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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ENCAINIDE AND ITS METABOLITES IN HUMAN BODY FLUIDS

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SUMMARY

Methods reported previously for the determination of encainide and its metabolites in biological fluids have not been extensively described and evaluated. We report an improved high-performance liquid chromatographic assay for the quantification of these compounds in plasma and urine with complete estimation of the accuracy and reproducibility of the analytical method. The major improvements consist of: (1) the use of ethaverine as an appropriate internal standard; (2) the use of the salting-out technique which improves the extraction recovery for the metabolites of encainide and the sensitivity of the assay; and (3) a shift of the ultraviolet absorption wavelength from 254 to 270 nm to increase the selectivity of the detection.

INTRODUCTION

Encainide (ENC), 4-methoxy-[2′-(N-methyl-2-piperidyl)ethyl]-benzanilide, is an antiarrhythmic agent with sodium channel blocking activity used in the treatment of various ventricular [1–4] and supraventricular [5,6] arrhythmias. In man, the drug is predominantly excreted after biotransformation to a number of metabolites (Fig. 1) including O-desmethylencainide (ODE), 3-methoxy-O-desmethylencainide (MODE) and N-desmethylen-

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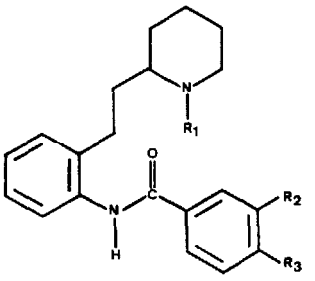

	R_1	R_2	R_3
ENC	CH_3	H	OCH_3
ODE	CH_3	H	OH
MODE	CH_3	OCH_3	OH
NDE	H	H	OCH_3

Fig. 1. Chemical structures of encainide and its metabolites.

cainide (NDE). Measurement of these metabolites in plasma is of clinical importance since they are at least as potent antiarrhythmic agents as the parent drug and since ODE and MODE are usually present at higher concentrations than ENC [7,8]. Moreover, biotransformation of ENC into ODE and further into MODE is genetically determined [1,9,10]. This genetic polymorphism of ENC disposition cosegregates with that of the antihypertensive agent debrisoquine [11,12]. Approximately 93% of the North American caucasian population extensively metabolize ENC into ODE and MODE, and they are referred to as having the extensive metabolizer (EM) phenotype. The other 7% functionally lack the enzyme responsible for the formation of ODE and MODE and are characterized as having the poor metabolizer (PM) phenotype.

The most recently reported high-performance liquid chromatographic (HPLC) method allows measurement of encainide and its three major metabolites in plasma and urine [13]. This method uses the external standard quantification technique which can account for loss of accuracy and precision. The sensitivity of the method was also not optimal since a single extraction procedure was used and therefore recovery was incomplete. Others have suggested the use of acebutolol as an internal standard with the same chromatographic technique [14]. Unfortunately, this product elutes early without baseline resolution from the solvent front peak. Also, acebutolol does not separate well from the NDE peak thus preventing quantification of this metabolite at low levels.

Most of the pharmacokinetic parameters reported with ENC have been based on drug concentrations determined with the assay reported by Mayol et al. [13]. Complete evaluation of this method including reproducibility, recovery and efficacy has, however, not been reported.

We describe herein improvements of the HPLC assay used for the analysis

of ENC and its metabolites in plasma and urine. The major improvements are: (1) the choice of an appropriate internal standard; (2) the modification of the extraction procedure to increase the recovery of ODE and MODE; and (3) shifting of the UV absorption wavelength to increase the selectivity of detection. Since antiarrhythmic drug therapy is often given to people with multiple pathologies and under numerous pharmacologic treatments, screening for potential interference from other drugs or their metabolites using plasma samples of patients receiving concomitant therapy with ENC is also reported.

EXPERIMENTAL

Chemicals and reagents

The base forms of ENC, ODE, MODE and NDE were provided by Bristol-Myers Pharmaceutical Research and Development Division (Wallingford, CT, U.S.A.). Ethaverine hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). Butyl chloride (HPLC grade), 2-propanol (HPLC grade) and tris(hydroxymethyl)aminomethane hydrochloride were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Methanesulfonic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals and reagents used were of analytical grade and obtained from the usual commercial sources.

Standard solutions

Stock standard solutions of ENC and its metabolites were prepared by dissolving 10 mg of each compound in 10 ml of ethanol. Working standard solutions were prepared from the stock solutions by suitable dilutions with ethanol to obtain final concentrations of 1 and 20 μg/ml for plasma and urine analysis, respectively. The stock and working solutions were stored at $-20\,^{\circ}$C in amber-colored flasks. The same procedure was repeated for the preparation of ethaverine solutions (internal standard) except that distilled water was used as the solvent. The final concentrations of ethaverine hydrochloride were 1 and 50 μg/ml for plasma and urine analysis, respectively.

Extraction procedure

To 1.0 ml of plasma or urine in a 15-ml Pyrex tube were added 200 μl of the internal standard solution (1 μg/ml for plasma or 50 μl/ml for urine), 0.5 *M* Tris–HCl pH 8.5 (100 μl for plasma and 500 μl for urine) and 2 g of sodium chloride. This mixture was then extracted twice by vortex-mixing with 5 ml of butyl chloride–2-propanol (95:5, v/v) for 15 s. After centrifugation at room temperature for 10 min at 3000 *g* in an IEC-Centra 8 centrifuge (International Equipment, Needham Heights, MA, U.S.A.) the organic layer was transferred into a conical tube. The combined organic extracts were evaporated to dryness in a Buchler vortex–evaporator at $45\,^{\circ}$C. The residue was dissolved in 50 μl of the mobile phase and then injected into the column.

Instrumentation

A Waters (Waters Chromatography Division, Milford, MA, U.S.A.) chromatographic system consisting of a Model M6000 pump, a U6K injector fitted with a 100-μl loop, a 710B Data Module integrator and a Model 450 variable-wavelength UV detector set at 270 nm were used. The chromatograms were also recorded simultaneously on a Linear double-pen recorder set at 2 and 20 mV resulting in 7.6-fold expansion and 1.33-fold contraction of the scale, respectively. Separation was performed on a Dupont Zorbax Sil silica column (25 cm $\times$ 4.6 mm I.D.; 5 μm particle size) using a mobile phase consisting of ethanol–water–methanesulfonic acid (500·25:0.25, v/v for plasma and 500:10:0.25, v/v for urine). The flow-rate was set at 1.2 ml/min.

Calibration curves

Working solutions of ENC and its metabolites were added to 1.0 ml of plasma or urine. The range of concentrations was 5–500 ng/ml in plasma and 0.5–10 μg/ml in urine. All samples were extracted and analyzed as above. Calibration curves based on the peak-height ratios of each metabolite or ENC to the internal standard were constructed using seven different concentrations of each product analyzed in duplicate. The data were then subjected to linear regression analysis to give the appropriate calibration factor.

RESULTS AND DISCUSSION

Fig. 2 represents typical chromatograms of extracts of drug-free plasma, of drug-free plasma spiked with 10 and 75 ng of each metabolite and ENC and of a subject's plasma sample each processed in presence of ethaverine as the internal standard. The retention times of the peaks of interest were 5.1 min (NDE), 6.9 min (ethaverine), 8.3 min (ODE), 9.9 min (MODE) and 11.6 min (ENC). Good resolution was obtained between each product and no endogenous compounds interfered significantly with these products. The limit of quantification and the limit of detection of ODE, MODE and ENC in plasma were of 2.5 and 1 ng as calculated by the method of Knoll [15]. For NDE, the limit of quantification was 5 ng and the limit of detection 2.5 ng at a signal-to-noise ratio of 3.

The sensitivity of the method was high enough to allow adequate measurement of ENC and its metabolites during single oral dose pharmacokinetic studies. Data shown in Fig. 3 were obtained in an EM and a PM subject, respectively, after administration of a single 60-mg oral dose of ENC [16]. Simultaneous recording using different attenuations was essential for accurate measurement of all these products with the same plasma sample since the metabolites and the parent drug are present at different and variable concentrations during a dosing interval. The elimination half-life of ENC calculated in the EM was 0.7 h and 9.3 h in the PM phenotype.

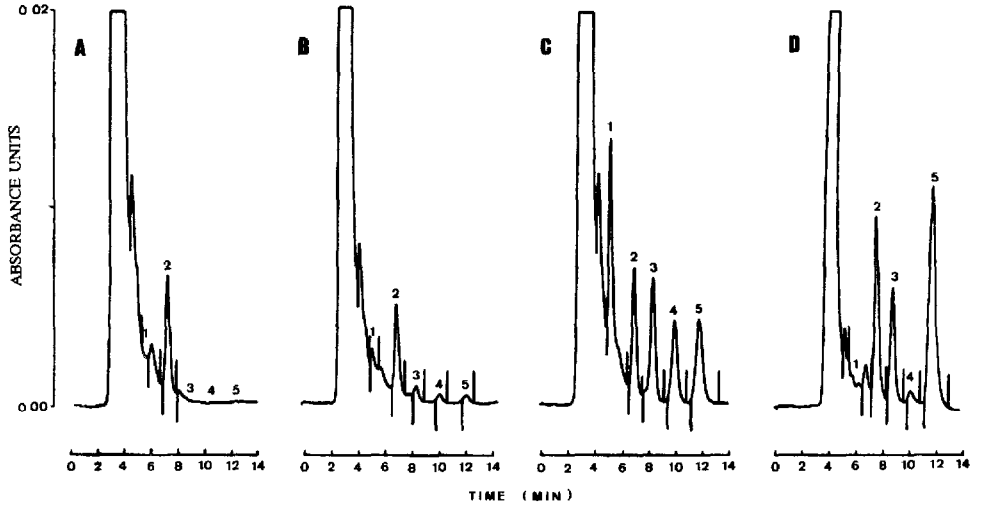

Fig. 2. Typical chromatograms obtained after extraction of blank plasma (A), blank plasma spiked with 10 ng (B) and 75 ng (C) of NDE (1), ODE (3), MODE (4) and ENC (5) and of a patient's sample containing an estimate of 51 ng of ODE, 5 ng of MODE and 130 ng of ENC (D). All these plasma samples were spiked with 200 ng of ethaverine hydrochloride (2) prior to extraction.

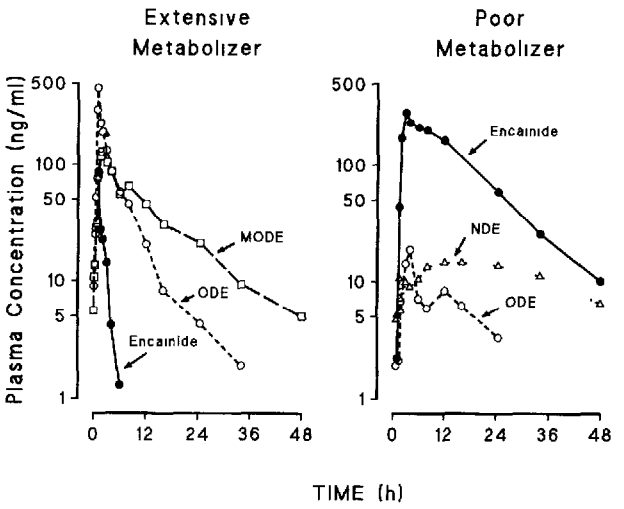

Fig 3. Concentration versus time profiles of NDE, ODE, MODE and ENC in an extensive (A) and a poor (B) metabolizer of encainide after administration of 60 mg of encainide orally.

Typical chromatograms of extracted drug-free urine, drug-free urine spiked with 6 μg of each metabolite and ENC and of a subject's urine sample are shown in Fig. 4. Before extraction, 10 μg of ethaverine hydrochloride was added to each sample. The retention time of NDE, ethaverine, ODE, MODE and ENC were 5.5, 7.6, 9.5, 11.3 and 13.4 min, respectively. A less polar mobile

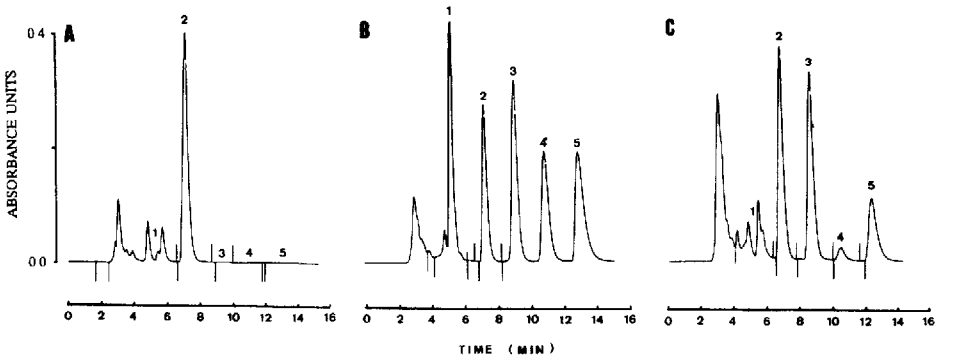

Fig. 4. Typical chromatograms obtained after extraction of (A) blank urine, (B) blank urine spiked with 6 μg of NDE (1), ODE (3), MODE (4) and ENC (5) and (C) of a patient's sample containing 4.6 μg of ODE, 0.5 μg of MODE and 4.3 μg of ENC. All these urine sample were spiked with 10 μg of ethaverine hydrochloride (2) prior to extraction.

phase was used for urine analysis to permit a better quantification of NDE which is present in appreciable amounts primarily in PM subjects. Nevertheless, partial interference from an unknown product (retention time of 5.8 min) was still encountered with NDE. Complete resolution of these peaks was not achievable without appreciable loss of time and accuracy in the measurement of the other metabolites. No interference was observed with any of the other peaks.

Table I shows that the extraction of ODE and MODE from plasma and urine is sensitive to the volume of the aqueous phase. Addition of a small volume of water decreased the percentage of extraction measured by the ratio of the UV signal amplitude for an extracted sample to a non-extracted standard. However, saturation of the aqueous phase with sodium chloride restored and increased this ratio leading to an extraction efficiency of approximately 90% for these metabolites. The same trend was observed with ENC and NDE although it was less significant. Thus, use of the salting-out technique during the extraction procedure led to increased accuracy of the method and greater sensitivity, mainly for ODE and MODE.

Linear calibration plots were obtained over the range 5–500 ng of each product in plasma and 0.5–10 μg in urine. All regression lines had correlation coefficients greater than or equal to 0.998. Intra- and inter-assay variations for ENC and its metabolites at different concentrations in plasma and urine are shown in Table II. In plasma, the mean ($\pm$S.D.) coefficient of variation (C.V.) of NDE, ODE, MODE and ENC for intra- and inter-assay analysis were 4.5 ± 2.7 and $5.5 \pm 2.1\%$, respectively, while in urine they were 3.8 ± 1.6 and $5.2 \pm 1.8\%$. For each product, mean recovery values calculated for the different concentrations were between 92.8 and 103.8% in plasma and 92.6 and 104.8% in urine.

TABLE I

PERCENTAGE EXTRACTION OF ENCAINIDE AND ITS METABOLITES FROM BIOLOGICAL FLUIDS

Compound	Extraction (mean ± S.D., $n=4$) (%)			
	Plasma alone (1 ml)	Plasma (1 ml) + NaCl	Plasma (1 ml) + water (1 ml)	Plasma (1 ml) + water (1 ml) + NaCl
NDE	97.5 ± 0.8	100.0 ± 1.0	98.5 ± 2.1	97.6 ± 2 5
ODE	79.8 ± 1.0	80.8 ± 0.1	59.6 ± 1.5	87.3 ± 0 1
MODE	78.7 ± 1.4	88.3 ± 2.0	79.7 ± 1.2	94.4 ± 2.4
ENC	101.4 ± 2.0	101.1 ± 4.0	97.0 ± 0.1	98.2 ± 2.3
	Urine alone (1 ml)	Urine (1 ml) + water (0.5 ml)	Urine (1 ml) + water (1 ml)	Urine (1 ml) + water (1 ml) + NaCl
NDE	83.6 ± 1.3	82.5 ± 1.4	79.8 ± 0.3	85.0 ± 1.2
ODE	70.3 ± 0.1	53.3 ± 1.3	39.9 ± 0.6	88.4 ± 1.5
MODE	81.3 ± 1.4	72.8 ± 1.6	66.3 ± 1.3	91.2 ± 1.3
ENC	93.8 ± 1.3	92.4 ± 1.7	90.2 ± 0.7	97.5 ± 1.0

TABLE II

INTRA- AND INTER-ASSAY VARIATIONS IN THE ANALYSIS OF ENCAINIDE AND ITS METABOLITES IN PLASMA AND URINE

Added (ng)	Recovered (mean ± S.D.) (ng)				Coefficient of variation (%)			
	NDE	ODE	MODE	ENC	NDE	ODE	MODE	ENC
Intra-assay variation (n=6)								
Plasma								
10	9.3 ± 0 9	9.8 ± 4 7	10.0 ± 0.6	9.8 ± 0.62	10.2	4.8	6.4	6.4
100	98.7 ± 2.6	98.3 ± 1.3	100.2 ± 3.7	98.1 ± 3.0	2.6	1.3	3.7	3.0
200	200.5 ± 1.5	202.0 ± 12.3	195.7 ± 11.8	197 5 ± 6.4	0.7	6.1	6.1	3.3
Urine								
1000	1027 ± 45	969 ± 43	926 ± 78	997 ± 31	4 4	4 4	8.4	3.2
2000	1982 ± 47	1986 ± 82	2038 ± 78	2095 ± 63	2 4	3.3	3.8	3.0
4000	4011 ± 93	3985 ± 160	3971 ± 144	3988 ± 119	2 3	4.0	3.6	3.0
Inter-assay variation (n=6)								
Plasma								
10	9 7 ± 1.0	10.2 ± 0.5	10.1 ± 0.7	10.0 ± 0 7	10.7	5.1	6.6	6.8
100	97 9 ± 3.5	101.0 ± 4.0	102.7 ± 4.8	100.5 ± 5.6	3.6	3.9	4.6	5.6
200	199 1 ± 5.1	202.6 ± 10.1	203.1 ± 11 7	206.3 ± 11.2	2.5	5.0	5.8	5.4
Urine								
1000	990 ± 80	1027 ± 65	991 ± 88	1013 ± 56	8.1	6.3	8.8	5.5
2000	2001 ± 67	2051 ± 104	2052 ± 93	2047 ± 115	3.4	5.1	4.5	5.6
4000	4111 ± 152	4082 ± 155	4068 ± 153	4075 ± 154	3.7	4.1	3.8	3.8

Standard curves were also performed with half and twice the amount of the internal standard normally used to assess whether the behavior of ethaverine was linear with the analysis procedure. Mean ($\pm$S.D.) ratios of the slopes so obtained to the respective slopes of each product calculated with the normal amount of the internal standard were 1.99 ± 0.05 with half the amount and 0.50 ± 0.01 when twice the amount of the internal standard was added. Such linearity indicates that ethaverine possesses the required properties for an internal standard.

Previously reported assays measured ENC and its metabolites at a wavelength of 254 nm. However, as shown in Fig. 5, ENC and all its metabolites can easily be monitored at 270 nm with only minor loss in sensitivity. When concomitant drugs are administered, detection at a more specific wavelength such as 270 nm allows the elimination of potential interference observed at 254 nm. Table III reports a series of drugs that were clinically used in concomitant therapy with encainide while the plasma and/or urine samples of these pa-

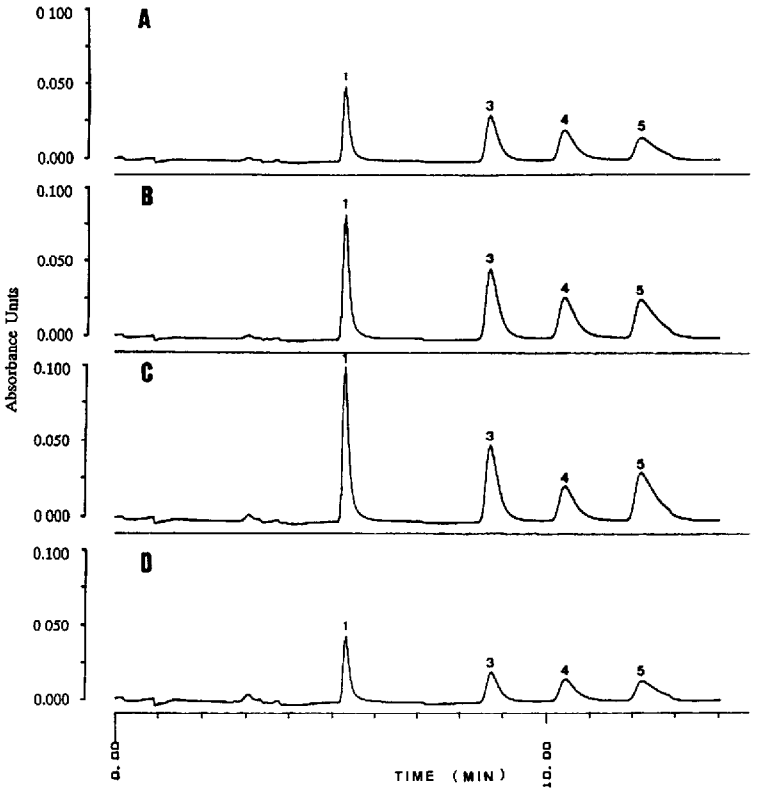

Fig. 5. Simultaneous analysis of NDE (1), ODE (3), MODE (4) and ENC (5) using a Hewlett Packard 1040A diode array detector with detection wavelengths set at 280 nm (A), 270 nm (B), 254 nm (C) and 235 nm (D). The flow-rate was set at 1.0 ml/min.

TABLE III

DRUGS WHICH DO NOT INTERFERE WITH THE DETERMINATION OF ENCAINIDE OR ITS METABOLITES BY THE DESCRIBED HPLC ASSAY

Acetaminophen	Isosorbide dinitrate
Alprazolam	Lidocaine
Aspirin	Lorazepam
Cephalexin	Meclizine
Chloral hydrate	Mexiletine
Diazepam	Nifidipine
Digoxin	Norfloxacine
Dipyridamole	Oxazepam
Docusate sodium	Ranitidine
Flurazepam	Tocainide
Furosemide	Triamterene
Hydrochlorothiazide	Triazolam
Ibuprofen	

tients were assessed for the determination of ENC and its metabolites. With the wavelength of detection set at 270 nm, analysis of these samples revealed minimal interference from other drugs. Of the drugs tested, only quinidine (in urine with MODE) or its metabolites caused limited interference. However, at 254 nm this interference was much greater in urine of patients taking quinidine and ENC simultaneously preventing adequate measurement of ENC and its metabolites.

The assay reported herein is based on that described by Mayol et al. [13]. Although the lifespan of the silica column is shorter (1000 samples) than can normally be expected because of the high amount of water in the mobile phase, this system remains the one allowing the most efficient separation of ENC and its metabolites within a 15-min analysis. We have found that neither C_{18} nor Cyano columns give good separations between ODE and MODE using conventional mobile phase (methanol–10 m*M* phosphate buffer, 65:35, v/v or acetonitrile–20 m*M* perchlorate buffer, 30:70, v/v). The chromatographic system used is indeed intermediate between normal bonded phase and reversed-phase chromatography in that it uses a polar mobile phase with a stationary phase normally designed to act as the polar constituent.

CONCLUSION

Use of ethaverine as the internal standard and of the salting-out technique during the extraction procedure with minor adjustment of the chromatographic system improves the accuracy and the sensitivity of the formerly reported assay of ENC [13]. The method is very convenient since ENC and its three major metabolites can be measured with the same plasma or urine sam-

ple. Shifting of the detection wavelength from 254 to 270 nm yields better selectivity without significant loss of sensitivity which is desirable for monitoring of ENC in patients with concomitant drug therapy.

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