Biochemical Pharmacology, Vol. 30, No. 24, pp. 3381-3384, 1981.
Printed in Great Britain.

0006-2952/81/243381-04$02.00/0

PRELIMINARY COMMUNICATIONS

PH DEPENDENT FORMATION OF β-GLUCURONIDASE RESISTANT CONJUGATES FROM THE BIOSYNTHETIC ESTER GLUCURONIDE OF ISOXEPAC

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(Received 14 September 1981 ; accepted 23 September 1981)

Isoxepac (6,11 dihydro-11-oxo-dibenz (b,e) oxepin-2-acetic acid) is a potent non-steroidal anti-inflammatory agent [1,2]. In man the drug is excreted mainly (>90%) in the urine, largely conjugated to glucuronic acid. The conjugate was identified as a glucuronide of isoxepac by hydrolysis by β-glucuronidase from Helix pomatia, and specific inhibition of the enzyme with the appropriate aldonolactone [3].

During the development of an HPLC procedure for the separation of isoxepac and its conjugates we noted the presence of up to four polar, drug related peaks in the "conjugate" portion of the chromatogram. The number of peaks observed seemed to depend, in part, on the age of the sample. Fresh urines were therefore obtained during a clinical trial (dose 100 - 400 mg orally), stored at -20°, and analysed immediately upon thawing. Two major peaks were observed, corresponding to isoxepac and its glucuronide. The glucuronide peak was identified using specific (aldonolactone-inhibited) hydrolysis with β-glucuronidase. On standing at room temperature for 3-4 hours, up to four additional conjugate peaks appeared, at the expense of the glucuronide (Figure 1). The amount of isoxepac remained relatively unchanged. Increasing pH accelerated the formation of these additional peaks, although at high pH (>8.0) hydrolysis to free isoxepac became more important (Figures 1 and 2). The rearrangements were apparently non-reversible on acidification (data not shown). All the products were readily hydrolysed to isoxepac on treatment with alkali (20 µl of 5M-NaOH ml^{-1}, 2-3 minutes at room temperature). Thus it appears that all the peaks were ester type conjugates of isoxepac and a base catalysed rearrangement took place before hydrolysis to the free drug.

Incubation of the conjugate mixture with β-glucuronidase resulted in the specific hydrolysis of only one peak, the principal conjugate present in the original sample (Peak 5, Figure 1), to isoxepac (Table 1).

As ester glucuronides are generally labile in the presence of mild alkali [4] increases in urinary pH, such as may occur on standing, or following antacid treatment, will result in both enzymically refractory products, and artefactually high levels of free drug. The formation of enzyme refractory products could result in misidentification of the nature of the conjugate if this depended on β-glucuronidase hydrolysis. In addition, use of enzymic hydrolysis would result in underestimation of total isoxepac, with important consequences for bioavailability studies.

The resistance of the rearranged conjugates to β-glucuronidase could be due to the specificity of the enzyme for 1-β-D glucuronides. The phenomenon being observed may be transacylation of the glucuronic acid. The endogenous compound, bilirubin, forms an ester glucuronide which, both in vitro

TABLE 1 : The effects of β-glucuronidase from H. pomatia on the conjugates of isoxepac present in urine

Peak No.	EXPERIMENT 1				EXPERIMENT 2					
	Peak area (arbitary units) at start	Time of Hydrolysis (hours)			Peak area (arbitary units)		Time of Hydrolysis (hours)			
		0.5	2.0	2.0(C)	before alkali	after alkali	0.5	2.0	4.0	4.0 (C)
		Peak area (% of initial value)					Peak area (% of initial value)			
1 + 2	0.5	← Not measured →			1.3	8.5	100	99	83	102
3	4.8	104	87	98	5.9	44.3	95	80	80	94
4	42.3	106	105	91	52.7	142.0	99	85	87	92
5	133.8	70	8	96	357.4	66.9	68	15	1	89
Isoxepac	52.6	154	241	95	76.4	69.0	127	152	163	104

Urines were brought to pH 5.2 with acetic acid and incubated with 2.0 $\mu l\ ml^{-1}$ of β-glucuronidase from H. pomatia (type H-2; Sigma Chem. Co., Poole, Dorset) at 37°. Control incubations (C) contained no enzyme. Aliquots were withdrawn at the times shown for HPLC analysis. Peak numbers are those assigned in Figure 1. In Experiment 2, urine was aged at pH 8.0 for 3 hours prior to treatment with β-glucuronidase.

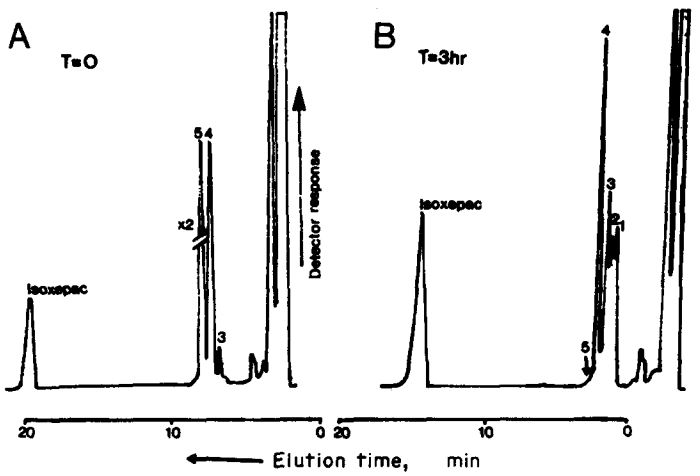

FIGURE 1 : HPLC of isoxepac and conjugates

Urine was brought to pH 8 with sodium hydroxide. Chromatogram (a) was before incubation, chromatogram (b) after 3 hours at 20°. Chromatographic conditions: column - 15 x 0.3 (i.d.) cm packed with 5μ ODS-Spherisorb; solvent - 72.8% water, 0.2% phosphoric acid, 27% acetonitrile detection - uv at 254 nm.
Peaks 1 - 5 are the isoxepac conjugates.

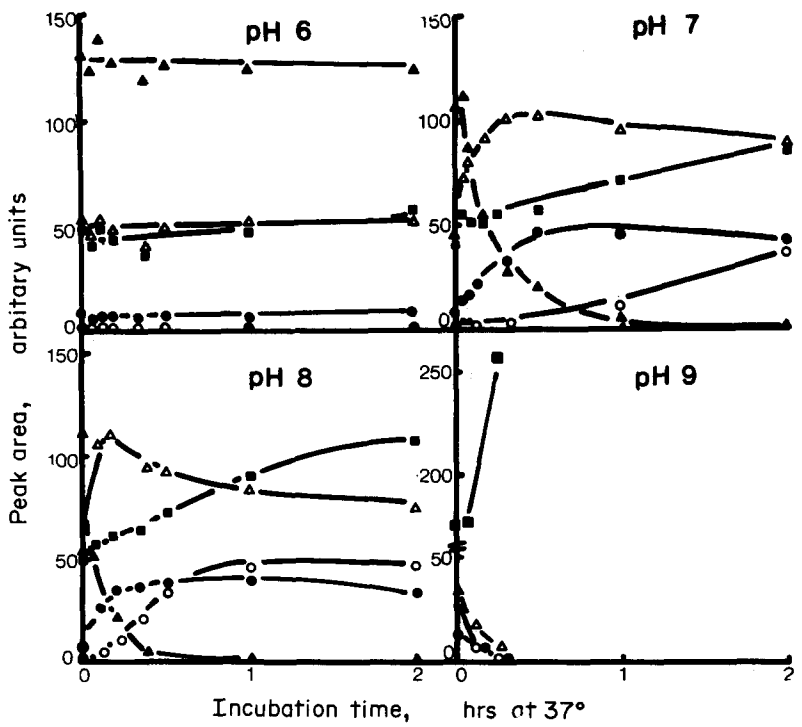

FIGURE 2 : The effect of pH on isoxepac conjugate HPLC patterns in urine

Urine was bought to the appropriate pH using hydrochloric acid or sodium hydroxide. Serial aliquots were analysed by HPLC (Figure 1). A Spectra-Physics integrator was coupled to the detector to measure peak areas. Peaks 1 and 2, ○ peak 3, ● peak 4, △ ▲ peak 5, ■ peak 6 (isoxepac)

and in bile, undergoes rearrangement from the 1-O-acyl to the 2-O-acyl, 3-O-acyl and the 4-O-acyl glucuronides all of which are resistant to enzymic hydrolysis [5,6,7]. Preliminary reports indicate that the 1-O-acyl glucuronide of clofibric acid may undergo similar rearrangements [8,9]. Such rearrangements appear, therefore, to be general properties of ester glucuronides and suitable precautions should be observed during their isolation and analysis.

Acknowledgements

The technical assistance of E. House and A. Bhatti is gratefully acknowledged.

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