxicity and carcinogenicity; thus, the consumption of medications containing this compound has been prohibited [3, 4].

The biotransformation of aristolochic acid is a complex process in which DNA undergoes multiple mutations and loses suppressor genes. Aristolochic acid possesses a mutational signature known as COSMIC 22, characterized by nonreversible transversions that cause continuous damage and affect multiple organs [3, 4]. Owing to its high toxicity, various investigations have been conducted to establish diagnostic methods for identifying this acid in biological samples from individuals and animals with suspected disease. However, the development of more extensive studies has been hindered by the need for substantial resources.

Considering the high consumption of medicinal herbs containing AA and the continuous increase in patients with nephrological diseases, the importance of acquiring knowledge about diagnostic methods is highlighted. This would allow their subsequent application to guarantee accurate and timely diagnoses.

A literature review was conducted through searches in PubMed, Scopus, the Wiley Online Library, Springer Nature, and Google Scholar, using terms such as aristolochic acid, diagnostic methods/diagnoses, and the reference lists of relevant articles. Studies published in English and Spanish from 1990 to the present were included. Moreover, the information gathered from these databases is primarily experimental since there is no established protocol for identifying aristolochic acid. After a substantial amount of clinical and scientific research is reviewed, the opinions presented reflect a consensus.

Biotransformation

Studies have shown that 8-methoxy-6-nitro-phenanthro-[3,4-d]-1,3-dioxole-5-carboxylic acid (aristolochic acid I, AAI) and its 8-demethoxylated derivative (aristolochic acid II, AAII) [5], which are found in several herbaceous plants, are responsible for aristolochic acid nephropathy (AAN) [5-8].

The biotransformation of AA has been investigated by analyzing tissues from patients with AAN [5, 9]. The major aristolochic acid-DNA adducts (AA-DNA) found in AA-exposed rodents and patients suffering from AAN were identified as 7-(deoxyadenosine-N-6-yl) aristo lactam I (dA-AAI), 7-(deoxyguanosine-N-6-yl) aristo lactam I (dG-AAI), and 7-(deoxyadenosine-N-6-yl) aristo lactam II (dA-AAII) [10].

In phase I, AA is transformed into N-hydroxyaristolactams through nitroreduction and then converted into the nitrenium ion aristolactam, which binds to purines in DNA, resulting in loss-of-function mutations in the tumor suppressor gene TP53 and functional mutations in the oncogenes FGFR3 and H-RAS. These aristolactam adducts are long-lived, allowing them to remain in the renal cortex for many years and facilitating the continued presence of AA [1, 11 12]. DNA adducts from AA are recognized as highly specific biomarkers. They are extremely mutagenic and inhibit DNA replication. Furthermore, they exhibit a mutational signature characterized by A → T transversions in tumor tissues of upper tract urothelial carcinoma. These adducts persist in humans due to inadequate recognition by global nucleotide excision repair of the genome, leading to more frequent development of predominant A → T transversions [11, 13]. These biomarkers are utilized to study the mutagenic and carcinogenic effects of AA. Therefore, their detection is vital for assessing disease risk [14].

Not everyone who consumes AA will experience long-term health effects. This may result from variations such as sex, age, genetic factors, and the dosage used, which can lead to toxicity from either a single high dose or several small doses taken continuously. Furthermore, susceptibility to this acid is linked to individual differences, including the enzymes involved in the biotransformation of AA. In the liver, AA is metabolized under two conditions: in anaerobic environments, it corresponds to aristolactams I and II, whereas in aerobic environments, the identified metabolite is the O-demethylated derivative [1].

In the complex metabolism of AA, the most significant enzyme that activates AA is NAD(P)H: quinone oxidoreductase (NQO1), which is found in the hepatic and renal cytosolic subcellular fractions. Other enzymes involved, though to a lesser degree, include hepatic microsomal cytochrome P450 (CYP) 1A1/2 and renal microsomal NADPH: CYP reductase (POR), which also reduce AA. Furthermore, factors that influence the expression and activity of these enzymes have been identified, such as genetic polymorphisms, drugs, smoking, and environmental and chemical pollution, which can help explain the susceptibility of individuals to AA [1].

In phase II, the metabolites formed through sulfation produce AA–DNA adducts. These sulfate conjugates are transported out of the liver by MRP membrane transporters and directed to the kidneys via organic anion transporters (OATs), especially OAT1 and OAT3, which facilitate the uptake of aristolochic acid by renal cells. This process results in renal damage due to the site selectivity of this acid [5]. Additionally, during this phase, NQO1 metabolism decreases, which is enhanced by SULT1B1 sulfonation after nitroreduction. This notably increases the mutation potential at the cellular level and the cytotoxicity of aristolochic acid [7].

Lesions resulting from AA biotransformation are progressive, and mutational events are irreversible. Consequently, a therapeutic regimen that demonstrates verifiable efficacy has not yet been established. [11].

AA-induced pathologies

Long-term exposure to foods containing AA or similar substances is considered a risk factor for the development of severe kidney disease, upper tract urothelial carcinoma, hepatobiliary cancer, and other types of cancer [15, 16]. In one study, the tissue of nine patients with upper urinary tract cancer who had previously consumed motherwort was evaluated, revealing approximately 1,500 genes with genetic alterations. This led to their genetic fingerprint, which is among the most complex compared with those of lung and skin cancer, referred to as signature 22 in COSMIC, suggesting the potential of "molecular fingerprints" in upper tract urothelial carcinoma and other types of cancer [15, 16].

Recent studies have shown a relationship between nephrotoxicity and carcinogenicity with this acid. The possible cause could be the toxic effects of aristolochic acid on proximal tubular epithelial cells, which induce the secretion of cytokines, the synthesis of the extracellular matrix, and the formation of new myofibroblastic epithelial cells [17].

Aristolochic acid nephropathy

This condition results in extensive interstitial fibrosis along with atrophy, loss of tubules, and hyperplasia in the urothelium found within the superficial cortex [11]. Nephropathy rapidly progresses to end-stage renal disease; most cases present a clinical picture marked by mild hypertension, elevated serum creatinine, proteinuria, severe anemia, leukocyturia, glucosuria, and a reduction in the glomerular filtration rate. Recent studies have confirmed that microalbuminuria and tubular proteinuria act as markers for the early detection of AA nephropathy [11].

Balkan endemic nephropathy

This disease is an endemic, noninherited familial chronic kidney disease that progresses slowly and frequently leads to upper tract urothelial carcinoma. The disease has a relatively high prevalence in agricultural villages along the Danube River [11]. A definitive connection between BEN and AA has been established, as AA-derived DNA adducts (AA-DNA) have been identified in renal cortical and urothelial tissues due to distinctive A → T transversions [11]. Key signs of this disease include tubular dysfunction with increased excretion of low-molecular-weight proteins, glucosuria, arterial hypertension, amino aciduria, and a decreased glomerular filtration rate [18].

Upper urothelial carcinoma

It is defined as a subset of urothelial malignancies located in the renal pelvis and upper ureter; it has multiple genetic, hereditary, and environmental causes, including a relation to AA poisoning, and most cases have been identified during inspections for AA nephropathy [11]. These urothelial carcinomas are generally bilateral synchronous or, in other instances, metachronous contralateral. Additionally, it is similar to AA-DNA adducts and TP53 mutations.

Other pathologies

Patients with hepatitis B have a greater predisposition to hepatocellular carcinoma when consuming herbs that contain aristolochic acid. This conclusion is supported by the complete sequencing of the exomes of hepatocellular carcinomas, which revealed a distinct mutational signature associated with aristolochic acid. These findings suggest a link between derivatives of aristolochic acid and the development of liver cancer [11]. Animal studies have also revealed other types of cancer related to aristolochic acid consumption, including tumors in the anterior stomach, the auditory canal, the kidney, the urothelial tract, the small intestine, and the bladder [11].

Diagnostic methods to identify AA

Whole-genome sequencing

Sequencing is a method that provides multiple advantages, such as the ability to analyze a large number of DNA sections simultaneously from a vast database, yielding extensive information about diseases linked to mutations. Another advantage is its ability to detect all types of genetic variations in a single experiment, and it can even identify DNA damage, facilitating data collection and interpretation. For these reasons, it is regarded as a fast, cost-effective method with high sensitivity and specificity for diagnosing genomic diseases [19, 21].

Sequencing is used to identify the genome-wide aristolochic acid (AA)-induced mutational signature, termed signature 22 in the Catalog of Somatic Mutations in Cancer (COSMIC). This mutational signature is characterized by high proportions of transversional mutations A:T>T:A [22, 23]. Samples can be obtained via urine or tumor biopsies stored in liquid nitrogen immediately after surgery. Diagnosis is made by comparing case/control variants. Aristolochic acid (AA)-associated mutations primarily affect the TP53 gene, which has been identified as the most frequently mutated gene [1, 9, 24]. Furthermore, other genes affected by recurrent mutations have been identified, such as KMT2A, KMT2C, KMT2D, ARID1A, TERT, FGFR3, ATRX, CREBBP, CHD2, CHD5, CHD8, LRRK2, AHNAK, SMCHD1, and XIRP2 [15, 17]. Sequencing has shown that multifocality is more common in patients with the AA-associated mutational signature. However, patients with this mutational signature define a low-risk subtype because a less aggressive behavior has been identified than other UTUC subtypes and, therefore, more favorable clinical outcomes [15, 25].

Dual spectroscopy

This technique integrates fluorescence spectroscopy, specifically known as surface-enhanced Raman spectroscopy (SERS), with graphene enrichment. Methods for detecting aristolochic acid I (AAI) from herbal remedies involve the transformation of AAI into fluorescence-active molecules, such as aristolactam (AAT), through derivatization [26] or the use of mass spectrometry (MS) following chromatographic separation [27]. A dual spectroscopic approach is employed to detect aristolochic acid (AA) in blood and tissue samples without the need for derivatization or chromatographic separation. Aristolochic acid I (AAI) is identified by surface-enhanced Raman spectroscopy (SERS) with graphene enrichment, whereas AAT, a byproduct formed by the metabolism of AAI in the body, is detected via fluorescence spectroscopy. Owing to the complex biological matrix of the samples, pretreatment was performed for extraction. The tissue samples were homogenized with saline solution, while the blood was used directly. The samples were first mixed with ethanol, followed by the addition of ammonium sulfate ($(NH_4)_2SO_4$), shaken, and allowed to stand for 3 minutes for phase separation. Equal proportions of ethanol (19–25%) and ammonium sulfate (16–24%) were prepared in the solution to maximize the extraction efficiency of the target compounds located in the upper phase.

After extraction, the mixture was centrifuged, and part of the upper phase was used for fluorescence analysis because AAT is detected immediately after extraction. Another portion is treated with previously synthesized magnetic nanoparticles (Fe_3O_4−Ag) and graphene for SERS and high-performance liquid chromatography (HPLC) analysis. Continuous metabolic monitoring indicates that the transformation of AAI to AAT occurs in the kidney. Furthermore, AAT has a longer lifetime in the kidney and liver. This dual spectroscopic strategy applies to long-term toxicological research and the direct diagnosis of AAI-induced organ damage [28].

Liquid chromatography in urothelial cells

The use of ultra-performance liquid chromatography–triple quadrupole mass spectrometry in exfoliated urothelial cells and the analysis of AA–DNA adducts at low levels within a complex biological matrix present the greatest analytical challenges. Several methods have been developed for detecting AA–DNA adducts, including 32P postlabeling analysis [2, 9]], high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) [26,27], and high-performance liquid chromatography with fluorescence detection (HPLC–FLD) [32]. However, 32P postlabeling analysis has certain limitations related to radioactivity, and HPLC-FLD has demonstrated low specificity.

Instead, this new method relies on ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Compared with conventional HPLC analysis, ultra-performance liquid chromatography (UPLC) provides improved chromatographic resolution and greater peak capacity through rapid elution on a column filled with 1.7 µm particles. The increased efficiency of small particles allows for shorter columns, which reduces the analysis time and solvent usage. For mass spectrometry analysis, multiple reaction monitoring (MRM) mode is used to select and track both the parent ion and one or more product ions simultaneously, thereby increasing specificity and sensitivity. Schmeiser et al. proposed that dA-AAI, a specific DNA adduct formed between aristolochic acid I and adenine, may be the critical mutation in the carcinogenic process in rodents. Therefore, this method serves to identify dA-AAI as a biomarker and monitor AA exposure [14, 33].

First, the dA-AAI adduct is synthesized to provide a reference standard. The purified dA-AAI was characterized by high-resolution mass spectrometry and fluorescence spectroscopy. In addition, exfoliated urothelial cells are isolated, and DNA digestion is performed. Owing to the low level of AA-DNA adducts in complex-digested DNA samples, sample clean-up and DNA adduct enrichment are necessary prior to UPLC–MS/MS analysis. After 10 µl of the digested solution was added for normal nucleoside determination, the remaining extract (approximately 1.5 ml) was subjected to solid-phase extraction (SPE). The digested DNA mixture was then applied to the column, and the mixture was washed with water and methanol. The methanol fraction containing the AA–DNA adducts was collected

and evaporated to dryness under a stream of nitrogen at 37 °C. The obtained residue was reconstituted in 100 µl of methanol for UPLC–MS–MS analysis. UPLC analysis was subsequently performed. Chromatographic separation was achieved on a Waters Acquity UPLC C18 bridged ethyl hybrid (BEH) column. The mobile phase consisted of water (A) and acetonitrile (B). The mobile phase flow rate was maintained at 400 µl/min, and the column temperature was 25 °C. The injection volume was 10 µl.

Mass spectrometry analysis was performed on a Waters triple quadrupole mass spectrometer equipped with an electrospray ionization source in positive ionization mode. Data analysis and quantification were performed via Waters MassLync software.

For quantitative analysis, accurately weighed dA-AAI and internal standards were dissolved in methanol and methanol/formic acid to prepare stock solutions. The collected urine samples were processed as described above and used as a blank sample matrix. Each solution was spiked with 10 ng/ml reserpine as an internal standard. Three replicate injections were performed for each calibration level. The calibration curve was established by plotting the peak area ratios of dA-AAI to the internal standard against known dA-AAI concentrations. Linear regression data derived from the average of the three graphs allow the quantification of dA-AAI in exfoliated urothelial cells. Generally, the concentration of the dA-AAI adduct is expressed as adducts per 109 normal nucleotides; therefore, the concentration of unmodified nucleosides is determined by diluting 10 µl of the digested DNA samples with 90 µl of methanol/water for UPLC–MS/MS analysis. This method is specific and rapid for the detection of low-level AA-DNA adducts in urothelial cells since it provides good accuracy and precision with a detection limit of 1 ng/ml [14].

PCR of the P53 gene

The P53 gene is also referred to as terminal transferase-dependent polymerase chain reaction. AA-producing carcinomas are associated with p53 overexpression, suggesting that the p53 gene is mutated in Chinese herb nephropathy-associated urothelial neoplasia (CHN) (30). The p53 gene is one of the most commonly mutated genes in human tumors and is mutated in 50% of all human cancers; therefore, this gene is expressed in urothelial carcinomas as well as renal and ureteral atypia [34].

The analysis of AA-DNA adducts involves four essential steps: enzymatic digestion of the DNA sample; enrichment of the adducts; radiolabeling of the adducts by T4 kinase-catalyzed transfer of 32P-orthophosphate; separation and identification of the labeled adducts; and detection and quantification by means of their radioactive decay [24].

Samples were obtained from breast carcinoma biopsies. Specific sequential detection starts by mapping the distribution of AA-DNA adducts at single-nucleotide resolution in exons 5--8 of the human p53 gene in genomic DNA via a polymerase-specific adduct-stop assay combined with terminal transferase-dependent PCR (TD–PCR) to amplify DNA fragments. AA–DNA adducts were reacted with human breast carcinoma DNA (MCF-7) in vitro, and the major DNA adducts formed were identified via the 32P postlabeling method. These AA-DNA adducts were used as templates for TD-PCR [35, 36].

As a result, the adduct pattern is qualitatively similar to that found in NCH patients, consisting of two major spots (spots 1 and 2) and one minor spot (spot 3). The two major adducts identified are dA-AAI (spot 1), dG-AAI (spot 2), and the minor adduct dA-AAII (spot 3). Furthermore, quantitative analysis via 32P postlabeling revealed the dose-dependent formation of aristolochic acid I-DNA adducts (AAI-DNA).

Chemical activation of exons 5--8 of the p53 gene revealed that certain sites in the sequence are more prone to DNA adduct formation with aristolochic acid I (AAI) and aristolochic acid II (AAII), but clear differences are also observed between the two nitrophenanthrenic carboxylic acids. The stop spectrum reveals that most stops are associated with purine residues in the sequence. This preference for reactions with purine bases is in agreement with the results of 32P labeling; therefore, purine adducts are the main reaction products of AAI and AAII with DNA. Furthermore, the mutational hotspot for urothelial carcinomas at codon 248 of exon 7 shows a strong AA–DNA adduct binding site within a cytosine–guanine (CpG)-rich sequence, especially with AAII. The other mutational hotspot within a CpG sequence at codon 282 of exon 8 has been identified as a marginal AA–DNA adduct binding site. In general, guanines at CpG sites in the p53 gene that correspond to mutational hotspots observed in many human cancers do not appear to be preferential targets for AAI or AAII. Using this method, it is not possible to predict AA-specific mutation hotspots in p53-related urothelial tumors [36].

Parametric magnetic resonance imaging

Magnetic resonance imaging (MRI) plays an important role in the investigation of renal disease. It is therefore useful for probing renal pathophysiology via functional renal MRI techniques such as blood oxygenation level-dependent (BOLD) imaging contrast, water diffusion-weighted imaging (DWI) and magnetic resonance relaxometry. On the other hand, quantitative T2 relaxation time mapping is a well-established technique for (pre)clinical research. (34,35) DWI has been shown to probe tissue microstructure through the displacement of water molecules. Furthermore, restricted water diffusion is manifested by a reduction in the apparent diffusion coefficient (ADC) [39].

When performing an experimental study in rats, two groups were considered: the AAN group, which received daily intraperitoneal injections of 40 mg/kg AAI dissolved in polyethylene glycol (PEG) for six days, and the control group, which received daily intraperitoneal injections of PEG at the same dose and schedule as the AAN group. 3D T2 images are observed in the coronal orientation parallel to both kidneys, and regions of interest (ROIs) are defined for the renal cortex (CO), the outer stripe of the outer medulla (OSOM), the inner stripe of the outer medulla (ISOM) and the inner medulla (IM). ROIs are manually extracted from the right kidney, and the mean T2 and apparent diffusion coefficient (ADC) values are estimated for each renal layer [40].

Statistical analysis via SPSS 22.0 revealed differences in T2 and CDA between the AAN group and the control group. The correlations between the parameters TIS (tissue injury score) and MRS (magnetic resonance spectroscopy) must present a p value less than 0.05 to be considered significant. Furthermore, the feasibility of the use of parametric magnetic resonance for the evaluation of renal injury in a rat model treated with AAI was examined via quantitative metrics of T2 and CDA magnetic resonance. T2-weighted magnetic resonance separates four renal layers from each other with adequate tissue contrast and shows microstructural changes in different renal layers at each time point. Severe renal injury is detected with CDA mapping. Similarly, T2 showed a significant positive correlation with TIS; OSOM T2 was able to reveal the highest correlation coefficient with damage at the level of the renal tubules. This finding suggests that T2 is a suitable imaging marker for the assessment of tubular injury during the acute phase of AAN [40].

Challenges

The main analytical challenge for the identification of AA has been the analysis of AA–DNA adducts at low levels in a complex biological matrix. In UPLC–MS/MS and terminal transferase–dependent PCR, it is essential to identify AA–DNA adducts. However, UPLC–MS/MS detects only one adduct, dA–AAI [14], whereas PCR detects two adducts, dA–AAI and dG–AAI [37]. In contrast, when the dual spectroscopic strategy is used, the detection of AAT is necessary [27]. On the other hand, in sequencing, it is not necessary to analyze DNA adducts since the pattern of mutations cataloged as a mutational signature is taken into consideration, thus obtaining relevant information about the genes involved, the type of mutation and the multifocality [15, 22, 23].

Regarding the terminal transferase-dependent PCR method for the P53 gene, there are certain limitations regarding postlabeling with 32P due to the radioactive nature of the isotope [24]. In addition, with PCR, it is not possible to predict specific AA mutation hotspots in urothelial tumors related to the p53 gene [37].

The samples required vary depending on the method used. For identification via UPLC–MS/MS, urine samples are needed; for PCR, tissue samples are used, and for the dual spectroscopic strategy, blood or tissue samples are used. Moreover, for sequencing, blood, tissue or urine samples are used [15, 28, 36]. The latter is the method closest to a reference standard because of the variety of types of biological samples used for the analysis.

Magnetic resonance imaging was used to detect signs of tubular damage in the acute phase of AA nephropathy via T2 [40]. However, this method does not detect the presence of AA within the body, unlike the methods mentioned above.

Conclusions

The methods presented here with different experimental approaches have proven to be highly relevant to the scientific community in the early diagnosis of AA-induced pathologies, such as nephropathies and urothelial carcinoma. However, currently, the use of next-generation sequencing has begun to be prioritized because of its advantages, such as greater sensitivity, specificity and ability to analyze multiple samples simultaneously. Despite this, the implementation of larger studies remains a challenge owing to the significant resources that these methods require.

Abbreviations

AA: Arachidonic acid.
DNA: Deoxyribonucleic acid.

Additional information

No supplementary materials have been declared.

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Authors' contributions

Daniela Jhulissa Rivas-Saca: Conceptualization, methodology, investigation, writing – original draft.
Lorena Viviana Mora Bravo: Conceptualization, project administration, supervision, validation, visualization, writing, review and editing.
Kerly Samantha Saquipay-Nieves: Conceptualization, data curation, formal analysis, funding acquisition, investigation and methodology.
All the authors read and approved the final version of the manuscript.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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