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Detection of Mesocarb in Human Urine by HPLC-Electrospray / Mass Spectrometry

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Abstract

A reliable method for detection of mesocarb, a psychostimulant which may be misused by athletes and forbidden by International Olympic Committee, is developed on HPLC-ES/MS. The conditions of separation and ionization for mesocarb detection in human urine was optimized. A p-hydroxylated metabolite could be detected in the urine after an oral administration of 2.5mg of mesocarb.

Keywords: mesocarb; psychostimulant; electrospray; liquid chromatography

Introduction

Mesocarb (3-(1-methyl-2-phenylethyl)-N-(phenylaminocarbolyl)sydnoneimine), also known as Sydnocarb, is a psychostimulant. Some events of misuse by athletes has been reported and the use of this drug has been banned by International Olympic Committee in sport competition. Since then, intensive studies on the metabolism of mesocarb and on the methodology was performed by many antidoping laboratories. Now, it is clear that hydroxylated metabolite free and conjugated seems to be the main metabolic pathway both in rat and in human (1) (2) (3). With regard to its analysis method mesocarb is still a question because it is labile and easily decomposed at higher temperature and this makes GC-MS analysis difficult to get the molecular ion as a detection indicator, only a pyrolysis product could be found in GC-MS analysis procedure (4). In our routine work of doping test this drug was controlled by using HPLC and GC-MS analysis respectively for screening and confirmation but the result is unsatisfactory from time to time (5).

HP59987A atmosphere ionization electrospray interface coupled with HP5989B mass spectrometer is a product newly improved by Hewlett Packard. As the other types of electrospray interface It is mainly applicated in large biomolecule analysis. Majority of the work with this new couple was concentrated on the biochemical and biomedical researches. The reports concerning the study on drug metabolism and doping test with this couple is

limited. As a new type of interface its capability to be used on-line with HPLC- mass spectrometer should be evaluated in every aspects of laboratory work. To demonstrate the capability and to develop a more reliable method for the confirmation of mesocarb in dope test we conducted this experiment. In this work, mesocarb, benzthiazide (diuretics), anabolic steroids and some other non-doping drugs, which are difficult to be detected by GC-MS, were involved. The work showed that on-line HPLC-electrospray/mass spectrometry could be a reliable method for confirmation of mesocarb in urine sample. For benzthiazide and steroids the result is inadequate to be used as a doping test method.

Experimental

Instrument and instrumentation:

HP5989B mass spectrometer with HP59987A electrospray interface
(Hewlett-Packard Company, Palo Alto 1601, California, USA)
HP1050 liquid chromatograph, (Hewlett-Packard GmbH, Waldbronn D-7517, FRG)
tune marker: a mixture of valine, tri-valine and hexa-tyrosine (Hewlett-Packard Company, Palo Alto 1601, California, USA) target ions: m/z 118.08, m/z 508.20, m/z 997.39
CID (collision-induced dissociation) setting: CapEx=120V
Electron-Multiplier voltage: 1800V
Drying nitrogen flow: 8000 ml/min
Drying nitrogen temperature: 350 ^{o}C
Nebulizing nitrogen pressure: 550 kPa (80 psi)
HPLC column: Spherisorb C-18 reversed-phase column, 5μm, 100x2.0mm (Iliter Company, Dalian 116011, P.R. China)
Mobile phase: A: formic acid solution, pH2.8 B: acetonitrile
Gradient: 0-8min: B=0-65 % 8-20min: B=65-80%
flow rate: 0.5ml/min

Reagent and solvent

Mesocarb standard was presented by Russia doping laboratory and checked by UV spectrum on HPLC. The stocking solution was made by dissolving it in methanol, the final concentration is 50ng/μl. All solvents and reagents used in experiment are chromatographic grade (Beijing Chemicals Company, Beijing 100089, P.R. China)

Drug Administration and urine collection:

2.5mg (one tablet and single dose) of mesocarb was orally taken by a volunteer. The urine was collected till 82h. No apparent clinic sign was observed.

Chemical treatment of urine sample

2ml of the positive urine was hydrolyzed with 6 m/l HCl at 90 ^{o}C for 1 h. After clearance by 4ml of ethyl ether the urine was extracted at pH9.0 ($NaHCO_3$:K_2CO_3=3:2, w/w) by 4ml of ethyl acetate. Dried with slow nitrogen stream the residue was redissolved in 300μl of methanol. 10μl of the solution was subjected to HPLC-ES/MS analysis.

Result

Mesocarb standard

2μl of the mesocarb stocking methanol solution (50ng/μl) was injected by the autosampler of HPLC. Scan mode was used to acquire the signal. The mass spectrum showed that the molecular ion peak of mesocarb is m/z 323 (M+1) and other fragments ion m/z 91, m/z 119, m/z 177 were obtained (Fig.1). Adding of natrium ion could be shown by m/z 345 (M+Na). Among these ions m/z 91 is tropylium which was formed by cleavage of the methylbenzene in mesocarb molecule. m/z 119 is resulted in a cleavage accompanied by one hydrogen rearrangement to form $(-CONHC_6H_5)^+$, and/or possibly by the cleavage of isopropyl benzene in the parent structure. m/z 177 proved to be a fragment of mesocarb by comparison with reagent blank but the fragmentation route is very difficult to be revealed.

Positive urine of mesocarb

With acidic hydrolysis and basic extraction by ethyl acetate a hydroxylated metabolite of mesocarb could be detected in the mesocarb positive urine (Fig.2). Same ions as those in spectrum of mesocarb standard could be seen in the spectrum of this metabolite, i.e. m/z 91 and m/z 119. As the spectrum of mesocarb standard the route of m/z 193 (seems to be 173+OH) was left unknown. The molecular ion is m/z 339 (323+OH). The substitute position of the hydroxyl failed to be located, but it should be a p-substitution at the ring - $CONHC_6H_5$ according to the Polgar's work. The other metabolites such as the dihydroxylated and amphetamine proved to exist in rat and human urine by Polgar was not found in our experiment.

Discussion

Reliability of the method

On-line HPLC-ES/MS can provide a acceptable method for the confirmation of this drug in human urine by the procedure as described above. In this experiment only the monohydroxyl mesocarb in conjugated state has been detected after the acidic hydrolysis. The free parent and the dihydroxylated were not found, which have been reported by Polgar et al. Possibly this is due to the administration dosage is rather small (2.5mg) and

the positive urine collected about two years ago, so further study on the metabolism of mesocarb with HPLC-ES/MS should be done to promote the reliability. Anyhow, the obtaining of the molecular ion peak with electrospray-mass spectrometer is a little progress for mesocarb detection.

CID test

In ES/MS procedure, to get fragmentation of a molecule, CID (collision-induced dissociation) will be performed instead of electronic impact. The collision energy can be changed by different setting of the voltage applied on the capillary exit (CapEx). In the CID test on mesocarb, significant changes in the relative abundances of molecular ion and other fragments could be expressed by altering the voltage. As shown in the spectra the base peak m/z 323 was replaced by m/z 177 when the CapEx voltage altered from 85V to 140V and also peak m/z 91, m/z 117 was strengthened (Fig.1, Fig.3). This means CID is an effective way for the fragmentation of mesocarb. For the fragmentation of some other compounds like steroids and benzthiazide CID is inefficient. In the CID test on these drugs the voltage 400V (the maximum setting) used to be applied to but no significant fragments could be acquired. Obviously, this is because of the very stable chemical structure of such compounds and the energy provided only by collision is insufficient. However, as the other types of soft ionization, electrospray has some advantages for the acquirement of molecular weight information and this is very valuable and important for the analysis of labile compounds.

Optimum of HPLC flow rate

Selection of HPLC flow rate for HPLC-ES/MS is rather contradictory, a compromise must be made between a good separation efficiency of column and a high-enough ionization efficiency by electrospray, both of which are closely related to the flow rate of mobile phase. According to the results of our experiment, if a column of diameter 2.0mm adopted and in case of mesocarb, the abundance in peak area of acquired signal was reduced by 8 times as the flow rate increased from 200μl/min to 800μl/min. To get a best separation of mesocarb on the column the flow rate should be 500μl/min at least. For 100ng of injected mesocarb, the S/N (ratio of signal to noise) is 920 at the flow rate 200μl/min and 260 at the flow rate 800μl/min respectively.

Solvent Interference

The solvents used in HPLC separation (chromatography grade), except for deionized water, will severely interfere with detection of mesocarb, sometimes to a degree of sheltering the expected signal in TIC and the function of "extract ion" had to be operated on to line out the TIC peak. Among the solvents used in our test acetonitrile will have m/z 282 and

methanol will have m/z 279, m/z 205 and m/z 149 which may come from the manufacture and cause a very strong interference. Distillation of the solvents may partially solve the problem and all the remaining interference should be excluded from the interpretation of mass spectrum.

Memory Effection

At very low flow rate, e.g. 10μl/min, when sample introduced by syringe pump the memory effection is apparent. The effection is mainly caused by the sample residue on the spray shield, capillary entrance and even in transport tube. So, it is necessary to flush the residue off after every injection. At higher flow rate, e.g. 200μl/min, no significant memory effection from the residue could be seen.

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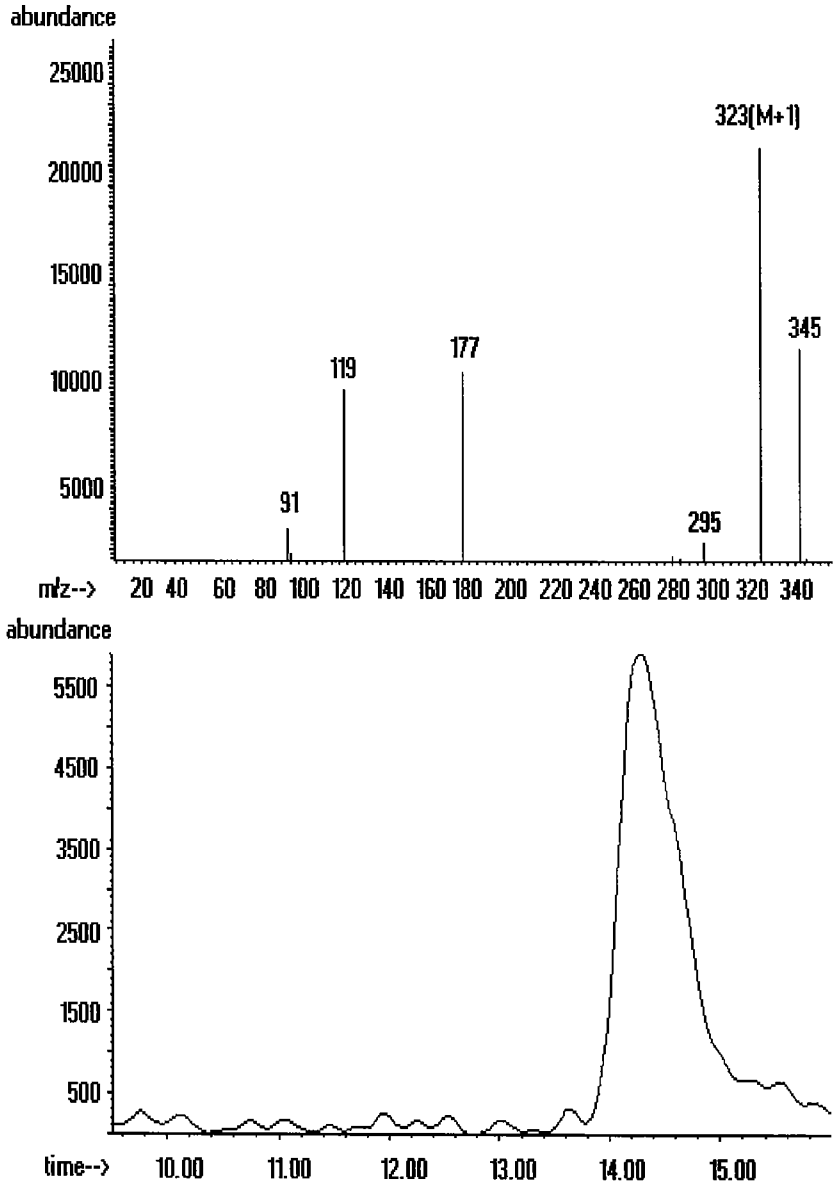

Fig.1 TIC and mass spectrum of mesocarb standard
CapEx: 85v

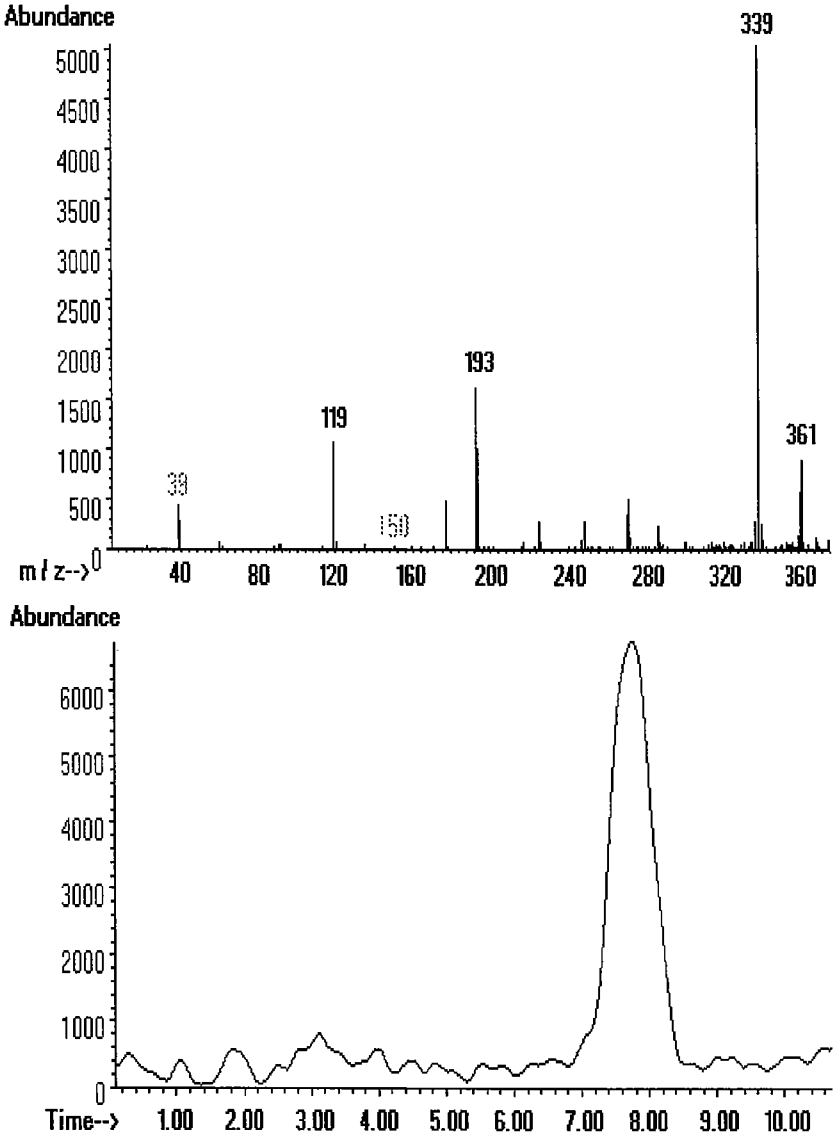

Fig.2 TIC and mass spectrum of mesocarb positive urine (10h)

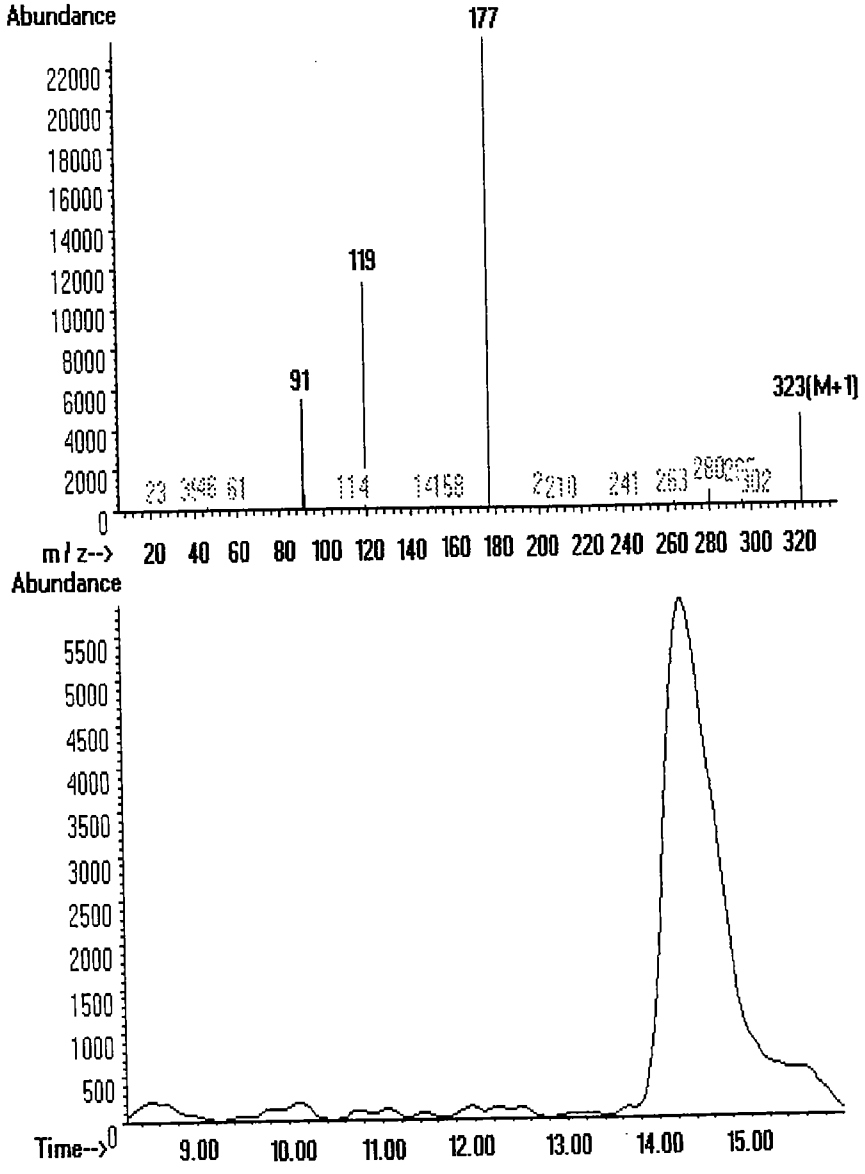

Fig.3 TIC and mass spectrum of mesocarb standard
CapEx: 140V