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SEPARATION AND IDENTIFICATION OF URINARY METABOLITES OF ^{14}C-*p*-*n*-BUTOXYPHENYLACETHYDROXAMIC ACID IN MAN*

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Abstract—The metabolic fate of ^{14}C-*p*-*n*-butoxyphenylacethydroxamic acid (Droxaryl)® is studied in Man after oral and rectal administration of the drug. The fractionation of the urinary metabolites was undertaken by ion-exchange chromatography on DEAE-Sephadex A-25 columns (0·01 M Tris-Cl buffer, pH 7·2, elution with a linear gradient of NaCl). The identification of the degradation compounds shows that the main metabolic routes seem to be the conjugation with glucuronic acid (about 70%) and with sulfuric acid (about 5–10%). Hybrid compounds cannot be excluded. Important degradation reactions occur also in the functional group of Droxaryl. The main difference in the metabolic behaviour of Droxaryl after oral or rectal administration in Man is the higher amount of unchanged drug in the urine of rectally treated patients.

EARLIER studies on the metabolic behaviour of *p*-*n*-butoxyphenylacethydroxamic acid (code name: CP 1044 J3 or DROXARYL®‡) carried out in animals orally treated by the ^{14}C-labelled compound, have shown that many metabolites can be expected in urine.[1]

These assays were undertaken by thin layer chromatography (TLC). Unfortunately this technique does not allow a good isolation and a good quantitation of these derivatives in the total urine and consequently other methods of separation have to be used.

Drugs containing aromatic rings in their molecule are very often metabolically converted into their hydroxylated derivatives; these appear in urine essentially as glucuronic and sulfuric acid water soluble esters. This may be expected in the case of Droxaryl and implies thus the choice of specific methods.

According to Weisburger[2] and Grantham[3] a good resolution of the urinary metabolites of some drugs could be obtained by ion-exchange chromatographic procedures. Grantham[3] reviewing this problem recently suggests a new chromatographic system which involves DEAE-cellulose as exchanger and formic acid-ammonium formate as buffer and gradient at pH 3·5. This system has the following main advantages: (1) generally irreversible adsorptions do not take place, (2) water-insoluble derivatives are practically not fixed on the exchanger, (3) buffer components can be easily removed by sublimation. We adopted similar methods for the study of the metabolic fate of Droxaryl in urine of Man.

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TABLE I. HUMAN URINE. CHROMATOGRAPHIC SYSTEMS USED WITH DEAE-SEPHADEX A-25

Volume of chromatographed urine* (ml)	Total radioactivity applied to the column (D.P.M.)	Buffer	pH	Shape and limits of the gradient	Pre-development with dist. water (ml)	Development with the gradient (ml)	Flow-rate (ml/hr)	Volume of the collected fractions (ml)
100	$1{\cdot}56 \times 10^6$ for capsules	(1) Tris-HCl 0·01 M.	7·2	linear, NaCl 0·05→0·5 M.	200	1600	80	10
(reduced to 10 by lyophilization)	$1{\cdot}11 \times 10^6$ for suppositoria	(2) Formic acid—Ammonium formate	3·6	linear, Ammonium formate 0·05→0·5 M.				

* Urine is adjusted to the pH of the starting buffer.

In this paper we shall discuss the separation and the identification of the major classes of Droxaryl metabolites in Man. We will distinguish: unconjugated or free metabolites and conjugated forms, that is to say, glucuronate and sulphate esters.

EXPERIMENTAL

Biological sampling is undertaken as follows[4]: specimens of urine for chromatographic purposes were available from a male patient, 69-yr-old, with normal hepatic and renal functions who received a single 750 mg dose of Droxaryl in capsules and a week later the same dose in suppositoria. In both cases, each micturition was collected and the urine pooled for a 48-hr-excretion period. Samples were kept in a freezer. Chromatographic methods used are summarized in Table 1.

DEAE-Sephadex A-25 is prepared as advised by Pharmacia (Sweden). The exchanger is suspended in the chosen buffer at its starting concentration and then washed until the resistivity value of the washing is close to that of distilled water. The glass column (2·5 × 45 cm) is filled by a continuous flow of the gel. This flow is stopped when the cake reaches a height of about 27 cm. A sample applicator is used to load the column with the buffered urine, this device remains at the top of the column until the end of the elution. The columns are developed by a sodium chloride or an ammonium formate linear gradient at pH 7·2 or 3·6 respectively. These gradients are obtained in a nine-chamber Varigrad (Buchler Instruments, New York), using only 4 of these chambers. The schedules are 0·01, 0·01, 0·5, 0·5, or 0·05, 0·05, 0·5, 0·5 $M.l^{-1}$ of NaCl or Ammonium formate respectively. 400 Ml of the chosen buffer are poured in each chamber. A predevelopment with 200 ml of distilled water is adopted in every case. The elution flow-rates indicated in Table 1 are kept constant by means of a micropump (Buchler Instruments, New York). 10 ml fractions are collected.

Radioassays are performed on 0·2 ml aliquots of each fraction. These aliquots are poured into 10 ml of counting medium containing 100 g of naphtalene, 7 g P.P.O., 0·3 g P.O.P.O.P. for 1 l of 1,4-dioxane. The same mixture is used for every other kind of sample counted. Quenching corrections are made by the external standardization system included in the Packard Tri-Carb liquid Spectrometer, model 3375, which is used for the counting.

In order to separate the main classes of the above mentioned metabolites, the aqueous phase, corresponding to each peak, determined by ^{14}C, is submitted to the following procedure (Fig. 1): free metabolites are obtained by ether extraction (2 vol., 5 times) at pH 6. Glucuronates are split by the β-glucuronidase action and the corresponding radioactive material is extracted by ether as above. Sulphates are hydrolyzed by HCl and the liberated free compounds are extracted in the same way. We observe that the split material is less water-soluble than the corresponding conjugated form and becomes easily ether-extractable at pH 6. The determination of the hydroxamic function present in the native drug is performed by the colorimetric method described by Bergmann and Segal.[5] The determination of the glucuronic acid is undertaken using the Tollens reaction.[6] The determination of sulphate ions is carried out with the Merck's "Thorine" reagent.

Assays for glucuronic and sulfuric acid are performed on aliquots of the aqueous phase after HCl-hydrolysis.

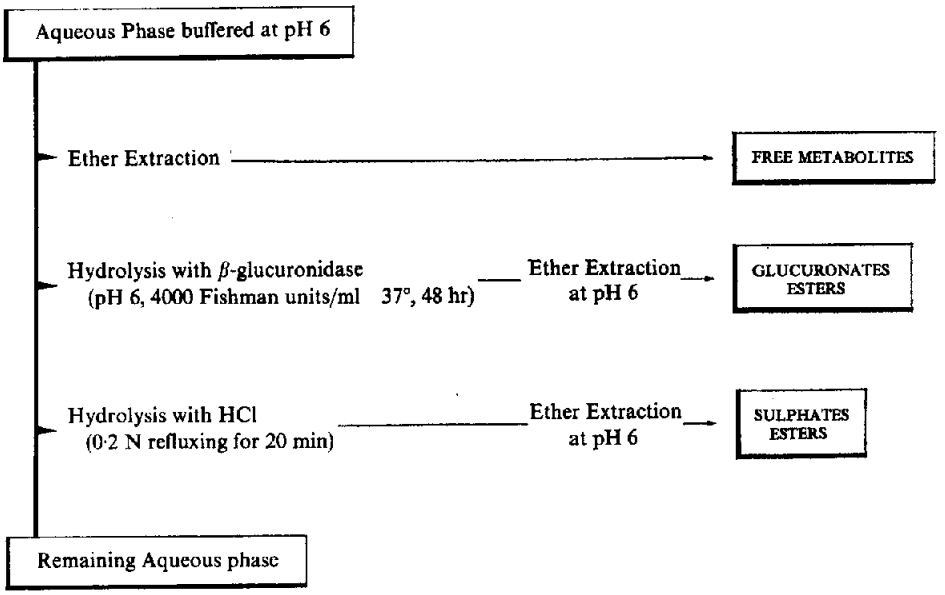

FIG. 1. Procedure used for the separation of the main classes of metabolites in the chromatographic fractions.

RESULTS AND DISCUSSION

The elution curves are reported in a semilogarithmic system. The dotted line shows the evolution of the gradient. The elution curve at pH 7 for human urine after oral administration of Droxaryl is shown in Fig. 2.

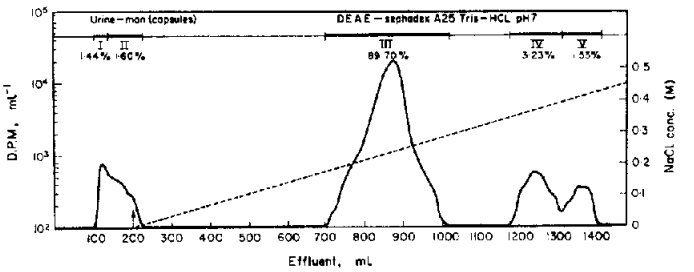

FIG. 2. Chromatographic pattern at pH 7·2 of human urine after oral administration of Droxaryl.

Five fractions are distinctly resolved. It must also be noted that these fractions are roughly distributed in 3 zones. The first zone includes metabolites which are eluted at very low NaCl concentrations. The second one contains metabolites eluted between about 0·15 and 0·30 M. l^{-1} of NaCl. In the last zone metabolites leave the column between 0·32 and 0·45 M. l^{-1} of NaCl. The amount of urinary-^{14}C is indicated above each peak.

After rectal administration of the drug the chromatographic pattern (Fig. 3) is almost the same as that reported for capsules.

Comparing the results obtained after the two routes of administration, one may note that fraction II is definitely higher in the case of rectal administration, the other fractions being lower or almost equal.

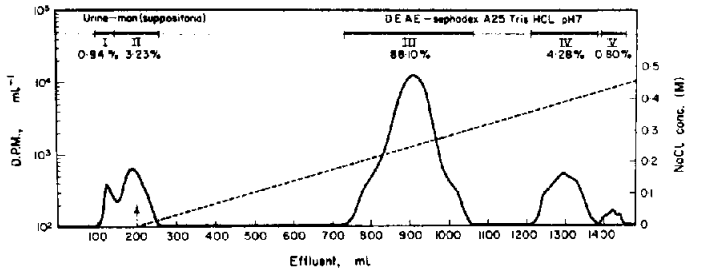

FIG. 3. Chromatographic pattern at pH 7·2 of human urine after rectal administration of Droxyaryl

The comparison of the amounts found in each peack is shown in the first column of Table 2.

The unchanged hydroxamic function is determined in all the fractions and the results are reported as percent of the total radioactivity in the second column of Table 2. Significant amounts are found only in the first three fractions in both cases

TABLE 2. HUMAN URINE. IDENTIFICATION OF THE MAIN CLASSES OF METABOLITES ON THE ELUTION CURVES

Peak	Repartition of urinary-^{14}C		*Percentage of unchanged Droxaryl		†Estimation of Glucuronic acid		‡Estimation of SO_4	
	capsules	suppositoria	capsules	suppositoria	capsules	suppositoria	capsules	suppositoria
I	1·4	0·9	0·2	0·4	+	+	—	—
II	1·6	3·2	0·4	2·7	+	+	—	—
III	89·7	88·1	1·2	1·7	++++	++++	++	++
IV	3·2	4·3	0	0	++	++	++++	++++
V	1·5	0·8	0	0	—	—	—	+
Total	97·4	97·3	1·8	4·8	—	—	—	—

* Results obtained by the Bergman and Segal's colorimetric method converted into ^{14}C-unchanged-Droxaryl.

† Glucuronic acid determined by the Tollens reaction after HCl-hydrolysis.

‡ Sulphate ions determined by the "Thorine" reagent (Merck, Darmstadt).

and it clearly appears that the rate of unchanged hydroxamic acid excreted is fairly higher after rectal administration. Columns 3 and 4 of the Table 2 show the location of glucuronic and sulfuric acids on the elution curve. On the basis of these results it seems reasonable to think that glucuronic esters are essentially located in the third fraction while sulphate esters in the fourth.

Further evidence for this interpretation is given by the elution pattern of human urine at pH 3·6 (Figs. 4 and 5). In these condition sulphates (peaks 4 and 5) have to emerge earlier from the column. Indeed, at this pH the ionization of these compounds must be less important.

Fig. 4 shows the elution curve of human urine after oral administration of Droxaryl using formic acid-ammonium formate buffer at pH 3·6. As expected the above-mentioned peaks 4 and 5 are now shifted to the left. The same thing occurs in the case of urine collected after rectal administration (Fig. 5).

Another way of understanding this shift is that there is hydroxyl hydrogen binding or some such chelating process which would render the molecules eluted with peaks 4 and 5 more neutral at low pH. Perhaps these two phenomena (decrease in ionization and chelating process) are involved simultaneously.

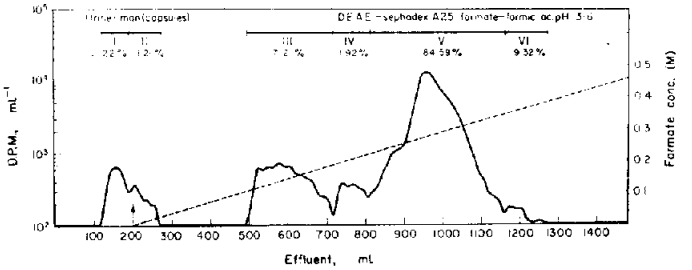

FIG. 4. Chromatographic pattern at pH 3·6 of human urine after oral administration of Droxaryl.

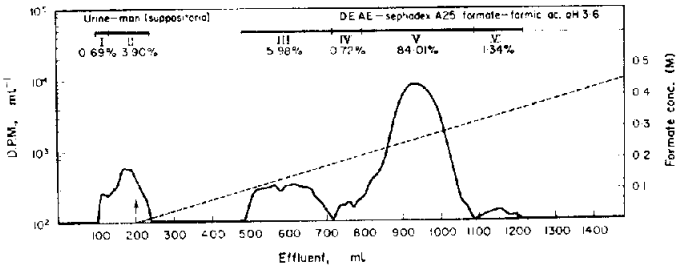

FIG. 5. Chromatographic pattern at pH 3·6 of human urine after rectal administration of Droxaryl.

Using the technique described in Fig. 2, the amount of unconjugated and conjugated compounds is determined in each fraction resolved after DEAE-Sephadex chromatography at pH 7. Results are reported in Table 3.

TABLE 3. HUMAN URINE. DETERMINATION OF THE MAIN CLASSES OF METABOLITES IN EACH ^{14}C-FRACTION AFTER DEAE-SEPHADEX SEPARATION

Class	Unconjugated (free compounds) %		Glucuronates %		Sulphates %		Total	
Peak	capsules	suppositoria	capsules	suppositoria	capsules	suppositoria	capsules	suppositoria
I	0·2	0·2	1·1	0·7	0·1	0	1·4	0·9
II	0·5	2·4	0·6	0·3	0·3	0·1	1·4	2·8
III	2·4	2·7	64·2	68·2	7·6	3·3	74·2	74·2
IV	0·1	0·2	0·9	1·6	0·7	1·2	1·7	3·0
V	0·1	0·1	0·2	0	1·0	0·4	1·3	0·5
Total	3·3	5·6	67·0	70·8	9·7	5·0	—	—

In the first column one can see the amount of free compounds. After administration by oral or rectal route about the same percentage is found in each peak except for the second fraction where the figures are clearly higher in the case of suppositoria. This agrees with the mentioned results for the rate of unchanged drug (Table 2). It appeared

thus that in the second peak, at least in the case of suppositoria, the radioactivity is practically due to the native compound only. The amounts of glucuronate esters are reported in the second column. It appears immediately that the third fractions present the highest percentage of these kind of esters. Nevertheless, a significant amount of sulphates is also detected in these peaks as one can see in the third column of the table. This fact leads us to suppose that hybrid metabolites are involved in the metabolic fate of Droxaryl. One can also observe that the occurrence of this kind of metabolites seems to be less important in the case of suppositoria.

The low percentage of the hydroxyamic function recovered in the third peak and the fact that this function does not appear even after enzymatic or acid-hydrolysis lead us to suppose that the hydroxamic group is largely degradated *in vivo*. Anyway, it seems probable that many different metabolites having various functions and conjugated with the glucuronic acid can be expected. The poor chromatographic separation of these kind of compounds on DEAE-Sephadex may be due to the small differences in the value of their pK. No significant differences can be noted between capsules and suppositoria in the sulphate contents of the fourth fraction. The nature of the compounds located in the first and the fifth peak is still unknown.

CONCLUSIONS

The idea we have up to now about the metabolism of Droxaryl, is summarized in Fig. 6.

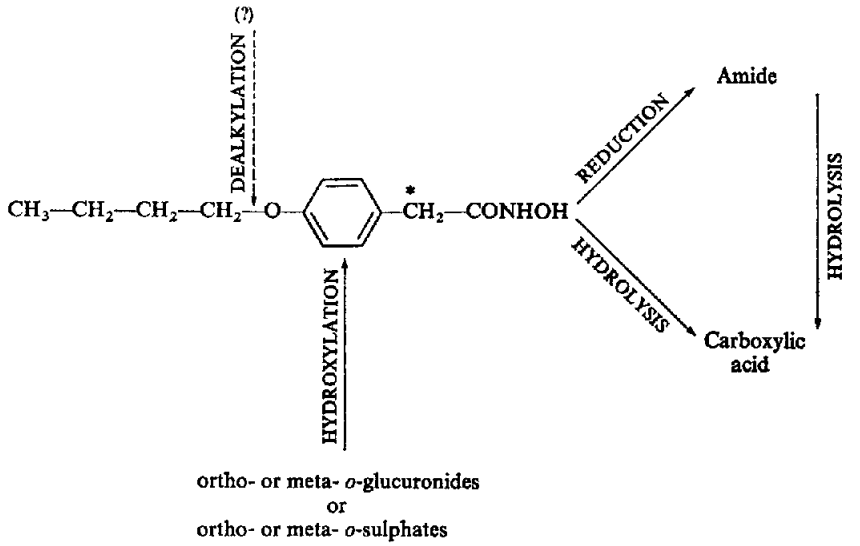

FIG. 6. Proposed main metabolic pathways of *p-n*-butoxyphenylacethydroxamic acid.

In Man, the main metabolic routes seem to be the conjugation with glucuronic (about 70%) and sulfuric acid (about 5–10%). Hybrid compounds cannot be excluded. Finally, compounds containing the native hydroxamic function account for 3–5 per cent only in urine. This means that important degradation reactions occur in the functional group leading perhaps to the formation of the corresponding amide and carboxylic acid.

If we compare the metabolic fate of Droxaryl after oral or rectal administration in Man, the main difference is obviously the higher amount of unchanged drug in the urine of rectally treated patients.

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