Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (9)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 2001

L.M.P. DAMASCENO, R.M.A. BENTO, L.N.L.F. GOMES, R. DE LA TORRE, F.R. AQUINO NETO:
Mesocarb Analysis Revisited
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln, (2001) 253-257

L.M.P. Damasceno[1], R.M.A. Bento[1], L.N.L.F. Gomes[1], R. de La Torre[2], F.R. Aquino Neto[1]

Mesocarb Analysis Revisited

[1]Doping Control Laboratory, Ladetec, Rio de Janeiro, Brazil.

[2]Pharmacology Unit, IMIM, Barcelona, Spain

Introduction

Mesocarb (Sydnocarb®; 3-(1-methyl-2-phenylethyl)-N-(phenylaminocarbonyl)-1,2,3-oxadiazolium-5-aminide) is an amphetamine-like compound with activity on the central nervous system. Since the inclusion of this compound in the list of banned substances by the Medical Commission of the IOC, several analytical methodologies have been developed for its detection in human urine [1-4].

Studies on the metabolism of mesocarb developed by several antidoping laboratories pointed out that the sulphate conjugate of p-hydroxymesocarb is the major metabolite present in human urine [1,5].

Different sample preparation procedures using liquid-liquid extraction have been described for its isolation from urine. Extracts are usually analyzed by GC or GC/MS (as its N-fluoroacyl or N-trimethylsilyl derivative) or by HPLC or HPLC/MS (without derivatization).

The metabolite of mesocarb is highly polar and thermally unstable. Both properties have been major drawbacks for its incorporation in already existing screening procedures in doping control. The present study compares different extraction procedures and optimizes a method for the detection of mesocarb ingestion. Additional aspects also investigated were the role of the acidic hydrolysis and the derivatization step in GC analysis.

Experimental

Administration: One tablet of Sydnocarb® (10 mg) to one healthy female volunteer.

Urine collection: Samples were collected at predose (blank sample) and up to 72h after administration.

Extraction Procedures:

Method I: Screening procedure for diuretics [1,2]

To 2.5mL of urine sample, 100µL of a methanolic solution of 7-propyltheophylline (1mg/mL), used as internal standard, were added. The urines were made alkaline with 100µL of ammonium chloride buffer (pH 9.5) and extracted with 7mL of ethyl acetate. After mixing (20min) and centrifugation (5 minutes, 3500rpm), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Method II: Screening procedure for diuretics with acidic hydrolysis [1]

To 2.5mL of urine sample, 100µL of a methanolic solution of 7-propyltheophylline (1mg/mL), used as internal standard, were added. The urines were acidified with 500µL of HCl 6M and the samples were incubated at 80°C for 1h. After the acidic hydrolysis, the pH was adjusted to 7 with KOH 5M and afterwards to pH 9.5 with 100µL of ammonium chloride buffer and extracted with 7mL of ethyl acetate. After mixing (20min) and centrifugation (5 minutes, 3500rpm), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Method III: Screening procedure for anabolic steroids (Free Fraction)

To 5mL of urine sample, 100µL of a methanolic solution of 7-propyltheophylline (1mg/mL), used as internal standard, were added. The urines were made alkaline (pH 9.5) with $NaHCO_3/K_2CO_3$ buffer (2:1) and extracted with 5mL of tert-buthyl-methylether. After mixing (20min) and centrifugation (5 minutes, 3500rpm), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Derivatization Conditions:

Condition I

40 µL of the mixture MSHFBA/TMSIm (100:2, v/v) were added to the dried residues, and they were incubated at 80°C for 5 min. After the mixture was cooled to room temperature, 10 µL MBHFBA were added, and the residues were incubated for 20 min at 80°C.

Condition II

Same as Condition I but samples were not incubated at 80°C.

Instrumentation:	GC/MSD analysis
Instrument:	HP 5890 GC with HP 5972 MSD
Capillary column:	5% crosslinked phenyl methylsiloxane (15m x 0.2mm i.d., 0.33μm)
Injection mode:	Splitless (1.0min delay)
Carrier gas:	helium (1,0mL/min)
Injector temp.:	280°C
Detector temp:	300°C
Sample volume:	2μl
Oven temperatures:	100°C (1min) - 25°C/min - 310°C (5min)
Ionization mode	EI
Acquisition mode	Scan (50-600 amu)

Results and Discussion

Derivatization

Mesocarb was analyzed as its N-fluoroacyl derivative pyrolysis product. The mass spectrum showed significant diagnostic ions (Figure 1).

Comparison between derivatized (MSHFBA/TMSIm and MBHFBA) and non-derivatized extracts showed best chromatographic responses for the derivatized product.

As already reported[2,5], due to thermal lability, a pyrolysis product common to mesocarb and p-hydroxymesocarb was detected with best chromatographic responses obtained for the derivatized product.

Derivatization experiments under different incubation conditions pointed out that the incubation step in the derivatization process seems to have no relevance to the formation of the derivatized pyrolysis product, with most of this process occurring in the injection port.

Extraction Procedures

Comparison between different extraction procedures showed that the screening procedure for diuretics based on ethyl acetate extraction (pH 9.5) without previous hydrolysis (*Method I*) gave best responses for the mesocarb metabolite (Figure 2).

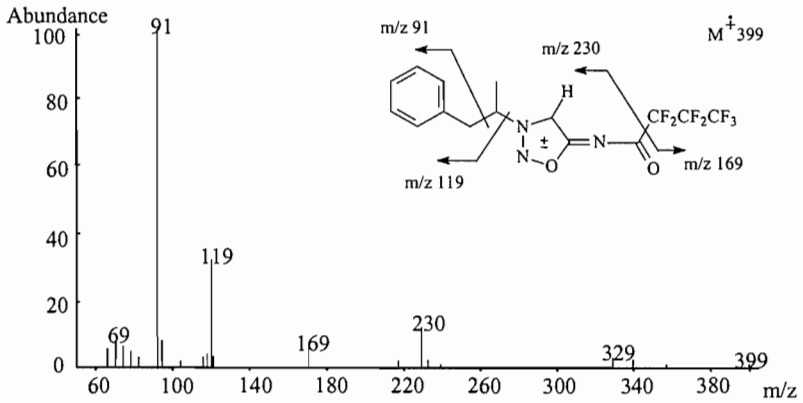

Figure 1. Mass spectrum of mesocarb pyrolysis product N-fluoroacyl derivative.

VENTURA *et al.*[1] has shown that sulphate conjugate of p-OH-mesocarb is the major metabolite present in human urine. It is however unstable under acidic hydrolysis conditions resulting in poor recoveries[1]. Ours results showed that the acidic hydrolysis step before liquid-liquid extraction did not have significant influence on the recovery of mesocarb metabolite but gave dirtier extracts (Figures 2A and 2B).

Extraction using the screening procedure for anabolic steroids was not successful (Figure 2C).

Urine Samples From Excretion Study

Chromatograms of the diagnostic ion obtained in the analysis of a urine from excretion study is also shown in Figure 2. The N-fluoroacyl derivative was detected in the whole excretion study interval (0-72h).

Conclusion

Best recoveries, faster and cleaner chromatographic results were obtained using the screening procedure for diuretics and derivatization with MSHFBA/TMSIm and MBHFBA without the need of the incubation step.

Using the optimized methodology, detection of p-hydroxymesocarb degradation product was possible up to 72h after drug ingestion.

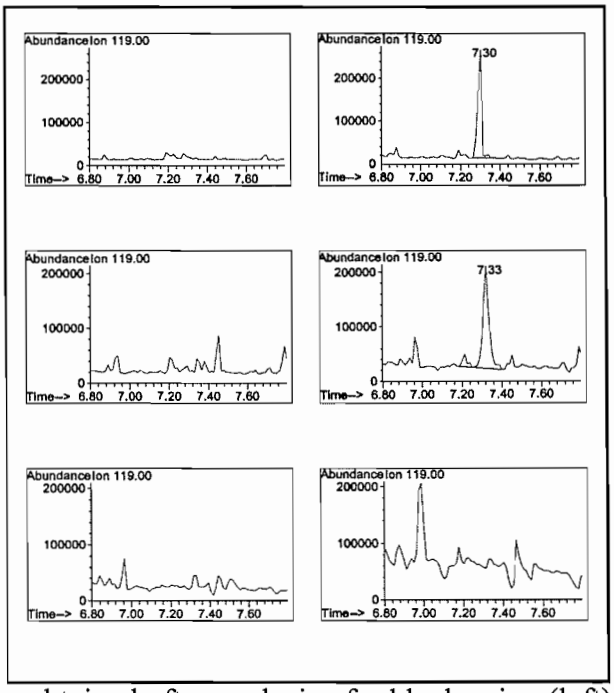

Figure 2. Chromatograms obtained after analysis of a blank urine (left) and a urine obtained from an excretion study (right) of mesocarb (6-12h). A - Screening procedure for diuretics; B Screening procedure for diuretics with acidic hydrolysis; C - Screening procedure for anabolic steroids.

Acknowledgements

We are grateful to Drs Kimmo Kuoppasalmi and Antti Leinonen, Doping Control Laboratory, United Laboratories Ltd, Finland, for a gift of Sydnocarb and mesocarb, and to Dr Rosa Ventura, IMIM, Spain, for background material and helpful discussions.

References

1 Ventura, R.; Nadal, T.; Alcalde, P.; Segura, J. *J. of Chromatogr.* **1993**, 647:203-10.

2 Ventura, R.; Nadal, T.; Alcalde, P.; Pascual, J.; Segura, J. *J. of Chromatogr. A* **1993**, 655:233-42.

3 Breidbach, A.; Sigmund, G.; Donike N. In: *Proceedings of the 12th Cologne Workshop on Dope Analysis* **1994**, 301-4.

4 Shumin, Y.; Zhu, S.; Yang, Z. In: *Proceedings of the 14th Cologne Workshop on Dope Analysis* **1996**, 377-84.

5 de Boer, D.; van Ooijen, R.D.; Maes, R.A.A. In: *Proceedings of the 12th Cologne Workshop on Dope Analysis* **1994**, 305-16.