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Determination of methyl 5-hydroxy-2-benzimidazole carbamate in urine by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay for methyl 5-hydroxy-2-benzimidazole carbamate (5-HBC) in urine was developed in order to assess the exposure of workers to the pesticide carbendazim. 5-HBC is measured in urine after hydrolysis, sample clean-up through a strong cation-exchange (SCX) column and extraction with ethyl acetate. HPLC with electrochemical detection provides selective and sensitive determination of 5-HBC with a detection limit of 5 μg/l. A C_{18} reversed-phase column was used with 0.06 *M* ammonium acetate solution (pH 8)–methanol (73:27) as mobile phase. The method was validated with respect to hydrolysis of urine samples, analytical recovery of spiked 5-HBC, stability of 5-HBC conjugates, limit of detection, background and precision. The overall analytical recovery from urine was better than 60%. 5-HBC, excreted in urine as a conjugate, was stable for at least one year when stored at −20°C. A background of *ca.* 5 μg/l was detected in urine from some non-occupationally exposed persons. Between-day coefficients of variation as calculated from the results of the stability test were 7, 4 and 4% for concentrations of 61, 244 and 295 μg/l 5-HBC, respectively (n = 16).

INTRODUCTION

In The Netherlands *ca.* 30 000 workers are involved in culturing of ornamental plants in greenhouses. They are occupationally exposed to a wide variety of pesticides during application and crop activities, which may represent a health hazard. In order to assess these health risks, the Dutch government formulated the need for exposure studies in these greenhouses. The fungicide carbendazim (methyl 2-benzimidazolecarbamate) was chosen as one of the compounds, as it is frequently used for the protection of flowers and flower bulbs.

Recent studies showed that dermal exposure to pesticides is often 100–1000 times higher than inhalatory exposure [1–3]. To estimate the risk caused by dermal exposure, a knowledge of the rate and amount of skin penetration of pesticides is essential. For this purpose, methods for assessing absorbed amounts of pesticides in humans are needed. With biological monitoring it is possible to assess the actual absorbed amount of a compound in the body independent of its route of entrance. To this end, the concentration of the compound or a relevant metabolite in blood or urine is measured. For the interpretation of biological monitoring data, laboratory studies with volunteers were performed to examine the relationship between dose and excretion. An analytical method for carbendazim or one of its metabolites in urine that could be applied in the laboratory study and subsequently in field studies

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was required. Most of the metabolic studies in animals concerning carbendazim have been performed with benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] which easily decomposes to carbendazim [4–6]. Metabolism studies in rats with [2-^{14}C]carbendazim showed that after oral administration 88% of the radioactivity was eliminated in urine and 11% in the faeces within 48 h [4]. Krechniak and Klosowska [7] reported that 71% of a radiolabelled dose in rat experiments was excreted in urine after intravenous injection within 12 h. The major metabolite in these experiments was methyl 5-hydroxy-2-benzimidazole carbamate (5-HBC), which was present in urine as glucuronide and/or sulphate conjugates, while other possible degradation products in animal systems include methyl 4-hydroxy-2-benzimidazole carbamate (4-HBC) and 2-aminobenzimidazole (2-AB) [5,6]. In addition, 5-HBC also appeared to be the principal metabolite in animal studies with the fungicide thiophanate-methyl [8]. No studies in humans have been published so far.

For the biological monitoring of carbendazim, and if desired benomyl or thiophanate-methyl, a method for determining 5-HBC in urine was developed. Only one paper has been published on the determination of 5-HBC, concerning the matrices cow milk, urine, faeces and tissues [9]. A high-speed liquid chromatographic system consisting of strong cation-exchange resin and a UV detector was used. However, only two to four samples could be analysed per day and the detection limit was 100 μg/l. In the present study, the aim was to develop a method with a detection limit of *ca.* 5 μg/l. This paper describes the development and validation of a high-performance liquid chromatographic (HPLC) method using a reversed-phase column combined with an electrochemical detector for the determination of 5-HBC in urine.

EXPERIMENTAL

Chemicals and materials

Analytical-grade hydrochloric acid (37%), ammonia, ammonium acetate, disodium hydrogenphosphate dihydrate, ethyl acetate, methanol and sodium hydroxide were obtained from Merck (Amsterdam, Netherlands) and 2-aminobenzimidazole (97%) from Aldrich (Bornem, Belgium). Methyl 5-hydroxy-2-benzimidazole carbamate (100%) and methyl 4-hydroxy-2-benzimidazole carbamate were kindly supplied by Nippon Soda (Tokyo, Japan) and DuPont (Wilmington, DE, USA), respectively.

All stock solutions of 5-HBC (100 mg/l) were prepared in methanol. Buffer (0.7 *M*) was prepared by dissolving 125 g of Na_2HPO_4 in 800 ml of water. This was adjusted to pH 11 using 6 *M* NaOH and diluted to 1 l. Mobile phase was prepared by dissolving 5 g of ammonium acetate (0.06 *M*) in 600 ml of water and adjusting the pH to 8 using ammonia (2.5%). Thereafter 270 ml of methanol were added and the mixture was diluted to 1 l with water. Strong cation-exchange cartridges (SCX, 100 mg) (Analytichem International) were purchased from Bètron (Rotterdam, Netherlands).

Apparatus

The HPLC equipment consisted of a Model 6000A pump, an SSI pulse damper (Interscience, Breda, Netherlands), a WISP 712 automatic sampler, a Valco 0.5-μm frit-filter and an RCM-8 × 10 cartridge holder with a 4-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) in series (all except the pulse damper from Millipore/Waters, Etten Leur, Netherlands). The conditions used were mobile phase 0.06 *M* ammonium acetate buffer (pH 8)–methanol (73:27), flow-rate 1.3 ml/min and injection volume 50 μl. Before use, the mobile phase was degassed with helium while stirring with a magnetic stirrer. Detection was carried out with a Model 5100 A coulometric detector from ESA provided with a Model 5010 analytical cell (Interscience). An SSI 0.2-μm carbon filter was placed before the detector (Interscience). The oxidation potential was 0.22 V. Chromatographic data were processed with an SP 4290 integrator coupled via Labnet to a Chromnet data station (Spectra-Physics, Eindhoven, Netherlands). For the determination of pH a portable Model PHM 80 pH meter provided with

a glass electrode (GK 2402B) was used (Radiometer, Copenhagen, Denmark).

Sample preparation

After thawing and mixing, 1 ml of urine was pipetted into a glass test-tube fitted with a ground-glass stopper. Subsequently, 40 μl of concentrated HCl were added and the samples were mixed again (the pH should be between 0.8 and 1.0). The samples were hydrolysed at 100°C in a water-bath for 45 min. The strong cation-exchange columns were conditioned with 4 ml of methanol, 2 ml of water and 3 ml of 0.1 *M* HCl in succession. After hydrolysis and cooling to ambient temperature, the samples were diluted by adding 4 ml of 0.1 *M* HCl. Thereafter the samples were passed through the SCX columns. The columns were washed with 3 ml of 0.1 *M* HCl and eluted with 3 ml of the mobile phase buffer. The eluates were collected in glass test-tubes and mixed with 150 μl of sodium phosphate buffer (the pH of the solution should be between 7.0 and 8.0). Subsequently, extraction with 4 ml of ethyl acetate was carried out for 15 min on a rotary extractor. After centrifugation for 10 min at 1800 *g*, 3.5 ml of the organic layer were pipetted into a glass test-tube containing 0.5 ml of 0.1 *M* HCl. The extraction was repeated once and then 4 ml of ethyl acetate were added to the first extract. 5-HBC was extracted back into the 0.1 *M* HCl layer during 15 min on a rotary extractor. After centrifugation for 10 min at 1800 *g*, the organic phase was discarded. The HCl layer was placed under a gentle stream of nitrogen for 2 min to remove the remaining ethyl acetate. A 50-μl volume was used for HPLC analysis.

Calibration

Calibration graphs were made using blank urine (1 ml) by adding known amounts of 5-HBC stock solution diluted with water (1:100). The samples were taken through the whole analytical procedure as described above. The calibration graphs were constructed by plotting the peak areas against the concentration of 5-HBC in the range 20–1500 μg/l.

Validation of the method

First the retention behaviour and peak shape of 5-HBC were examined on a reversed-phase column with respect to pH (range 5–8) of the mobile phase. Subsequently three detectors were compared, namely a Model LC 95 UV–Vis detector (Perkin-Elmer, Gouda, Netherlands), a Model SFM 25 fluorescence detector (Kontron Instruments, VHI, Maarssen, Netherlands) and an ESA electrochemical detector with respect to sensitivity. The effect of pH (range 1–11) on the extraction recovery of spiked 5-HBC (1340 μg/l) from 0.7 *M* sodium phosphate buffer was examined with ethyl acetate. The recovery was determined by injecting both buffer and ethyl acetate phases. For this purpose, the ethyl acetate phase was evaporated to dryness and the residue was dissolved in 0.1 *M* HCl. In order to validate the method with respect to hydrolysis, limit of detection, stability and precision, urine samples containing 5-HBC conjugates were required. A volunteer (male, body mass 85 kg) was given 2.0 mg (10.46 μmol) of carbendazim suspended in 75 ml of drinking water. Urine was collected just before dosing and in fractions afterwards until 45 h after administration.

Deconjugation of 5-HBC was investigated with respect to pH and hydrolysis time in a water-bath at 100°C. Urine specimens (1 ml) were adjusted to establish a pH meter reading of −0.50 to 2.0 (in steps of 0.25 pH units) using concentrated HCl and hydrolysed for 1 h. In another experiment free 5-HBC was determined after hydrolysis in a water-bath at 100°C for 5, 10, 15, 20, 25, 30, 40, 50, 60 and 90 min. The pH of the highly acidic samples (*e.g.*, pH = −0.5) were not corrected for activity coefficients.

Strong cation-exchange columns were validated with respect to breakthrough and recovery to clean-up the urine samples after hydrolysis at a 5-HBC concentration of 1340 μg/l. Back-extraction from ethyl acetate into 0.1 *M* HCl was investigated at the same concentration.

The overall analytical recovery was investigated by comparing the slopes of calibration graphs in urine from non-occupationally exposed persons following the whole procedure with calibra-

tion graphs made in 0.1 *M* HCl. The stability of 5-HBC conjugates in urine during storage at −20°C was determined using three samples from the volunteer experiment at concentrations of 61, 244 and 295 μg/l. These samples were also used for quality control.

RESULTS AND DISCUSSION

A reversed-phase column instead of a cation-exchange column [9] was used for better control of the retention behaviour and peak shape of 5-HBC. As the mobile phase, 0.06 *M* ammonium acetate buffer (pH 8)–methanol (73:27) was used. Under these conditions the column lifetime is at least 1000 injections. At pH < 5, 5-HBC is protonated, which results in a poor peak shape. At pH 6, 7 and 8 a good peak shape was observed. Electrochemical detection (ED) was chosen because it provided the lowest detection limit, *viz.*, 0.1 ng of 5-HBC at an oxidation potential of +0.22 V. In addition, at pH 8 of the mobile phase, the ED response was increased by a factor of 2 as compared with that at pH 7. The detection limits obtained with the UV and the fluorescence detectors were 1.3 and 0.6 ng, respectively. Fig. 1 shows the voltammogram of 5-HBC.

The extraction recovery of spiked 5-HBC from buffer was better than 95% at pH 6–9 (Fig. 2). Fig. 2 further indicates that at pH > 9, 5-HBC is unstable and that at pH < 2 it is fully protonated and therefore cannot be extracted.

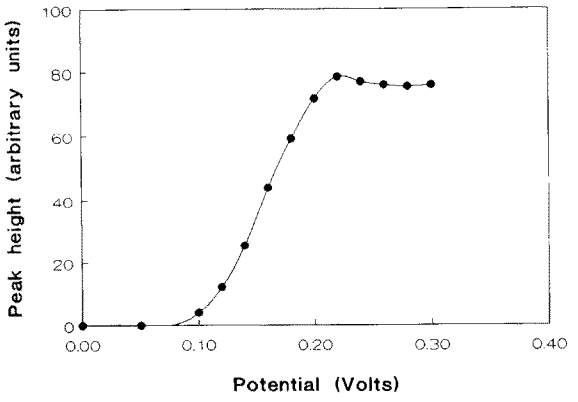

Fig. 1. Voltammogram of 5-HBC. The peak height of 5-HBC is plotted against the potential of the ESA electrochemical detector. For conditions, see text.

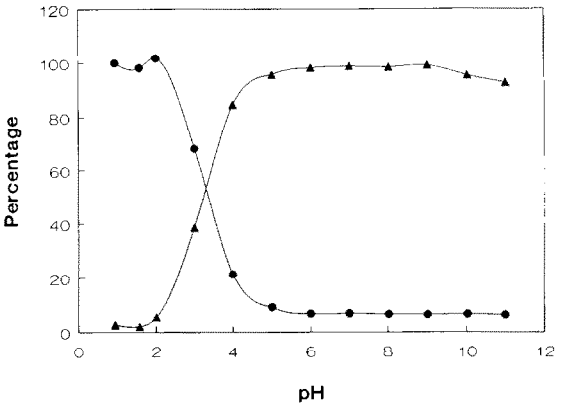

Fig. 2. Distribution of 5-HBC (1340 μg/l; $n = 2$) over phosphate buffer and ethyl acetate at different pH values. ▲ = Percentage of 5-HBC in ethyl acetate; ● = percentage of 5-HBC in buffer.

An optimum was found for deconjugation of 5-HBC at a pH just below 1 by hydrolysing samples from the pilot experiment at different pH values for 1 h in a water-bath at 100°C (Fig. 3). At this pH, 20 min were necessary to reach the maximum amount of liberated 5-HBC. For security a time of 45 min was chosen to deconjugate the samples (Fig. 4). Fig. 4 also indicates that 5-HBC is excreted in urine almost fully conjugated.

Validating the SCX columns with hydrolysed urine samples, breakthrough was observed between 2 and 5 ml of urine, depending on the constitution of the urines. Therefore, routinely 1 ml of the samples was applied to the SCX columns.

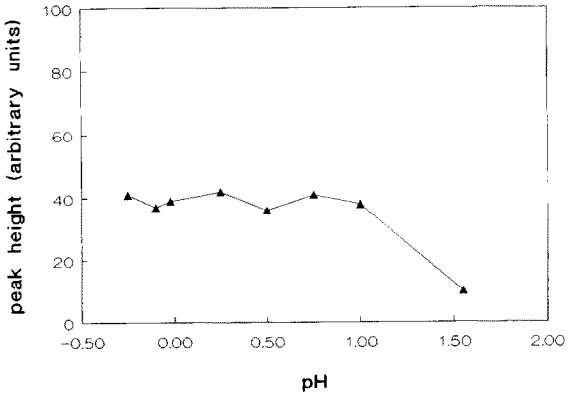

Fig. 3. Relationship between the pH of a urine sample and the amount of deconjugated 5-HBC after 1 h in a water-bath at 100°C.

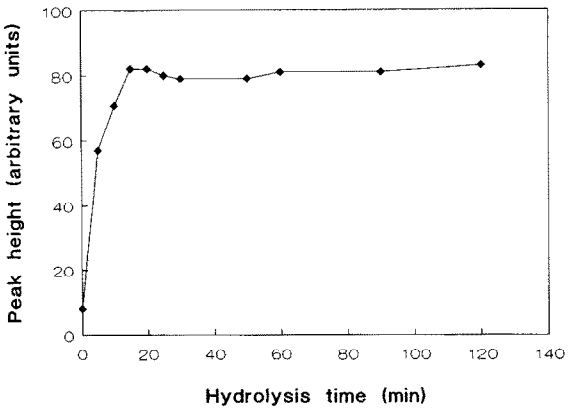

Fig. 4. Relationship between hydrolysis time of a urine sample and the amount of deconjugated 5-HBC in a water-bath at 100°C at a pH between 0.8 and 1.0.

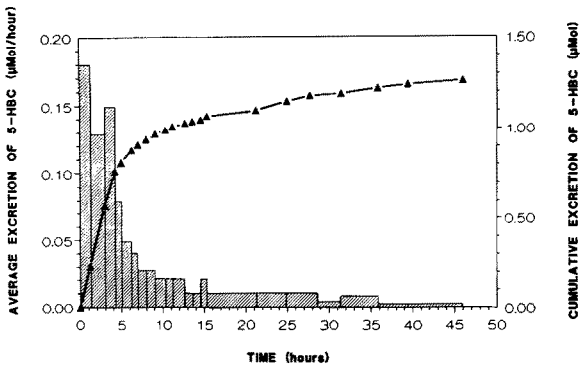

Fig. 5. Average urinary excretion of 5-HBC (μmol/h) (left-hand ordinate) and cumulative excretion of 5-HBC (μmol) in urine (right-hand ordinate) *versus* the time after taking an oral dose of carbendazim (2 mg; 10.46 μmol).

5-HBC was eluted with 3 ml of the mobile phase with a recovery of >90% (n = 2).

For 5-HBC back-extracted from ethyl acetate into 0.1 *M* HCl, a recovery of >80% (n = 2) was established.

Calibration graphs were prepared using blank urines and showed excellent linearity (r > 0.998) up to concentrations of 1.5 mg/l. The overall recovery from urine was >60% comparing the slopes of the calibration graphs for different blank urines (average slope = 0.29, S.D. = 0.03, n = 9) and those for 0.1 *M* HCl (average slope = 0.48, S.D. = 0.04, n = 9).

With some blank urine samples a peak was found with the same retention time as 5-HBC, corresponding to a 5-HBC concentration of *ca.* 5 μg/l. The use of carbendazim for controlling a wide range of fungal diseases in fruits, vegetables, etc., may possibly explain this background level.

The limit of detection, defined as three times the signal-to-noise ratio, was calculated to be *ca.* 5 μg/l. 5-HBC conjugates were stable for at least one year when stored in a freezer at −20°C. Standard solutions of 5-HBC in methanol were stable at room temperature for at least five months when kept in the dark. The between-day coefficients of variation as calculated from the results of the stability test were 7, 4 and 4% for 5-HBC concentrations of 61, 244 and 295 μg/l (n = 16), respectively.

For the subject who received an oral dose of carbendazim, *ca.* 12% of the dose was recovered as 5-HBC (Fig. 5). This percentage was also representative for three other volunteers who were examined. The results of the volunteer studies on the excretion of 5-HBC after oral and dermal administration will be published elsewhere.

A representative chromatogram of a urine sample from an exposed person, after sample treatment, is shown in Fig. 6. The peak with a

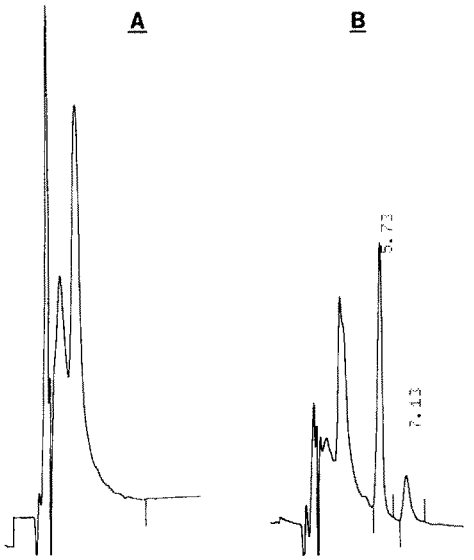

Fig. 6. (A) Chromatogram of a urine extract from a non-exposed person. (B) Chromatogram of a urine extract from an orally exposed person. The peak with a retention time of 5.73 min is 5-HBC (259 μg/l). The peak with a retention time of 7.13 min is probably another metabolite of carbendazim.

retention time of 5.73 min is 5-HBC and that with a retention time 7.13 min may be another metabolite of carbendazim, because it is not present in urine samples from non-occupationally exposed persons. In the urine from the orally exposed subject, we observed that the ratio of the peak height of this compound to the peak height of 5-HBC was *ca.* 1:7 just after dosing and 1:2 at the end of the experiment. Preliminary experiments indicate that this peak is not caused by 4-HBC or 2-AB.

The accuracy of the method cannot be fully evaluated because no external quality control samples are available for 5-HBC conjugates. In addition, pure 5-HBC conjugates were not available for conducting recovery experiments. However, Fig. 4 shows that 5-HBC is excreted almost fully conjugated and that after *ca.* 20 min the deconjugation is complete. This indicates that all conjugated 5-HBC is liberated after hydrolysis.

CONCLUSION

HPLC in combination with electrochemical detection offers a suitable method for the determination of low concentrations of 5-HBC in urine. Routinely 25 samples can be analysed per day. This procedure can be used for studying the toxicokinetic properties of carbendazim, for benomyl and thiophanate-methyl in man, in order to develop biological monitoring methods for these compounds.

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