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Determination of mesocarb metabolites by high-performance liquid chromatography with UV detection and with mass spectrometry using a particle-beam interface

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ABSTRACT

A method of screening for mesocarb ingestion in doping control is described. After alkaline extraction with ethyl acetate, samples are analysed by reversed-phase high-performance liquid chromatography (HPLC) with UV detection. A peak with a shorter retention time than and a UV spectrum identical with those of unchanged mesocarb was obtained when positive urine extracts were analysed. The metabolite was identified as the sulphate conjugate of *p*-hydroxymesocarb after HPLC–mass spectrometry with a particle-beam interface and hydrolysis studies. The compound was detected in urine until 48–72 h after administration of single doses of 10 mg. Unchanged mesocarb and free *p*-hydroxymesocarb were not detected in the samples studied.

INTRODUCTION

Mesocarb is a substance with stimulant activity recently added to the list of banned compounds in sport by the Medical Commission of the International Olympic Committee [1]. Hence methods to detect the presence of this compound or its metabolites in human urine are required. Information concerning human and animal metabolism and urinary excretion of mesocarb is limited. Free and conjugated hydroxylated metabolites are the main products described in rat urine [2,3].

In this paper, a method of screening for the presence of mesocarb metabolites in human urine based on high-performance liquid chromatographic (HPLC) separation and UV detection is described. Confirmation analysis is done by HPLC–mass spectrometry (MS) using a particle-beam interface. The application of the procedure to antidoping control in the 1992 Barcelona Olympic Games allowed the detection and confirmation of a real doping case.

EXPERIMENTAL

Chemical and reagents

7-Propyltheophylline, metandienone and diphenylamine were used as internal standards (ISTD). 7-Propyltheophylline was synthesized from theophylline and propyl iodide in alkaline medium. Metandienone and diphenylamine were purchased from Sigma (St. Louis, MO, USA).

Solutions of β-glucuronidase from *Escherichia coli* (Boehringer-Mannheim, Mannheim, Germany) and β-glucuronidase–arylsulphatase from *Helix pomatia* (Sigma) were used for enzymatic hydrolysis.

Water used in the HPLC eluent was of Milli-Q

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purity (Millipore Ibérica, Barcelona, Spain). Methanol, acetonitrile and ethyl acetate were of HPLC grade. Diethyl ether was of analytical-reagent grade and distilled before use. Other reagents were of analytical-reagent grade quality.

High-performance liquid chromatography with ultraviolet detection

HPLC–UV analyses were performed in a Series II 1090 liquid chromatograph equipped with a diode-array detector (Hewlett-Packard, Palo Alto, CA, USA) under the conditions described previously [4]. The column was Ultrasphere ODS (7.5 × 0.46 cm I.D.) with particle size 3 μm (Beckman, Fullerton, CA, USA).

The mobile phase was a mixture of 0.1 *M* ammonium acetate solution (adjusted to pH 3 with phosphoric acid) and acetonitrile with gradient elution. The acetonitrile content (initially 10%) was increased to 15% in 2 min, to 45% in 3 min, to 60% in 3 min, maintained for 1 min, decreased to the initial conditions in 1 min and stabilized for 2 min before the next injection. The flow-rate was 1 ml/min.

The detector was set to monitor the signals at 240, 270, 290, 300, 318 and 350 nm. In addition, the full spectrum between 200 and 400 nm for each detected peak was stored in the data system and plotted at the end of each run.

High-performance liquid chromatography–mass spectrometry

HPLC–MS analyses were performed in a Model 5989 mass spectrometer coupled to a Model 59980B particle-beam interface and a Series II 1090L liquid chromatograph, all from Hewlett-Packard. The operating parameters of the interface were desolvation chamber temperature 70°C, helium pressure 50 p.s.i. (1 p.s.i. = 6894.76 Pa) and nebulizer position −1.

The liquid chromatographic column was Ultrasphere ODS (25 × 0.2 cm I.D.) with particle size 5 μm (Beckman). The mobile phase was a mixture of 0.1 *M* ammonium acetate solution (containing 0.5% of formic acid) and acetonitrile, with gradient elution. The acetonitrile content (initially 38% for 3.5 min) was increased to 60% in 3 min, maintained for 1 min, decreased to the initial conditions in 1 min and stabilized for 2.5 min before the next injection. The flow-rate was 0.4 ml/min.

Electron impact (EI) ionization (70 eV) and scan acquisition (*m/z* 65–550) were used. The source temperature was kept at 250°C.

Gas chromatography

Gas chromatographic analyses were performed in a Series II 5890 gas chromatograph with a nitrogen–phosphorus-selective detection (GC–NPD) (Hewlett-Packard). The injection port and detector temperatures were 280°C. The column was 5% phenyl–methyl silicone (12.5 m × 0.2 mm I.D.) with film thickness 0.33 μm (Hewlett-Packard) and the temperature was programmed from 90 to 280°C at 20°C/min. Helium was used as the carrier gas at 0.6 ml/min.

Sample extraction

The samples were extracted using the procedure described previously [4] with some modifications. To 2.5 ml of urine sample, 25 μl of the ISTD solution (100 μg/ml methanolic solution of 7-propyltheophylline for HPLC–UV screening analysis and 100 μg/ml methanolic solution of metandienone for HPLC–MS confirmation analysis) were added. The sample was made alkaline (pH 9.5) with 100 μl of ammonium chloride buffer, salted with 1 g of sodium chloride and extracted with 8 ml of ethyl acetate. After agitation (tilt shaker, 40 movements/min for 20 min) and centrifugation (1100 *g* for 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen. The extract was reconstituted with 100 μl of water–acetonitrile (85:15, v/v) and analysed by HPLC–UV detection (20 μl) or HPLC–MS (10 μl).

For the determination of amphetamine (a potential metabolite of mesocarb), 25 μl of the ISTD solution (1 mg/ml methanolic solution of diphenylamine) were added to urine samples (5 ml). The samples were made alkaline with 0.5 ml of 5 *M* potassium hydroxide solution and 3 g of anhydrous sodium sulphate and 2 ml of distilled diethyl ether were added. After agitation (tilt shaker, 40 movements/min for 20 min) and centrifugation (800 *g* for 5 min), 700 μl of the

organic layer were mixed with 300 μl of methanol and 3 μl of the mixture were analysed by GC–NPD.

Acidic hydrolysis

To 2.5 ml of the sample, 0.5 ml of 6 *M* hydrochloric acid and 50 mg of cysteine were added. The sample was heated for 1 h at 80°C. After incubation, the sample was cooled to room temperature and 400 μl of 5 *M* potassium hydroxide solution were added before the application of the extraction procedure described above.

Enzymatic hydrolysis

Two enzymatic hydrolyses were used: (i) to 2.5 ml of the sample, 1 ml of 0.2 *M* sodium phosphate buffer (pH 7) and 50 μl of *E. coli* solution were added and the mixture was usually incubated at 55°C for 1 h; (ii) to 2.5 ml of the sample, 1 ml of sodium acetate buffer (pH 5.2) and 50 μl of *H. pomatia* solution were added and the mixture was usually incubated at 55°C for 3 h. Longer incubation times were used in special studies.

Excretion studies

Two excretion studies were performed with one healthy male (A) and one healthy female (B) volunteer. Mesocarb was administered as Sydnocarb® tablets (10 mg) in single doses. Urine samples were collected for a period of 72 h.

The metabolite detected in the non-hydrolysed urines was determined by HPLC–UV detection using the calibration graph for unchanged mesocarb (0, 1.24, 3.10, 6.20 and 12.40 nmol/ml; molecular mass of mesocarb = 322.37). The extraction recovery and the molar absorptivity of the metabolite were assumed to be the same as those of unchanged mesocarb.

RESULTS AND DISCUSSION

Methodological aspects

The development of a procedure to detect mesocarb ingestion is difficult because only information on rat metabolism has been described previously [2,3] and no pure samples of the metabolites are available.

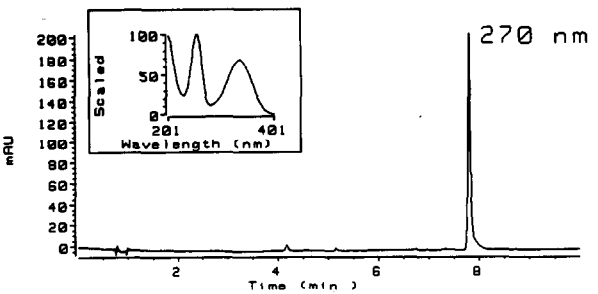

Fig. 1. Analysis of a methanolic solution of mesocarb by HPLC–UV detection and characteristic UV spectrum of mesocarb.

Normally, new compounds included in the list of banned substances in sport are tested by existing analytical procedures to avoid an increase in the complexity of doping control analysis. Most analytical methods used in doping control are based on gas chromatographic separations. The detection of mesocarb and its metabolic products is not directly amenable to gas chromatography because of their thermal lability [5,6]. Pyrolysis of mesocarb and its *p*-hydroxy metabolite to give N-nitroso-N-cyanomethylamphetamine takes place in the injection port.

Using the HPLC conditions of our routine screening method for diuretics and masking

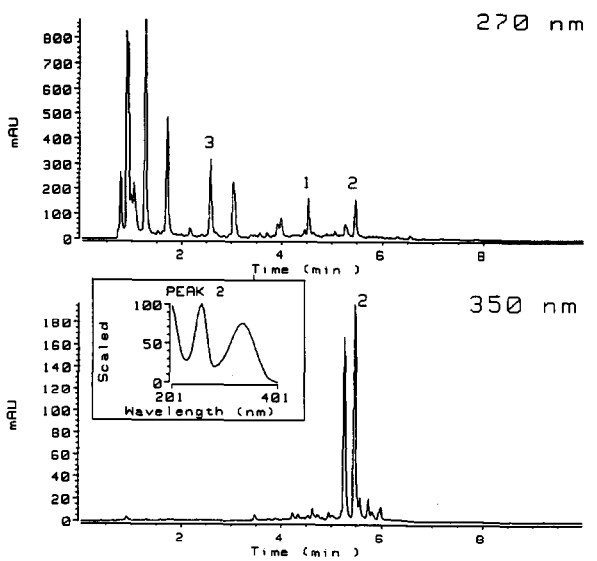

Fig. 2. HPLC–UV analysis of a urine sample obtained after mesocarb intake (real doping case). Peaks: 1 = ISTD; 2 = suspected mesocarb metabolite; 3 = caffeine.

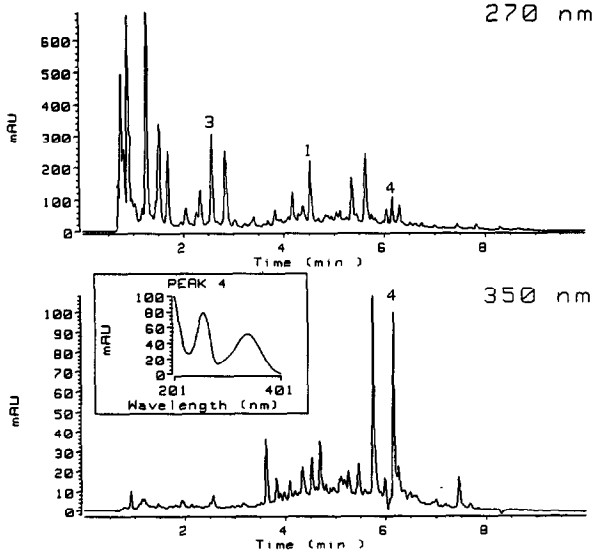

Fig. 3. HPLC–UV analysis of a hydrolysed urine sample obtained after mesocarb intake (real doping case). Peaks: 1 = ISTD; 3 = caffeine; 4 = suspected mesocarb metabolite.

agents [4], mesocarb gave a narrow peak at 7.8 min with a characteristic UV spectrum (Fig. 1). Therefore, this method was used to test urines obtained after mesocarb administration. A peak with the same UV spectrum as unchanged mesocarb was detected at 5.4 min after the analysis of urine extracts (Fig. 2). No unchanged mesocarb was present. After acidic hydrolysis and the same extraction procedure, this peak disappeared and another peak with the same UV spectrum appeared at 6.1 min (Fig. 3). Again, unchanged mesocarb was not detected.

On HPLC–EI-MS with a particle-beam interface and a source temperature of 250°C (Fig. 4), mesocarb showed a mass spectrum identical with that described by other workers using direct introduction of the compound into the mass spectrometer [2,3]. At lower source temperatures broader peaks appeared, indicating poor vaporization of the compound in the ion source.

Analysis by HPLC–MS of extracts from non-hydrolysed urines (Fig. 5) gave a peak with a mass spectrum related to that of the *p*-hydroxymesocarb previously reported in rat urine [2,3]. The mass spectrum showed a characteristic peak at *m/z* 135, indicating *p*-hydroxylation of the phenylisocyanate moiety, but the relative abundance between the ions of *m/z* 91 and 135 did not fit with that of *p*-hydroxymesocarb [2,3].

When extracts from urines subjected to acidic hydrolysis were analysed by HPLC–MS, a peak showing a mass spectrum corresponding to *p*-

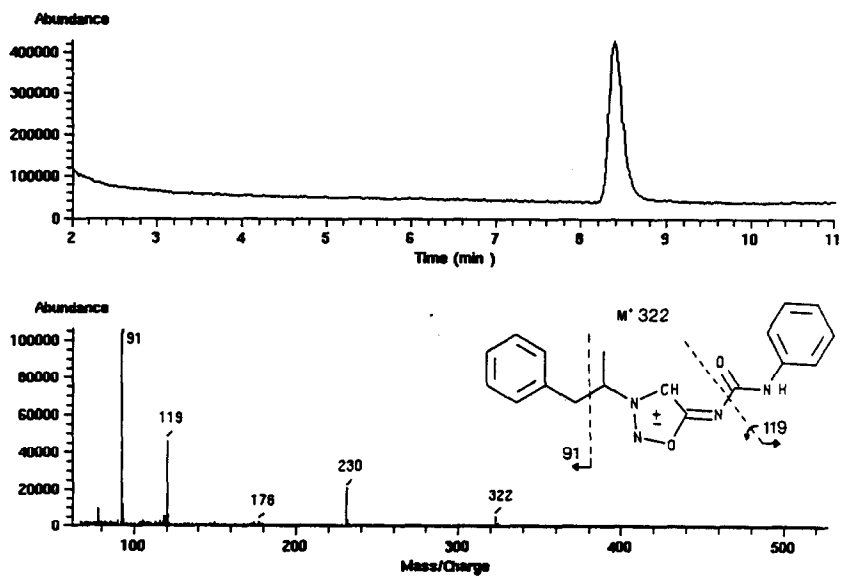

Fig. 4. Analysis by HPLC–MS of a methanolic solution of mesocarb and mass spectrum of the peak obtained.

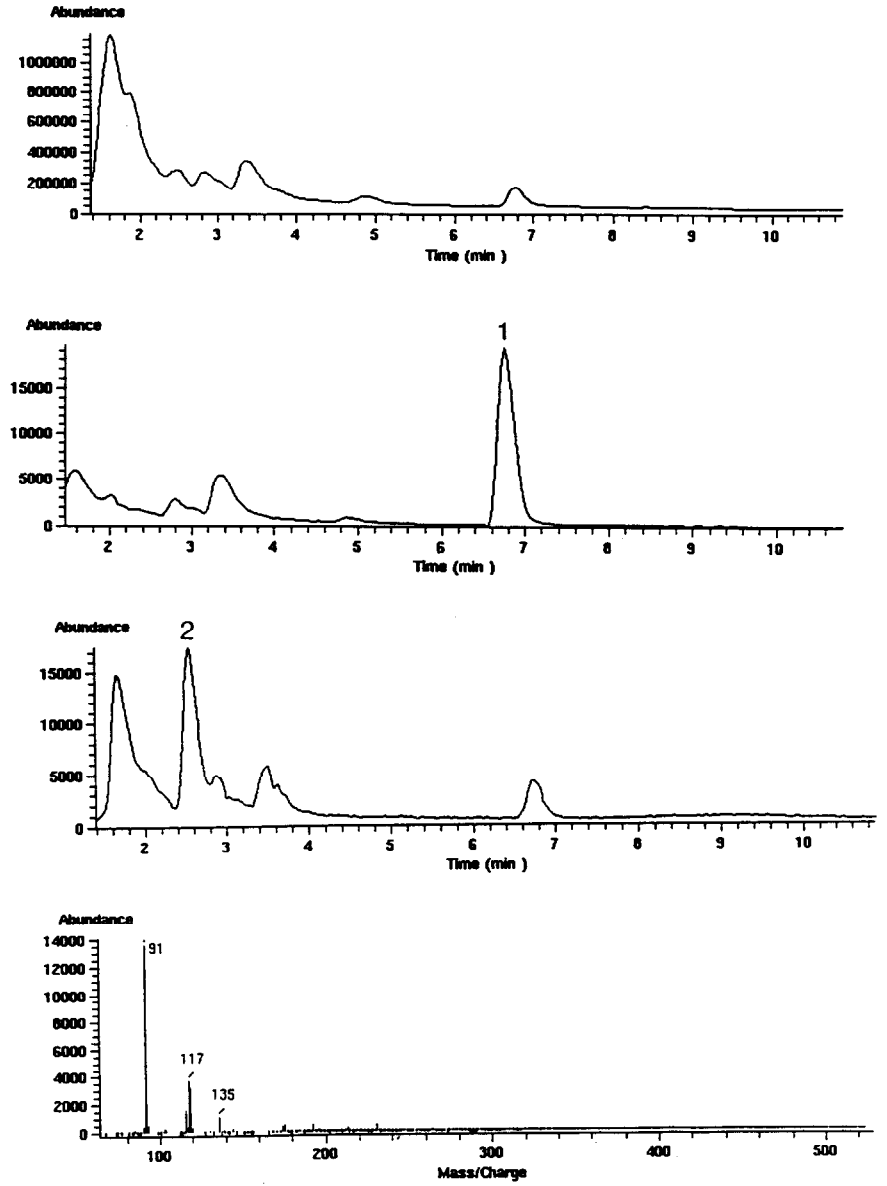

Fig. 5. Analysis by HPLC–MS of a positive urine sample. From top to bottom: total ion chromatogram; chromatogram of m/z 122 (characteristic ion of the ISTD); chromatogram of m/z 91; and mass spectrum of the peak detected at 2.5 min. Peaks: 1 = ISTD; 2 = mesocarb metabolite.

hydroxymesocarb was detected (Fig. 6). In addition to the ions described [2,3], a low-molecular-mass ion (m/z 338) was also observed.

Hence the compound detected in the HPLC–UV screening of non-hydrolysed urines appears to be a conjugate of *p*-hydroxymesocarb. Concentrations of this compound in urines from excretion studies were calculated using the calibration graph for mesocarb ($y = 342.35x + 0.006$, $r = 0.999$, where y is the peak-area ratio between mesocarb and the ISTD and x is the concentration). Results are presented in Table I. The compound can be detected in urine until 48–72 h after intake and accounts for 26.5–

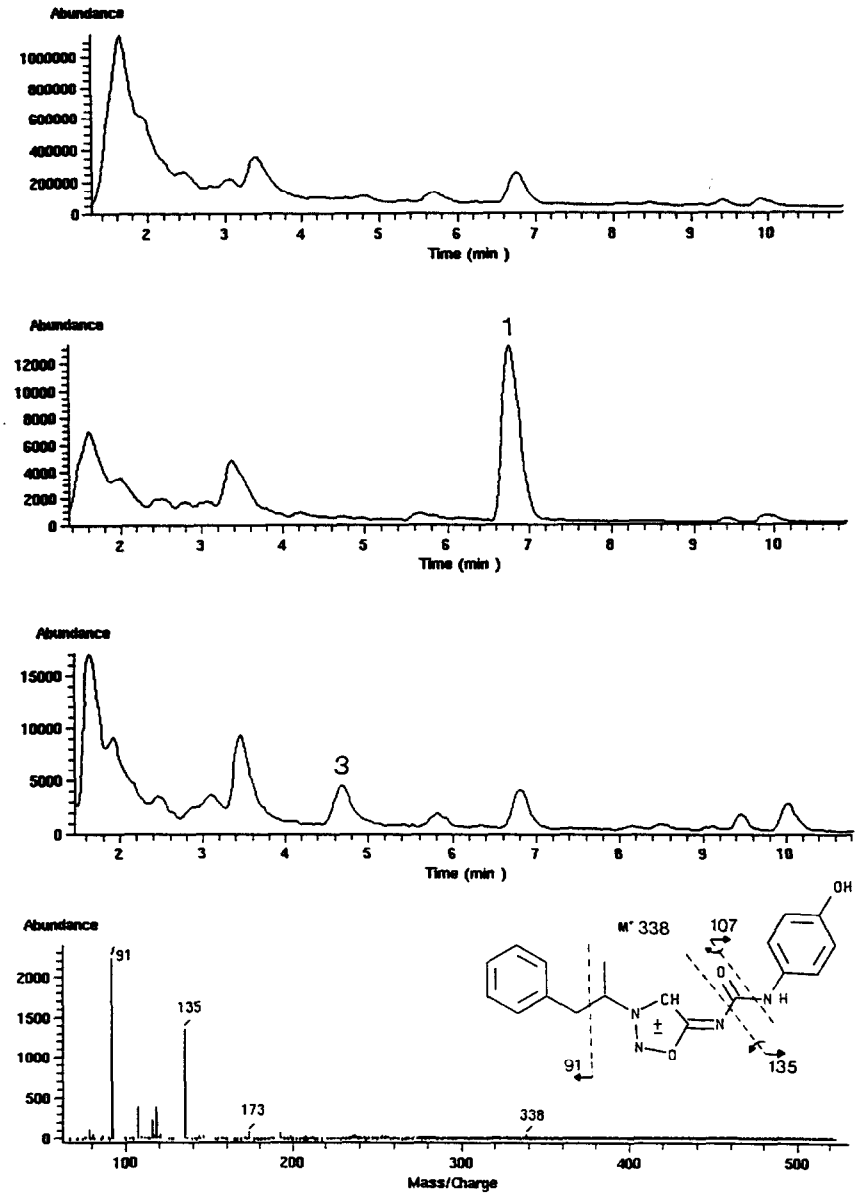

Fig. 6. Analysis by HPLC–MS of an extract from a hydrolysed urine sample. From top to bottom: total ion chromatogram; chromatogram of m/z 122 (characteristic ion of the ISTD); chromatogram of m/z 91; and mass spectrum of the peak detected at 4.6 min corresponding to *p*-hydroxymesocarb. Peaks: 1 = ISTD; 3 = *p*-hydroxymesocarb.

29.4% of the administered dose. Other metabolites found in rat urine, such as free *p*-hydroxymesocarb, unchanged mesocarb and amphetamine [3], were not detected in the samples studied here.

Additional studies

In order to identify the conjugated metabolite, different HPLC–MS assays were performed. EI ionization at low electron energy (12 eV) and chemical ionization (CI) with methane, ammonia

TABLE I

CONCENTRATIONS OF CONJUGATED *p*-HYDROXYMESOCARB DETECTED IN HUMAN URINE AFTER ADMINISTRATION OF 10 mg (31.02 μmol) OF MESOCARB TO HEALTHY VOLUNTEERS

Volunteer	Time (h)	Volume (ml)	Concentration (nmol/ml)	Total excreted (nmol)	% of dose excreted	Cumulative %
A	0–6	400	4.93	1972	6.36	6.36
	6–12	880	1.98	1742	5.62	11.98
	12–24	625	3.69	2306	7.43	19.41
	24–48	1450	2.14	3103	10.00	29.41
	48–72	1180	ND[a]	–	–	29.41
B	0–6	1300	0.99	1287	4.14	4.14
	6–12	1000	1.86	1860	5.99	10.13
	12–24	900	2.17	1953	6.29	16.42
	24–48	1800	1.08	1944	6.26	22.68
	48–72	1300	0.93	1209	3.89	26.57

[a] Not detected.

or isobutane were tested in order to obtain a mass spectrum with the molecular ion of the compound or a characteristic fragment of the conjugated moiety (*e.g.*, *m/z* 194 characteristic fragment of glucuronides described using ammonia CI [7]). No relevant results were obtained.

The hydrolysis of the conjugate was also studied (Fig. 7). Enzymatic hydrolyses under conditions used routinely in our laboratory gave lower yields than acidic hydrolysis (Fig. 7A). After acidic hydrolysis, only *p*-hydroxymesocarb was detected with a recovery of 38.9% and no conjugated *p*-hydroxymesocarb was left in the sample (Fig. 7A). When unchanged mesocarb was subjected to the same conditions of acidic hydrolysis, only 49.9% of the compound was recovered. Mesocarb, and probably *p*-hydroxymesocarb formed after acidic hydrolysis, are thus unstable under these conditions, which explains the low recovery of the acidic hydrolysis.

After enzymatic hydrolysis under our routine conditions, both free and conjugated *p*-hydroxymesocarb were detected; *p*-hydroxymesocarb accounted only for 11.4 and 14.2%, depending on the enzyme used (Fig. 7A). When the sample was incubated in the same conditions without enzyme, no free *p*-hydroxymesocarb was detected, indicating that this compound is formed due to the enzymatic activity.

After longer incubation times (up to 144 h) with β-glucuronidase from *E. coli*, no increase in the amount of free *p*-hydroxymesocarb was observed. However, similar incubation of the urines with β-glucuronidase–arylsulphatase from *H. pomatia* led to complete hydrolysis of the conjugate to *p*-hydroxymesocarb.

When the samples were hydrolysed after extraction of conjugated *p*-hydroxymesocarb from the urines (Fig. 7B), only traces of *p*-hydroxymesocarb were detected when conventional incubation (55°C, 1 h) with *E. coli* was used. On the other hand, conventional incubation (55°C, 3 h) with *H. pomatia* led to nearly complete hydrolysis of the conjugate to *p*-hydroxymesocarb. Further studies are needed to determine the reason (*i.e.*, the absence of interfering salts) for the higher rate obtained when the hydrolysis takes place after extraction of the conjugate compared with that obtained when the hydrolysis is performed directly in the urine.

Based on these results, conjugation of *p*-hydroxymesocarb with glucuronic acid appears to be a minor metabolic route and the conjugate of *p*-hydroxymesocarb extracted from the urines

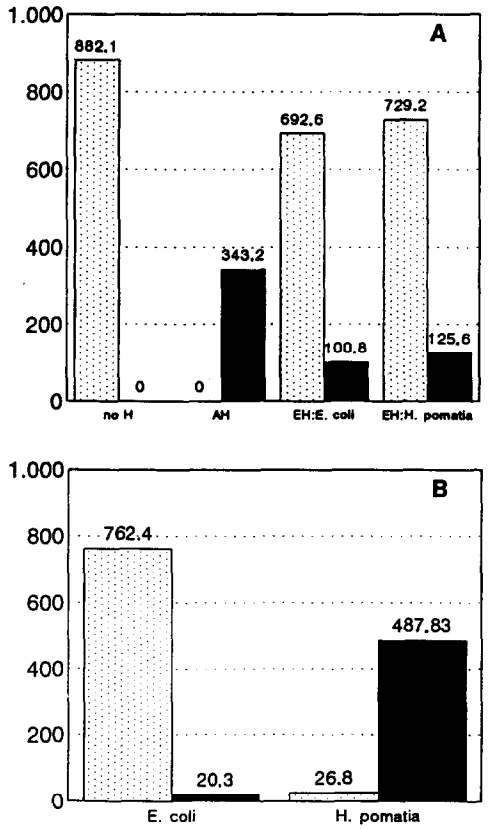

Fig. 7. Mean ($n = 3$) of the areas of the peaks of conjugated (dotted area) and free (solid area) *p*-hydroxymesocarb obtained after hydrolysis studies. (A) Comparison of different hydrolyses of the urines (no H = no hydrolysis; AH = acidic hydrolysis; EH = enzymatic hydrolysis); (B) enzymatic hydrolysis with *E. coli* or *H. pomatia* of the conjugate of *p*-hydroxymesocarb previously extracted from the urine.

and detected in the HPLC–UV screening appears to be a sulphate.

CONCLUSIONS

A HPLC–UV screening method is used routinely in our laboratory to screen for the presence of mesocarb, diuretics and other banned compounds in urine [4]. Its application during the 1992 Barcelona Olympic Games allowed the detection of a real positive case. The confirmation was performed HPLC–MS of the non-hydrolysed and hydrolysed urines, and also by GC–MS with identification of the pyrolysis product. Figs. 2, 3, 5 and 6 correspond to this positive urine. Similar results to those presented in these figures were obtained with control urines from the above-cited excretion studies carried out in the same analytical batch. These results illustrate the utility of the described method to detect the ingestion of mesocarb during routine doping control analyses.

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REFERENCES

1 Medical Commission, International Olympic Committee, *International Olympic Charter Against Doping in Sport*, IOC, Lausanne, 1990, updated December 1991.
2 J. Tamás, M. Polgár, G. Czira and L. Vereczkey, in *Proceedings of the 5th International Symposium on Mass Spectrometry in Biochemistry and Medicine, Rimini (Italy), 1978*, p. 43.
3 M. Polgár, L. Vereczkey, L. Szporny, G. Czira, J. Tamás, E. Gács-Baitz and S. Holly, *Xenobiotica*, 9 (1979) 511.
4 R. Ventura, T. Nadal, P. Alcalde, J.A. Pascual and J. Segura, *J. Chromatogr.*, in press.
5 M. Donike, personal communication.
6 R. Ventura, T. Nadal, M.J. Pretel, A. Solans, J.A. Pascual and J. Segura, in M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke and S. Rauth (Editors), *Proceedings of the 10th Cologne Workshop on Doping Analysis, Cologne (Germany), 1992*, Sport und Buch Strauss, Edition Sport, Cologne, 1993, pp. 231–248.
7 T. Cairns and E.G. Siegmund, *Anal. Chem.*, 54 (1982) 2456.