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DETERMINATION OF THE METABOLITES OF BEZITRAMIDE IN URINE

II. THE BASIC METABOLITE

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SUMMARY

A high-performance liquid chromatographic method for the determination of low levels (less than 1 μg/ml) of the basic metabolite of bezitramide, 1-(4-piperidinyl)-1,3-dihydro-2H-benzimidazol-2-one, in human urine is described. Special attention is given to the separation from the basic metabolite of droperidol, a drug frequently co-administered with bezitramide.

INTRODUCTION

Bezitramide (Burgodin[R]) is a potent, long-acting, orally active analgesic. As the relief of pain induced by the drug and experienced by the patient is a complex matter, depending on such factors as resorption and metabolic fate of the drug, drug therapy can be evaluated by monitoring the drug in the body and measuring the final accumulation of its metabolites in the urine. These measurements require quantitative methods for the assay of the drug, its hydrolysis product and its metabolites I and II [1-(4-piperidinyl)-1,3-dihydro-2H-benzimidazol-2-one] (Fig. 1).

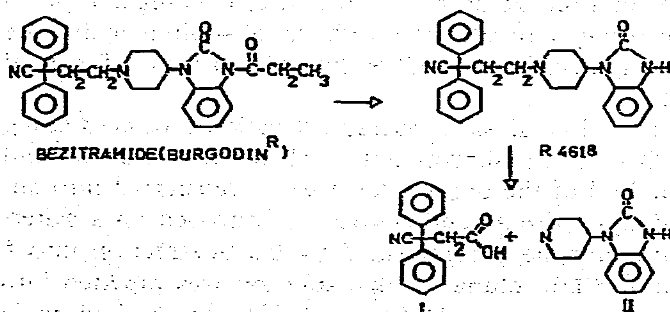

Fig. 1. Main metabolic degradation of bezitramide.

Fig. 1 is based on a report by Heykants[1] in which the distribution and metabolism of bezitramide in the Wistar rat are described. The present paper describes the determination of II in the urine of patients and volunteers. An assay is described that makes use of the high molecular extinction coefficient at 280 nm. Separation is achieved by means of reversed-phase high-performance liquid chromatography (HPLC) using a modified silica as adsorbent. No other method for the assay of II has hitherto been reported.

MATERIALS AND METHODS

Metabolite II and its analogues III and IV (Fig. 2), which are metabolites from the neuroleptic drugs droperidol and clopimozide, respectively, were obtained from Janssen Pharmaceutica (Beerse, Belgium) (see also Fig. 3).

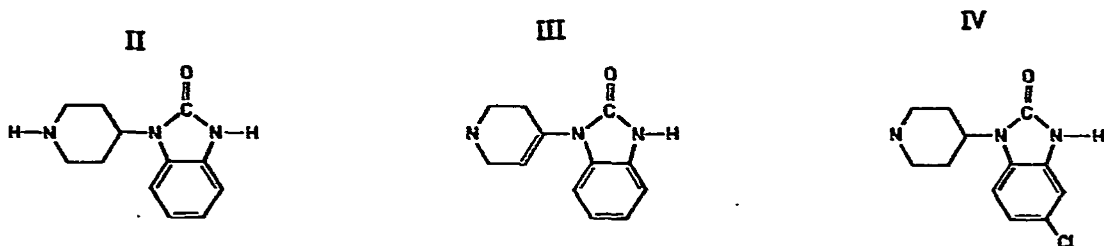

Fig. 2. Chemical structures of the basic metabolite of bezitramide and its analogues.

All organic solvents used were of analytical-reagent grade or better. LiChrosorb RP-2 and LiChrosorb Si-60 (5 and 10 μm) were obtained from Merck (Darmstadt, G.F.R.). Buffer solutions were prepared from Merck Titrisols. A buffer solution of pH 10.6 was prepared by adding 0.1 *N* sodium hydroxide to 0.05 *M* sodium hydrogen carbonate solution.

Stock solutions of II and its analogues (30 mg per 100 ml) were prepared by first wetting the substances with a drop of methanol and subsequently adding the required volume of water.

Sample extraction procedure

Urine samples were stored in a refrigerator at −10°. After thawing and ultrasonication, 30 ml of urine were brought to pH 10.6 with 0.1 *N* sodium hydroxide solution or with 0.1 *N* hydrochloric acid in case of the hydrolysed urine. The final volume was made up to 50.0 ml.

A 5-ml volume was pipetted into a glass-stoppered extraction tube containing 5 ml of an extraction solvent [chloroform–isopropanol (85:15)]. After shaking and centrifuging at 1732 *g* for 5 min, 3 ml of the organic layer were transferred into another tube and evaporated to dryness with a gentle current of nitrogen on a water-bath of 60°. Sample purification could be accomplished by back-extraction into 5 ml of 0.01 *N* hydrochloric acid. A 3-ml volume of this solution was pipetted into an extraction tube and, after alkalinization to pH 10.6 with 4 *N* sodium hydroxide solution and adding 2 ml of buffer solution (pH 10.6), the mixture was extracted

with 5 ml of chloroform–isopropanol (85:15). A 3-ml volume of this organic layer was evaporated to dryness with a gentle current of nitrogen on a water-bath at 60°. To the residue 100 μl of mobile phase were added and, after ultrasonication, 10 μl of this solution were injected into the chromatograph. Standardization of the extraction procedure was accomplished by adding a known amount of II to a pooled blank urine sample. A standard solution of II in the mobile phase was injected several times a day.

High-performance liquid chromatography

HPLC was performed by using an M6000 pump, a U6K injector and a multi-wavelength UV detector, Type 440 (Waters Assoc., Milford, Mass., U.S.A.). The column length was 250 mm and the inner diameter 3 mm. The signal was recorded on a Philips PM 8100 recorder.

The columns were packed with LiChrosorb RP-2 (10 μm) using a balanced slurry technique as described by Kraak *et al.*[2]. The mobile phase used in the final determination of II was acetonitrile–methanol–isopropylamine (930:70:5). The mobile phase flow-rate was 6.9 mm/sec. The detector wavelength was 280 nm.

pK_a determination

The pK_{a_2} determination of the benzimidazolone part of the molecule was carried out on a Unicam SP800 UV spectrophotometer and on a Zeiss PMQ II spectrophotometer.

A titration assembly consisting of the following parts was used for the determination of the pK_{a_1} value of the piperidine part of the molecule. A microburette (Metrohm E457), capable of delivering microlitre volumes was filled with 0.1000 *N* hydrochloric acid. A titration vessel containing the glass electrode and the calomel reference electrode was thermostated at 20.0°. Nitrogen could be led into the vessel. The nitrogen was purified by bubbling it through Fieser's solution, 2 *N* sulphuric acid and water (twice). The pH was measured using a Radiometer Type 26 pH meter.

Hydrolysis of conjugates

Alkaline hydrolysis using 1 ml of 4 *N* sodium hydroxide solution and 3 ml of urine was used for the determination of the percentage of conjugate products. Hydrolysis was carried out at 95° for 1 h.

Column clean-up of the urine

A column clean-up method using Extrelut (Merck) was applied to 10 ml of urine. After elution with about 40 ml of chloroform–isopropanol (3:1), the eluate was evaporated to dryness and the residue was treated as described in the sample extraction procedure.

RESULTS AND DISCUSSION

To determine the amount of II in urine, it is necessary to know such properties as the pK_a, extractability and chromatographic behaviour. It is also essential to compare the selectivity of the method with respect to the structurally related compounds III and IV. III is the basic metabolite of droperidol, a neuroleptic drug which fre-

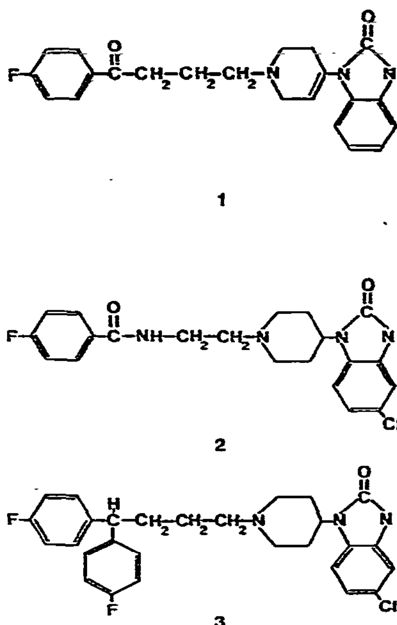

Fig. 3. Chemical structures of droperidol (1), halopemide (2) and clopimozide (3).

quently is co-administered with bezitramide. IV is the basic metabolite of the anti-psychotic drug halopemide[3] (see also Fig. 3).

A second consideration in the determination of II is the detection limit of the method in relation to the dose of the parent compound. The daily dose of bezitramide varies from 5 to 15 mg, giving urine concentrations of the metabolite in the range 3–10 μg/ml, assuming a mean 24-h urine production of 750 ml. This assumption is based on the complete absorption of the parent drug from the gastro-intestinal tract. It should be mentioned, however that the parent drug, and also its hydrolysis product, is insoluble in water and in 0.01 *N* hydrochloric acid. This means that the dissolution rate could be a determining factor in the absorption of the parent drug. As the absorption will be less than 100%, lower concentrations of II in urine can be expected.

An indication of this slow release of the parent drug from the dosage form is the long delay in the onset of pharmacological action. This delay is 1.5–2 h and can be regarded as long in comparison with water-soluble drugs such as atropine sulphate. This means that the concentration range in urine can be substantially lowered as a result of this slow release of the parent compound, especially when rapid intestinal passage occurs. A range of 0.3–10 μg/ml must therefore be considered when developing an analytical method.

pK_a values

II is an amphoteric substance possessing a basic nitrogen and an amide function which can enolize (Fig. 4). Table I gives the pK_a values of the three substances. The pK_{a_2} value of the keto–enol group can easily be determined by using UV spec-

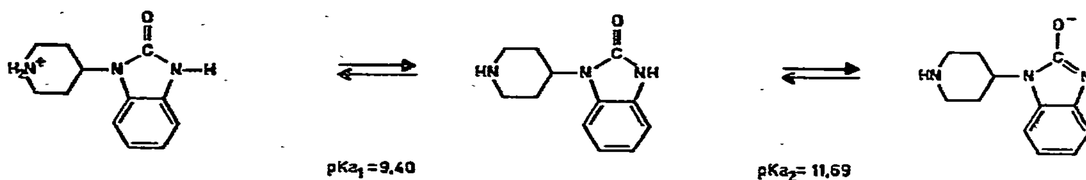

Fig. 4. Ionization of metabolite II.

TABLE I

pK_a VALUES OF COMPOUNDS II, III AND IV

Compound	pK_{a_1}	pK_{a_2}
II	9.40	11.69
III	9.12	11.74
IV	8.60	11.52

troscopy in three different buffer solutions according to Albert and Serjeant[4]. Fig. 5 shows the UV spectrum of II. It is clear that the ionization of the benzimidazolone part of the molecule causes a bathochromic shift at higher pH. This part of the molecule is a fairly strong chromophoric group, which has a molar extinction coefficient of about 6600 at 280 nm. This will permit a detection limit of the order of 1–10 ng, depending on the noise level of the detector.

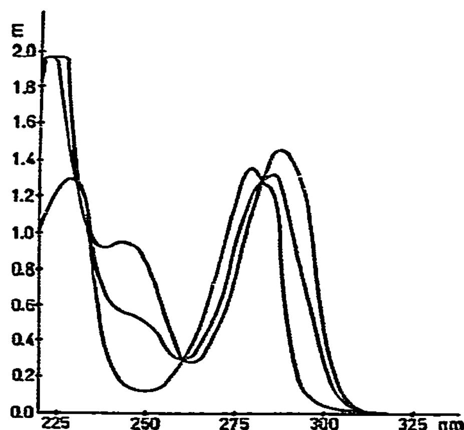

Fig. 5. UV spectrum of metabolite II in three different buffer solutions (pH 9, 11 and 13). Note the bathochromic shift from pH 9 to pH 13.

Selection of chromatographic system

Straight-phase systems gave no separation of the three compounds of interest. A reversed-phase separation using LiChrosorb RP-2 was therefore considered.

Acetonitrile containing 0.25% of ammonia separated the three substances, but the capacity factor of metabolite II was too high ($k = 9.25$) and a strongly tailing peak resulted in this mobile phase. Therefore, we replaced the ammonia with *n*-

propylamine and isopropylamine[5]. The resulting peak shape was much more satisfactory than with ammonia as the base, but with n-propylamine a considerable decrease in the selectivity factor was observed which was independent of the concentration of the base. With isopropylamine the selectivity factor was adequate, but was dependent on the concentration of the base. Adequate selectivity was obtained at a base concentration of 0.05%, but the capacity factor of II was too high to permit low detection levels.

A third component was therefore desirable. Methanol appeared to be a good choice as it did not have a strong influence on the selectivity and improved the peak shape. Table II shows the capacity factors and the selectivity parameter, α. On the basis of this table, we chose the mobile phase for the determination of II.

TABLE II

CAPACITY FACTORS (k') AND SELECTIVITY PARAMETERS (α) WITH ACETONITRILE–METHANOL MIXTURES CONTAINING 0.05% OF ISOPROPYLAMINE

Compound	*Acetonitrile:methanol ratio*									
	100:0		*97:3*		*94:6*		*90:10*		*80:20*	
	α_{ij}	k'	α_{ij}	k'	α_{ij}	k'	α_{ij}	k'	α_{ij}	k'
II		3.8		2.7		2.0		1.4		1.0
	1.44		1.48		1.50		1.50		1.40	
II		5.5		4.0		3.0		2.1		1.4
	1.12		1.14		1.11		1.10		1.08	
IV		4.9		3.5		2.7		1.9		1.3

Extraction and purification

The extraction of II, III and IV could be accomplished by using polar solvents such as butanol or chloroform–isopropanol. Diethyl ether and ethyl acetate did not appear to be useful extractants because of their relatively large amounts of impurities and the relatively high solubility of water in them. The extraction was carried out at pH 10.6, the mid-point between the two pK_a values, where the substance is in the unchanged form. Acid backwashing into 0.01 N hydrochloric acid appeared to be favourable in relation to the background of the urine (Fig. 6). Isolation from urine using an Extrelut column resulted in irreproducible losses of 30–40%.

The recovery of II from urine (1 μg/ml) buffered at pH 10.6, using a single extraction with an equal volume of chloroform–isopropanol (85:15), was 85.6% $\pm$ 2.5%. Using an acid wash, losses of II can be compensated for by taking a larger sample volume. The lower limit of the determination of II in urine is 150 ng/ml, which is sufficient for our purposes. Urine samples from patients receiving bezitramide contained the metabolite in concentrations varying from 0.150 to 0.300 μg/ml. No conjugation of II was observed.

CONCLUSION

The results of the assay of the bezitramide metabolite II show that this metabolite can easily be determined down to 150 ng/ml in urine. The method can be

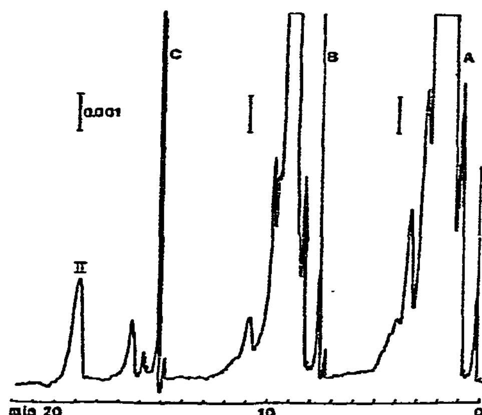

Fig. 6. Three chromatograms obtained by a single extraction of urine (A), a single extraction of urine with an acid backwashing (B) and by a standard solution containing 100 ng of II (C).

used in the evaluation of the fate of Burgodin in the human body. This method is to be preferred to the previously described method[6] for the determination of the acidic metabolite in urine as it is more sensitive.

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