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DETERMINATION OF THE METABOLITES OF BEZITRAMIDE IN URINE

I. ACIDIC METABOLITE

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SUMMARY

Two methylation methods are compared in relation to the determination of low levels (< μg/ml) of the acidic metabolite of bezitramide in human urine. It was necessary to use alkali flame ionisation detector, which specifically detects nitrogen-containing compounds. Several difficulties associated with the use of this detector are described.

INTRODUCTION

Bezitramide (Burgodin®, Janssen Pharmaceutica, Beerse, Belgium) is a potent, long-acting, orally active analgesic. As the experience of pain by the patient is a complex matter, therapy can be evaluated by following the metabolic fate of the drug. This requires quantitative assay of the drug, its hydrolysis product and its metabolites I and II (see Fig. 1). This figure is based on a research report of Janssen Pharmaceutica[1], in which the distribution and metabolism of bezitramide in the Wistar rat is described. This report describes the determination of metabolite I (3-cyano-3,3-diphenylpropionic acid) in the urine of patients and volunteers.

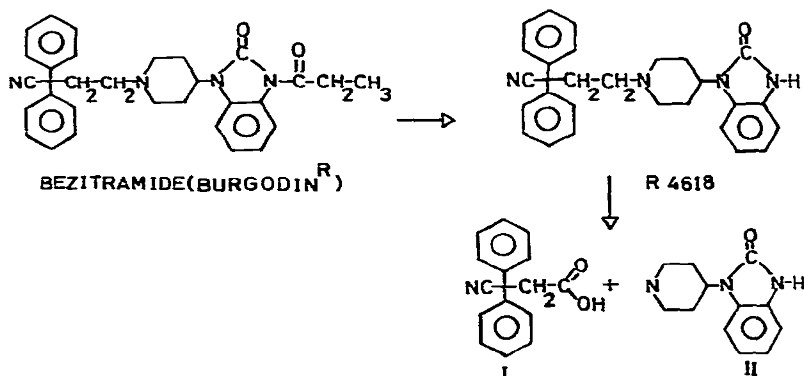

Fig. 1. Main metabolic degradation of bezitramide.

Quantitative procedures for carboxylic acids generally involve gas–liquid chromatography with flame ionisation detection (GLC–FID) after esterification. Many derivative-forming reagents have been described; examples are diazomethane, a highly toxic and dangerous agent, boron trifluoride in methanol and "Methyl 8" (dimethylformamide dimethyl acetal). Conditions for microgram-scale derivative formation are described in this report on the assay of I in urine after administration of therapeutic doses of bezitramide, and two reagents (boron trifluoride in methanol, and "Methyl 8") are compared.

MATERIALS AND METHODS

Reagents

The compound I was obtained from Janssen Pharmaceutica and methaqualone from Merck (Darmstadt, G.F.R.). Pure dimethylformamide dimethyl acetal ("Methyl 8", or DMA) was obtained from Pierce (Rockford, Ill., U.S.A.).

Neutral aluminium oxide (Woelm) and silica gel 60 (70–230 mesh) were Merck products. A 13% solution of the boron trifluoride–methanol complex was obtained from BDH (Poole, Great Britain) and from Merck. The water content was assayed by a modified Karl Fischer procedure[2]. All other chemicals were of analytical grade and were used as purchased.

Stock solutions

A stock aqueous solution of I (40 μg/ml) was prepared by ultrasonification. A stock solution of methaqualone (31.2 μg/ml) was prepared in methanol.

Gas–liquid chromatography

The GLC was performed on a Becker 409 gas chromatograph, equipped with dual FIDs and on a Perkin-Elmer 3920 gas chromatograph equipped with an alkali FID (AFID). Nitrogen (40 ml/min) was used as carrier gas in the Becker instrument, and helium (30 ml/min) in the AFID apparatus. Glass columns (210 $\times$ 0.4 cm I.D.), packed with 3% of OV-17 on Chromosorb W HP (100–120 mesh) (Chrompack, Middelburg, The Netherlands) were used. The injection port of each instrument was maintained at 275°, and the interface temperature in the Perkin-Elmer chromatograph was kept at 300°.

Infrared analysis

The infrared (IR) spectra of I and of its methyl ester were obtained on a Beckman IR20A spectrometer with use of the potassium bromide disc technique. The effects of acid and alkaline hydrolysis on the disappearance of the cyano-group was examined using IR spectroscopy.

Microgram-scale derivative formation of metabolite I

Procedure 1. In a conical glass-stoppered tube containing about 20 μg of I and a known amount of methaqualone as internal standard was placed 25 μl/ml of DMA and 100 μl of dichloromethane as solvent. After the reaction had been allowed to proceed at room temperature for 20 min, 2–3 μl of the mixture were injected into the FID chromatograph by means of a Hamilton syringe.

Procedure 2. In a conical glass-stoppered tube containing 5–10 μg of I and a known amount of methaqualone were placed 10 ml of 14% boron trifluoride solution in anhydrous methanol, and the mixture was heated under reflux for 15 min at 60°, then cooled in ice. After adding 3 ml 0.1 *N* NaOH and 1 ml of benzene, shaking and centrifuging, the benzene layer was removed, 20 mg of silica gel were added, and 5–10 μl of the benzene were injected into the AFID instrument by means of a Hamilton syringe.

Urine samples

Urine samples from patients receiving Burgodin were obtained (through Dr. W. Jansen) from the Department of Anaesthesiology of the Academisch Ziekenhuis, Leiden (Head, Prof. Dr. J. Spierdijk). A 10-ml portion of urine, containing at least 5 μg of I was made alkaline with 2 ml of 4 *N* NaOH, and hydrolysis was allowed to proceed for 1 h at 95° in a glass-stoppered centrifuge tube.

After cooling the mixture, 15 ml of dichloromethane were added, the mixture was vigorously shaken for 1 min, and, after centrifugation at 1732 *g*, 8 ml of the upper aqueous layer was removed and transferred to another centrifuge tube; the dichloromethane layer was evaporated, and the residue was set aside for determination of metabolite II. After adding 1 ml of 7.3% hydrochloric acid and 15 ml of dichloromethane to the aqueous solution, another extraction was made; after centrifugation at 1732 *g* to obtain phase separation, the organic layer was evaporated in a water bath at 50° by means of a stream of nitrogen. The residue was subjected to derivative formation.

Purification of the extract

The above-mentioned residue containing I was dissolved in 0.5 ml of dichloromethane, and the solution is applied to a column (10 × 1 cm) of 3 g of aluminium oxide (neutral). After elution with 20 ml of dichloromethane to remove impurities, elution was continued with 15 ml of dichloromethane containing 5% of glacial acetic acid. This eluate was evaporated to dryness at 40° with nitrogen, and derivative formation was performed as described above.

RESULTS AND DISCUSSION

Derivative formation

As I is non-volatile, derivative formation is necessary. The methyl ester of I gives a symmetrical peak on the GLC column used, and reproducible quantitative assay of microgram and sub-microgram amounts is possible. At 270°, the methyl ester has a retention, relative to the internal standard methaqualone, of 0.688.

The identity of the eluted peak was confirmed by comparing its retention time with that of the methyl ester prepared on the milligram scale with use of the DMA reagent or the boron trifluoride–methanol reagent. The IR analysis of the acid and the ester showed the presence of a CN band at 2260 cm^{-1} in both substances, and in the spectrum of acid a carbonyl-group peak occurred at 1700 cm^{-1}, which was shifted to 1740 cm^{-1} in the ester. One of the problems appeared to be the choice of solvent for the derivative-forming reaction. Methyl acetate gave a clear solution, but a recovery of only 50%; methanol also gave a clear solution, but low recovery and many

minor peaks, and chloroform gave a turbid solution and low recovery (30%). Small drops of fatty material appeared on the glass surface and could not be dissolved by using ultrasonification. However, dichloromethane gave a clear solution and good recovery (85.3% ± 2.3% absolute standard deviation). The absolute standard deviation of the mean was 0.72% ($n = 11$).

The choice of reaction time appeared to be a minor problem; after 20 min at room temperature, the reaction had reached maximum yield. Increasing the temperature was not necessary when dichloromethane was used as solvent.

Hydrolysis of conjugated products

Hydrolysis of the conjugates of I can be accomplished enzymically or by acid or alkaline hydrolysis at 95° during 1 h. The presence of a cyano-group, however, vitiates such drastic treatment, as saponification to the amide and (eventually) decarboxylation may take place.

Investigation of hydrolysis of the acid by extraction and derivative formation after heating for 1 h at 95° in 2 *N* sodium hydroxide led us to the conclusion that only unidentified peaks with retention times shorter than that of the peak for the ester of I appeared; the areas of these peaks amounted to 3.2% of the area of the peak for the methyl ester of I. After 4 h at 95° in 2 *N* sodium hydroxide, the area of the unidentified peaks was 11% of that of the peak for the methyl ester of I, and the area of this peak was also diminished. Use of the nitrogen-selective AFID showed that the hydrolysis product contained a nitrogen atom. The residue after hydrolysis gave an IR spectrum identical to that of the methyl ester of I. Whether or not hydrolysis of the conjugates is complete is uncertain, as no reference material is available. Standardization of the procedure will at least allow comparison of the results from different urine samples.

Extraction and purification of the sample

We obtained clean extracts, which permitted us to determine a level of at least 2 μg/ml of I in urine; quantitation at lower levels was prevented by urinary excretion products.

Calculation of the yield by using direct methylation of a known amount of acid gives a recovery value of 92.6%. The amount of I in urine can be assayed by using a calibration graph of the peak heights of the methyl ester of I and methaqualone against the amount of I present in urine (see Fig. 2, in which the parameters of the calibration lines are also shown). In order to obtain cleaner extracts (while we were using the FID instrument), we tried to purify the urine by column chromatography as described by Brownsill *et al.*[3]; however, recovery of I was only *ca.* 66%, and removal of the background caused by urinary substances was much inferior to that achieved by solvent extraction.

By using the AFID, as little as 0.6 μg/ml can be determined. The explanation of this lower detection limit lies in the third extraction, in which benzene is used as solvent, as well as in the specificity and superior sensitivity of the nitrogen detector. Also, the removal of coloured substances by means of the silica gel lowers the detection limit.

A drawback of this method is the low yield (30 to 40%) of the methyl ester when boron trifluoride in methanol is used. Assay of the water content of the two reagents in order to get an explanation of the low yield yielded nothing conclusive,

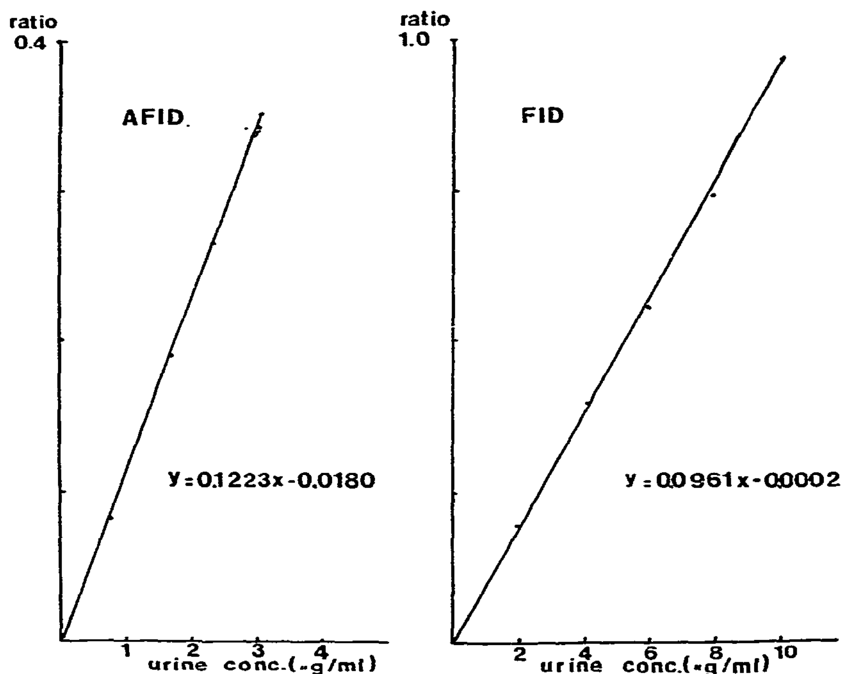

Fig. 2. Calibration graphs using FID and AFID detection. Statistical parameters obtained by linear regression are shown.

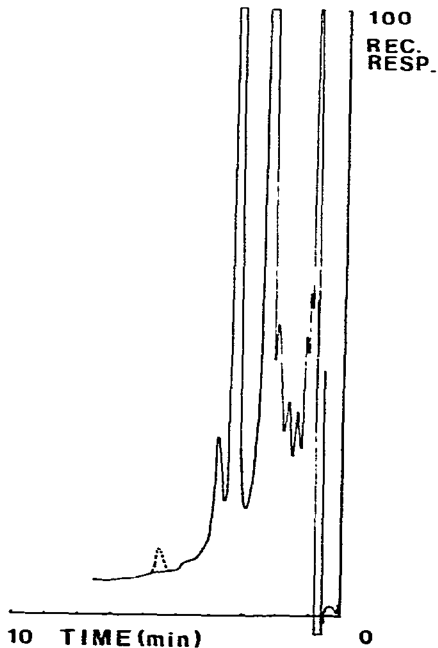

Fig. 3. Chromatogram of a urine sample using AFID detection. The dashed line shows the peak obtained from 0.5 μg/ml of I in urine. (Attenuation 32).

and increasing the reaction time produced no effect. Adaptation of the dimethyl acetal method for the AFID by evaporating the dichloromethane as well as the reagent itself led to serious loss of the methyl ester.

Fig. 3 shows a chromatogram of the background of the urine and a chromatogram showing the peak height of an amount of I in agreement with a urinary level of 0.5 μg/ml. Fig. 2 shows the calibration graph for 0–3 μg/ml of I in urine. From this graph, it can be calculated that the minimum amount of the dose excreted in urine in the form of I that can be detected is 10%. In this calculation, we assume a daily dose of 10 mg and a total 24-h urine production of 750 ml. Results of the excretion of I, together with the excretion of metabolite II, will be published in a forthcoming article.

ACKNOWLEDGEMENTS

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