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MASS FRAGMENTOGRAPHIC QUANTITATION OF ETHOTOIN AND SOME OF ITS METABOLITES IN HUMAN URINE

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SUMMARY

A method for the determination of ethotoin and its *p*-hydroxylated and dealkylated metabolites in urine has been developed. Ethotoin and the metabolites were extracted from acidified urine with ethyl acetate and silylated before injection into a combined gas chromatograph—mass spectrometer. Four partly identified metabolites were recorded, but their exact quantitation was not possible as pure reference substances were not available.

The limit of sensitivity was far below the amounts of ethotoin and of its metabolites found in urine from patients treated with therapeutic doses of ethotoin.

INTRODUCTION

Recently, the antiepileptic drug ethotoin (Peganone) was shown by our group to exhibit dose-dependent kinetics [1]. The purpose of the present investigation was to develop a method for the quantitative determination of ethotoin and its metabolites excreted in urine in order to clarify the mechanism behind the dose-dependent kinetics of ethotoin.

EXPERIMENTAL

Samples

Urine samples were collected from patients undergoing continuous treatment with ethotoin in therapeutic doses. Blank urines were collected from the same patients before ethotoin treatment was started.

Reagents and solvents

Ethyl acetate and toluene from E. Merck (Darmstadt, G.F.R.) were of analytical-reagent grade. N,O-Bis(trimethylsilyl)acetamide (BTSA) from Pierce

(Rockford, Ill., U.S.A.) was of specially purified grade. Glucuronidase/arylsulphatase was obtained from Boehringer (Mannheim, G.F.R.).

Reference substances

The structures of ethotoin and its metabolites are shown in Fig.1. Ethotoin and its dealkylated metabolite (5-phenylhydantoin) were donated by Abbott Labs. (North Chicago, Ill., U.S.A.). The two internal standards, mephenytoin and heptamal, were donated by Sandoz (Basle, Switzerland) and Ciba-Geigy (Basle, Switzerland), respectively. The *p*-hydroxylated metabolite was isolated from human urine and purified. Metabolites III, IV, V and VI were extracted from urine and purified, but were not isolated in a pure state.

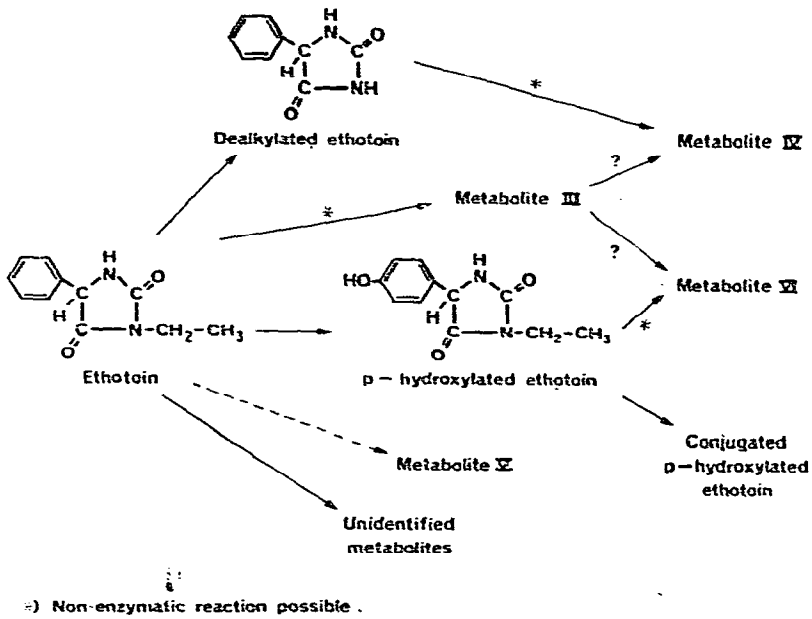

Fig.1. Metabolic pathways of ethotoin in man.

Isolation and purification. Metabolites III, IV, V and VI were extracted from urine samples with ethyl acetate and cleaned by column chromatography on silica gel with ethyl acetate—*n*-hexane (1:1).

The urine remaining after this extraction was hydrolysed with glucuronidase/arylsulphatase. The *p*-hydroxylated metabolite released was extracted with ethyl acetate and cleaned by column chromatography on silica gel with ethyl acetate—*n*-hexane (1:1), followed by two recrystallizations from ethyl acetate—*n*-hexane (1:1). The melting point of the compound and its UV spectrum in ethanol, as well as the acid properties and the mass spectra of the compound and some of its derivatives, were determined.

Identification. The identity of the isolated *p*-hydroxylated ethotoin was confirmed by the following analytical data. The main peaks in the mass spectrum were 16 *m/e* higher than the corresponding peaks in the mass spectrum of ethotoin. The compound could be silylated at two positions and acetylated at one. The unchanged compound was a weak acid, but its acetylated derivative was neutral. The UV spectrum and the melting point were in good agreement with those of synthetic 3-ethyl-5-(*p*-hydroxyphenyl)hydantoin [2]. No definite proof was obtained that the hydroxylation takes place in the *para*-position, but *p*-hydroxylation has been shown to be a major metabolic pathway in man for phenytoin [3,4] and other 5-phenylhydantoin derivatives [5], the *p*-hydroxylated metabolite was found in urine from dogs treated with ethotoin [2] and *m*-hydroxylation of phenytoin occurs to a slight extent [6].

The exact structural formulae of metabolites III, IV and VI have not yet been proved. The compounds could be formed in small amounts in vitro from ethotoin, dealkylated ethotoin and *p*-hydroxylated ethotoin, respectively, in alkaline solution. The mass spectra of the unchanged compounds (III, IV and VI) and of their silylates may point to an oxidation in position 5 in the hydantoin ring system [2].

The mass spectra of metabolite V and of its silylated derivative could be explained by the assumption that the metabolite had arisen from ethotoin in which the phenyl group had been converted into the 3,4-dihydroxy-1,5-cyclohexadien-1-yl group. This metabolic pathway has been shown to be open for phenytoin [7]. Furthermore, this would be in agreement with the fact that the metabolite had no acidic properties.

No compound having two phenolic hydroxyl groups, as shown for phenytoin [8] was observed.

Mass spectrometry. A combined gas chromatograph—mass spectrometer (LKB 9000) was used. The conditions used for the mass spectra were as follows: ionization energy, 70 eV; trap current, 60 μA; accelerating voltage, 3500 V. Tables I and II give the mass spectra of ethotoin, its metabolites and the internal standard as unchanged and as silylated compounds, respectively.

Quantitation

Extraction and derivatization of non-conjugated metabolites. To a 50-μl urine sample in a centrifuge tube 1 ml of 0.1 *N* acetic acid and 100 μl of mephenytoin solution (0.1 g/l in ethanol) as internal standard were added. The sample was mixed with 3 ml of ethyl acetate for 5 min. After centrifugation, the organic phase was transferred into a tapered tube and evaporated to dryness in a stream of nitrogen. The residue was derivatized with 50 μl of BTSA for 15 min and diluted with 100 μl of toluene. A volume of 1 μl was injected into the combined gas chromatograph—mass spectrometer.

Extraction and derivatization of total p-hydroxylated metabolites. To a 50 μl urine sample in a centrifuge tube, 200 μl 0.1 *N* acetic acid were added. After the addition of 5 μl of glucuronidase/arylsulphatase, the tube was left for 18 h at 37°. The subsequent procedure was as described for the non-conjugated compounds.

If only the *p*-hydroxylated metabolite was to be measured, gas chromatography with a flame-ionization detector could be used. The procedure was then

TABLE I

MASS SPECTRA OF ETHOTOIN (ETO), DEALKYLATED ETHOTOIN (DA-ETO), *p*-HYDROXYLATED ETHOTOIN (*p*-H-ETO) AND METABOLITES III, IV AND V

Fragments below 100 *m/e* and/or with intensities below 10% have been omitted. The results given are relative intensities.

m/e	ETO (mol.wt. 204)	DA-ETO (mol.wt. 176)	*p*-H-ETO (mol.wt. 220)	Metabolite III	Metabolite IV	Metabolite V
103				100	100	89
104	100	100		37	35	100
105	96	62				87
119						32
120			86			
121			100			99
132	10					
133	20	18				24
135						41
148			25			
149						29
174					13	
176		40				
191						26
192						30
202				22		25
204	56					59
220			59			
238						19

the same as described above, except that heptamal was used as the internal standard and after the extraction the residue was dissolved in 50 µl of ethyl acetate. Derivatization with BTSA was omitted. A volume of 3 µl was injected into the gas chromatograph.

Mass fragmentography and gas chromatography. The conditions for mass fragmentography were as follows: ionization energy, 20 eV; trap current, 60 µA; column, glass, 0.6 m × 2 mm I.D.; 3% OV-1 on Celite J.J. CQ, 100—120 mesh; pre-heater temperature 250°, column oven 180°, separator 270°, ion source 290°; helium flow-rate, 15 ml/min. The mass fragments used for analysis are given in Table II.

The gas chromatographic measurements were performed on a Pye Series 104 gas chromatograph equipped with a flame-ionization detector. The conditions were as follows: column, glass, 0.9 m × 4 mm I.D.; 1% OV-17 on Celite J.J. CQ, 100—120 mesh; pre-heater temperature 250°, detector oven 350°, column oven 230°; carrier gas, nitrogen at a flow-rate of 60 ml/min.

Samples for standard curves. Known amounts of ethotoin and its metabolites were added in increasing amounts to urine blanks.

For dealkylated ethotoin, for metabolite IV and for total *p*-hydroxylated ethotoin, standard curves from 100 to 1000 mg/l were used. For ethotoin, for non-conjugated *p*-hydroxylated ethotoin and for metabolites III, V and VI, standard curves from 5 to 50 mg/l were used. If higher concentrations were present, the urine samples were diluted with water.

Relative standard solutions of metabolites III, IV, V and VI were prepared,

because complete isolation of the compounds was not feasible. The concentrations were estimated by comparison of the areas under the peaks in the gas chromatograms of the metabolites with the area under the peak in a gas chromatogram of the dealkylated metabolite of ethotoin (5-phenylhydantoin). A flame-ionization detector was used.

RESULTS AND DISCUSSION

Extraction and formation of derivatives

Ethotoin itself could be extracted quantitatively with dichloromethane [9]. However, it was necessary to use a more polar extraction medium such as ethyl acetate, as some of the ethotoin metabolites are rather polar. The fact that several of the metabolites are weak acids could not be utilized in a cleaning procedure as ethotoin and most of its metabolites are unstable in alkaline solution.

For most of the compounds, the extraction recovery was approximately 100% (only 75% for metabolite V).

Silylation of ethotoin, of the internal standard, of *p*-hydroxylated ethotoin and of metabolite III proceeded readily at room temperature, but the dealkylated ethotoin and metabolites IV and V reacted more slowly, especially in low concentrations. This effect might be due to adsorption of the non-silylated compounds on the glass walls, but after 20 min the derivatizations were found to be almost quantitative.

Hydrolysis of conjugated metabolites

The only conjugated metabolite found in noticeable amounts was *p*-hydroxylated ethotoin. Enzymatic hydrolysis of conjugated *p*-hydroxylated ethotoin under the conditions described was found to be quantitative (or at least maximal) after 12—15 h. Acid hydrolysis with concentrated hydrochloric acid was not possible as it gave rise to some decomposition of the *p*-hydroxylated ethotoin released.

Urine analysis

Typical mass fragmentograms for the determination of ethotoin, dealkylated metabolite, metabolite IV and non-conjugated *p*-hydroxylated ethotoin, of standards and of urine extracts from blank urine and from urine from a patient treated with ethotoin are shown in Fig. 2. Metabolites III, IV, V and VI could not be measured exactly as no reference with known concentrations was available. The concentrations were estimated from the areas of the peaks in the gas chromatograms as described above.

OV-1 or OV-101 could be used as the column material. OV-17 yielded no separation of the silyl derivatives of ethotoin, of dealkylated ethotoin and of metabolite IV. Although they were measured at different channels, slight interference from the latter was observed at the channels measuring ethotoin and dealkylated ethotoin.

Concerning the conjugated metabolites, only *p*-hydroxylated ethotoin was found in noticeable amounts (a trace amount of conjugated metabolite V was observed). It was measured as total minus free compound (the free compound

TABLE II

MASS SPECTRA OF SILYLATED COMPOUNDS OF ETHOTOIN (ETO), DEALKYLATED ETHOTOIN (DA-ETO), *p*-HYDROXYLATED ETHOTOIN (*p*-H-ETO), METABOLITES III, IV, V AND VI AND MEPHENYTOIN (MPT)

Fragments below 100 *m/e* (except 73) and/or with intensities below 10% (except molar fragments or fragments used for mass fragmentography) have been omitted. The results given are relative intensities (I).

ETO		DA-ETO		*p*-H-ETO		Metabolite III		Metabolite IV		Metabolite V		Metabolite VI		MPT	
m/e	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*	*m/e*	*I*
73	50	73	98	73	100	73	100	73	100	73	100	73	100	135	29
136	10	162	100	223	22	105	33	105	21	191	15	147	21	176	13
162	100	163	15	250	31	147	47	147	20	292	4*	193	10	190	15
163	16	177	69	264	16*	162	14	264	13*	454	0.4	322	8*	261	100*
176	15	178	12	265	10	234	42	265	11			452	2	262	22
177	23	188	23	349	15	264	12	408	0.3					263	60
261	51*	190	15	364	55	287	28*							275	15
262	11	205	46			335	36							290	2
276	23	305	16*			336	11								
		320	3			349	12								
						364	5								

*Mass fragments used for mass fragmentographic measurements.

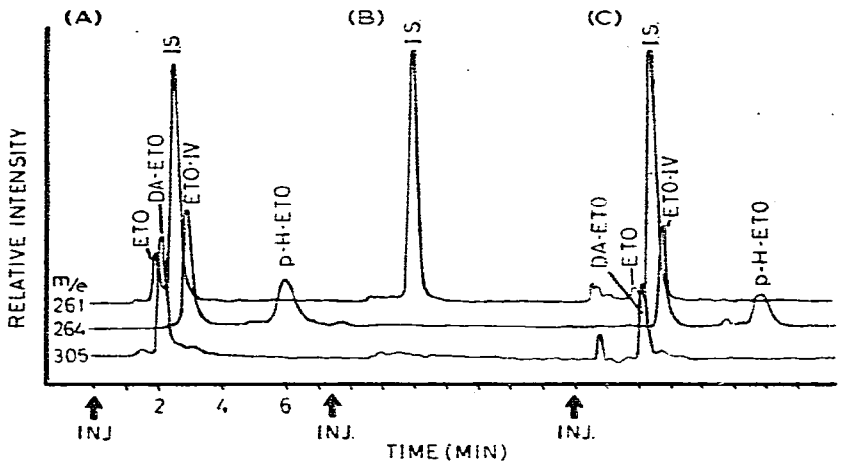

Fig. 2. Mass fragmentograms of urine extracts. (A), blank urine with added standards: ethotoin (ETO), non-conjugated *p*-hydroxylated ethotoin (*p*-H-ETO), dealkylated ethotoin (DA-ETO), metabolite IV (ETO-IV) and mephenytoin (internal standard, I.S.). (B), blank urine with added internal standard. (C), urine from patient treated with 2 g of ethotoin; the concentrations found 3.5, 5.5, 280 and 450 mg/l were for ethotoin, non-conjugated *p*-hydroxylated ethotoin, dealkylated ethotoin and metabolite IV, respectively.

being about 2% of the total). As no interfering compounds were found with the same retention time as *p*-hydroxylated ethotoin, it could be measured as accurately with a gas chromatograph equipped with a flame-ionization detector (see below).

Specificity

Interfering material in urine extracts from patients not treated with ethotoin was never observed.

Mass spectra of reference materials and mass spectra of peaks in gas chromatograms from urine extracts were in agreement (identical after subtraction of the background).

The specificity of the gas chromatographic method for the determination of total *p*-hydroxylated ethotoin was checked analyzing 20 urine samples by both gas chromatography and mass fragmentography [concentration range 300—1600 mg/l (1—5 mmole/l); coefficient of correlation 0.975; the slope was not significantly different from unity, and no sample concentration differed by more than 8% as determined by the two methods].

Accuracy

Urine from a patient treated with 2125 mg of ethotoin per day was analyzed 20 times by mass fragmentography and by gas chromatography. The standard deviation found by mass fragmentography was 4—5% for non-conjugated metabolites and ethotoin itself and 6% for total *p*-hydroxylated ethotoin (both by mass fragmentography and by gas chromatography).

Recovery

Recoveries of ethotoin and its non-conjugated metabolites through the whole procedure were not investigated intensively as they varied in parallel with the recovery of the internal standard (range 75—90%), and no effort was made to increase the recoveries. The recovery of the conjugated *p*-hydroxylated metabolite could not be determined exactly as no reference material was available, but the same amounts were found whether the urine samples were hydrolyzed for 15, 18 or 24 h under the conditions described.

Sensitivity

Less than 1 ng (5 nmole), corresponding to 5 mg/l (25 μmole/l), in 50 μl of urine could easily be measured quantitatively by mass fragmentography for ethotoin and all of the metabolites mentioned. The gas chromatographic method was reliable down to 50 mg/l (lower concentrations, in some urine samples after single doses, were measured by mass fragmentography).

Quantitative determination

Standard curves were prepared from gas chromatograms or mass fragmentograms of urine extracts to which known amounts of ethotoin and its metabolites had been added. The ratio of the peak height of the compound determined to that of the corresponding internal standard was plotted against the concentration. A linear relationship was obtained for all of the compounds (see Fig.3).

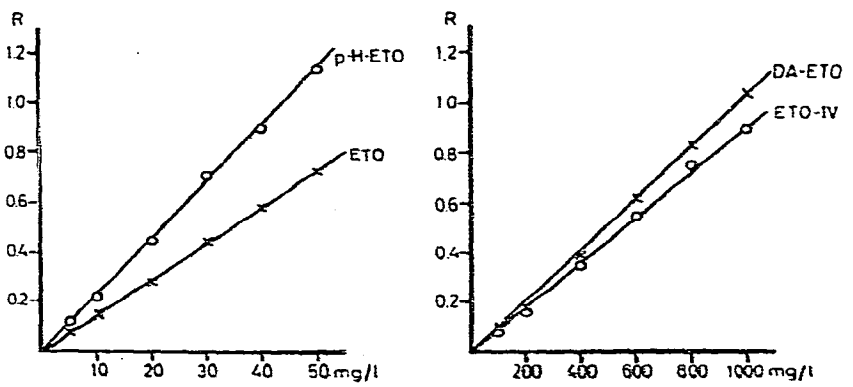

Fig.3. Standard curves for ethotoin (ETO), non-conjugated *p*-hydroxylated ethotoin (*p*-H-ETO), dealkylated ethotoin (DA-ETO) and metabolite IV (ETO-IV). R = ratio between the peak heights of the compounds analysed and that of the internal standard.

The concentration range found after application of the dose range 1.75—4.25 g of ethotoin per day is shown in Table III.

The details and their significance for dose-dependent kinetics of ethotoin will be published elsewhere [10].

TABLE III

EXCRETION RANGE OF ETHOTOIN AND ITS METABOLITES IN URINE FROM FIVE PATIENTS UNDERGOING CONTINUOUS TREATMENT WITH ETHOTOIN (2—4 g PER DAY)

Compound	Concentration range (mg/l)	Excreted amounts (% of applied dose)
Ethotoin	3— 38	0.2— 1.9
Total *p*-hydroxylated ethotoin	300—1600	14 —32
Non-conjugated *p*-hydroxylated ethotoin	8— 30	0.4— 0.8
Dealkylated ethotoin	90—1445	5.4—14.3
Metabolite IV	300—1200	17 —34
Metabolites III + V + VI		0.8— 2.2
Total		44 —76

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