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HPLC DETERMINATION OF ACONITINE , HYPACONITINE AND MESACONITINE IN ACONITE EXTRACT

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ABSTRACT

A sensitive method is presented for the determination of aconitine, mesaconitine and hypaconitine in both extract and biological samples. The cited drugs were extracted using the usual alkaloidal extraction methods and purified then determined by HPLC using methanol - H_2O - $CHCL_3$- triethylamine (700 : 300 : 10 : 1) as a mobile phase and UV detector at 254 nm. The sensitivity of the method ranged between 0.017 to 0.46 µg / ml , and a coefficient of variation 1.29 to 2.43 . The method was successfully applied for the quantitative determination of aconitine and its analogues in aconite extract and animal tissues.

Injection of verapamil to the experimental animals (mice & rats) significantly increased LD_{50} in mice to 0.74 mg/kg and in rats to 4.8 mg/kg.

INTRODUCTION

Aconitine is a highly toxic diterpene alkaloid , it was formerly used either as a tincture of *Aconite* species or prescribed in the far East herbal preparations for relief of minor musculoskeletal pain (1), rheumatic arthritis, bruises , fractures and cardiac complaints (2).

Traditional chinese medicine often contains "chuanwu and caowu", the roots of certain *Aconitum* species which are thought to have an anti-inflammatory effect . Excessive amounts of these materials, which contain diterpene alkaloids particularly aconitine, can produce toxic effects and occasional fatalities (3).

Aconitine poisoning (following the ingestion of chuanwu) led to typical gastrointestinal nausea and vomiting and neurological generalized weakness, numbness and paraesthesia. There was evidence of cardiotoxicity (hypotension and ventricular ectopics) (4).

Aconitine is a potent Na^+ channel agonist so class I antiarrythmic drugs were used as a protective measure against many cases of aconitine induced toxicity (1).

Aconitine never gave a specific color reaction , so most of the methods reported for its analysis usually depended on the detrmination of its relative toxicity (5-7) . Other previous assay methods were gravimetric (8), aqueous (9,10) and non aqueous titrimetric (11) and spectrophotometric (12) techniques. Paper chromatography in combination with ultraviolet spectrophotometry(13) was also reported for the detection of different aconite alkaloids.

This drug is consumed traditionally in the far East or ingested by mistake and has been recently used by many people for suicidal and homocidal purposes.

Thus, the aim of the present work is to develop a simple and sensitive method for simultaneous determination of aconitine and its analogues in biological samples as well as in aconite extract .

EXPERIMENTAL

Apparatus and chromatographic conditions:

Analyses were performed on a liquid chromatograph from (Waters) equiped with automated controller - Model 680 ; Pump and solvent delivery system Model M-45; An automatic injector WISP 712; UV detector Model 481 and data module Model 730. The column was a stainless steel column (3.9 mm x 36 cm) packed with µ Bonda pack C_{18} : Mobile phase methanol - H_2O - $CHCL_3$- triethylamine (700 : 300: 10: 1) ; flow rate : 0.5 ml/min . Samples were introduced into the column through a fixed volume injector with a 50 µl sample loop at an ambient temperature. Chromatograms were traced on a strip chart recorder at a speed of 0.2 cm / min.

Chemicals and reagents :

Aconitine (Fluka, Germany) was used without further purification mesaconitine and hypaconitine from (Kihida, Osaka , Japan). Aconite extract (Les Laboratoires Givaudan, lavirotte and Cie, Lyon, France). Water was distilled and deionized. All other chemicals and reagents were USP or ACS quality and were used as received. Verapamil hydrochloride (Knoll, Germany).

Standard solutions :

Stock solutions :

Stock solutions were prepared by dissolving seprately 5 mg of each of aconitine , mesaconitine and hypaconitine in 50 ml of the mobile phase .

Working solutions :

1 ml of the stock solution of each aconitine , mesaconitine and hypaconitine were diluted separately to 100 ml of the mobile phase .

Procedure :

Aliquots of the previous working soltuions equivalent to 0.15 µg till 3.5 µg aconitine , 0.25 µg till 4.0 µg of mesacontine and 0.20 till 5.0 µg of hypaconitine were added separately to 10 ml measuring flasks. The flasks were brought to volume using mobile phase and mixed throughtly .Three 50 µl injections of each standard solution were made to prepare standard plots. The peak area of aconitine, mesaconitine and hypaconitine were plotted against its concentrations. Least square linear regression analysis was used to determine the correlation coefficient of the standard plots. .

Determination of aconitine and related alkaloids in aconite extract.

Accurately measured 1 ml of extract was diluted to 50 ml with distilled water and rendered alkaline with dilute ammonia solution, then extracted with 20, 10, 10 and 10 ml portions of ether .The combined ether extract was dried with anhydrous sodium sulfate the ether distilled to dryness and the residue dissolved in the 10 ml volume of the mobile phase .

LD_{50} determination of aconitine :

Adult male mice (50 animals) 25-30g body weight and 50 male rats 200-300 g body weight were used to study the behavioral and toxic effects of aconitine LD_{50} was determined according to **Sperman Karber** procedure (14).

Another group of animals 50 mice and 50 rats were injected I.P. with 2 mg / kg of verapamil hydrochloride. The LD_{50} of aconitine was determined according to the previous procdure (14).

Determination of aconitine, mesaconitine and hypaconitine in animal tissues (rats):

100 male rats were used for each of aconitine, mesaconitine and hypaconitine (Ca. 250 g average body weight) were given sublethal doses (1 ml prepared solution = 0.04 mg/ kg for each of aconitine, mesaconitine and hypaconitine) intraperitoneally and were divided into 5 subgroups. One hour after administration of the dose , the members of the first subgroup were killed by a sharp blow on the head and immediately dissected ; the heart , stomach , kidney and liver were removed for analysis . The other 4 subgroups were treated similarly after 2,5, 8 and 10 hrs. Another 100 female rats were treated in a similar manner.

Isolation of aconitine and related alkaloids from biological samples:

Each organ (liver, heart , kidney and stomach) was immediately treated after necropsy with 50ml 1% HCl and blended in a homogenizer. Enough $(NH_4)_2SO_4$ was added to each homogenized sample to make saturated solution. The mixture was warmed to 65°C until the proteins coagulated . The mixture was filtered through Büchner funnel and the residue washed with hot water. The combined filtrate and washings were cooled and made alkaline with dil NH_4OH, saturated with NaCl and extracted twice with 20 ml ether . Cautiously ether was evaporated and the resulting extract was dried and dissolved in 0.5 ml of mobile phase.

RESULTS AND DISCUSSION

A small amount of aconite root extract or even ingestion of small piece of aconite tubers taken by mistake for edible grass can cause pharmacological effects and they developed symptoms of aconite toxicity (15). A large amount of either the extract or the tubers cause neurotoxic and cardiotoxic effects and can lead to death resulting from ventricular arrhythmia or direct paralysis of the heart . These toxic effects are based on aconitum alkaloids. The molecular mechanism of the toxicity is the permanent activation of the voltage - sensitive sodium channels of exitable membrane (16).

In the present study aconitine produced marked writhing in mice. The writhing was previously reported (17) . Furthermore animals showed convulsion before death.

The results revealed that the LD_{50} of aconitine alkaloid after I. P. injection in mice was 0.32 mg/kg while in rats, it was 1.07 mg/kg. Reported LD_{50} for mice after I.P. administration was 0.308 mg/kg (18). Therefore detection of low levels of toxic alkaloids either in body fluids or body tissues seems to be difficult. **Mizugaki et al.** (19) reported a GC-MS method for the detection of aconitine alkaloid using trimethylsilyl derivatization and they found that detection limit by SIM mode to be as low as 10 pg. **Yoshioka et al.** (20) reported a case of aconitine poisoning after ingestion of aconitine but due to the refuse of the victim's family to do autopsy, they reported an analysis of blood and urine but they registered a coma and a brain oedema leading to death.

Ohta et al. (21) reported an HPLC-MS method for the analysis of body fluids samples because the alkaloids under investigation are water soluble, polar and labile.

Zagazig J. Pharm. Sci., Dec. 1999
Vol. 8 , No. 2 , pp 50 - 53

Kitae et al. [22] reported an improved analysis of aconitine and mesaconitine , in body fluids by gas chromatography selected ion monitoring with their deuterium - labelled analogues as internal standard in a concentration range of 50 pg to 50 ng. Their recovery rates ranged from 97.6 to 101.3%.

Tracing the literature no use of HPLC method was reported for either the detection or the quantitative estimation of *Aconitum* alkaloids in the tissue samples.

Verapamil by I. P. injection markedly increased LD_{50} in mice to 1.074 mg/kg and in rats to 4.8 mg/kg . Previous studies documented that acontine is a potent sodium channel agonist and increase Na^+ and Ca^{++} tissue content. The results indicated that verapamil may be used in some cases of aconitine poisoning as class I antiarrhythmic drugs [1, 23].

Table (1, 2) showed that the HPLC method is sensitive and efficient and the calibration graph is linear and the results were preferable to a previously reported method [24].

Certainly, the combination of the presented extraction and purification method with the HPLC technique has the advantages over the previous techniques by the simplicity , reproducibility and rapidity in addition to no interference with biological metabolites .

The cited method present a new and only done method for the identification and detection of aconitine alkaloid and its analogues in the tissue samples by comparison of their retention times and by calculations. The toxicity analysis presented in this paper was intended to detect aconitine and its analogues in the body organs and extract in both cases of acute poisoning and the post - mortum toxicity analysis .

Aconitine could be traced in the heart tissue for up to 8 hours after injection at a concentration of about 88.6% of the dose injected. This result may explain the previously reported cardiac complains [1]. Aconitine was detcted in the liver in trace amounts while the kidney and stomach showed nill amounts.

Although mesaconitine could be traced in the stomach while hypaconitne was traced in all the biological samples in relative amounts of the original amounts injected .

In conclusion from the previous findings it is indicated that the toxicity of aconitine is almost due to its effect on the heart tissue.

The proposed method showed good recovery and easily applicable for both tissues and extract in addition to availability of the apparatus (HPLC) and short time (about 30 minuts) which showed previously tedious and time consuming methods.

Table (1): Sensitivity , recovery and coefficient of variation of aconitine, mesaconitine and hypaconitine "alkaloids" using HPLC technique.

Alkaloid	Aconitine	Mesaconitine	Hypaconitine
Detection limits µg/ml	0.0174-0.348	0.0258-0.348	0.023 - 0.46
Recovery %	99.3	97.4	97.2
Coefficient of variation	2.43	1.29	1.54

Table (2) : Assay of aconitine in aconite extract by HPLC method (results average of 5 replicates).

Preparation	Stated conc. mg/ml	Amount found mg/ml	Amount added mg/ml	Amount found mg/ml	Recovery %
Extract of aconite average recovery	3.00	3.2	2.00	5.16	99.2

SD 0.0301

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تقدير الأكونتين والهيباكونتين والميزاكونتين فى الخلاصات والأنسجة الحيوية باستخدام كروماتوجرافيا الضغط العالى

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يقدم البحث طريقة حساسة لتقدير الأكونتين والهيباكونتين والميزاكونتين فى الخلاصات والأنسجة الحيوية وذلك باستخلاص المركبات السابق ذكرها بالطريقة العامة لاستخلاص القلوانيات ثم تنقيتها وتقدر باستخدام كروماتوجرافيا الضغط العالى باستخدام مزيج مكون من كحول البيوتيل - الماء - الكلوروفورم وثلاثى اثيل الامين بنسبة (٢٠٠ : ٣٠٠ : ١٠ : ١) ويتم القياس باستخدام ومض الأشعة فوق البنفسجية عند ٢٣٤ نانوميتر . وحساسية الطريقة تقع بين ١٢ [illegible] ، ٣٥ [illegible] ميكروجرام /سم٣ واستخدمت الطريقة بنجاح فى تقدير الأكونتين والهيباكونتين والميزاكونتين فى الأنسجة الحيوية وكذا وجد أن حقن الهيباكونتين فى حيوانات التجارب قبل حقنها بالأكونتين يزيد من الجرعة [illegible] [illegible] إلى [illegible] [illegible] [illegible] فى [illegible] .