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Gas-liquid chromatographic estimation of lignocaine, ethylglycylxylidide, glycylxylidide and 4-hydroxyxylidine in plasma and urine

K. K. ADJEPON-YAMOAH AND L. F. PRESCOTT

University Department of Therapeutics, The Royal Infirmary, Edinburgh EH3 9YW, U.K.

Gas-liquid chromatographic methods have been developed for the estimation of lignocaine and its metabolites ethylglycylxylidide (EGX), glycylxylidide (GX) and 4-hydroxyxylidine in plasma and urine. Lignocaine, EGX and GX were extracted from alkaline solution, the metabolites were acetylated and the three compounds were chromatographed simultaneously using single column temperature programming with a nitrogen-sensitive flame ionization detector. The limits of detection were 10–30 ng ml^{-1}. Conjugated 4-hydroxyxylidine in urine was hydrolysed with glusulase and chromatographed as the acetyl derivative.

Lignocaine is a widely used antiarrhythmic local anaesthetic agent. Its *N*-dealkylated metabolites, ethylglycylxylidide (EGX) and glycylxylidide (GX), have pharmacological actions qualitatively similar to lignocaine (Ehrenberg, 1948; Åström, 1971; Smith & Duce, 1971; Boyes, 1972 personal communication), and have been thought to cause cns toxicity after the administration of lignocaine (Boyes & Keenaghan, 1971; Strong & Atkinson, 1972; Strong, Parker & Atkinson, 1973). In studying the relation between plasma lignocaine concentrations and therapeutic and toxic effects, it is necessary to measure concentrations of EGX and GX. 4-hydroxyxylidine, the major urinary metabolite of lignocaine, is thought to be inactive.

Di Fazio & Brown (1971) described a method for the assay of EGX and GX by direct chromatography of chloroform extracts on 10% UCW 98 on Chromosorb W. The ranges of linearity for EGX and GX were stated to be 0·08 to 40 and 0·4 to 40 $\mu l\ ml^{-1}$ respectively, but few details were given and no mention was made of the reproducibility. It is most unlikely that EGX and GX could be chromatographed directly in the concentrations found in plasma on a 'non-polar' liquid phase without serious adsorption losses. Keenaghan & Boyes (1972) extracted lignocaine and its metabolites from urine and chromatographed the heptafluorobutyryl derivatives without the use of an internal standard. Details of precision, reproducibility, recovery and sensitivity were not given. Strong & Atkinson (1972) and Strong & others (1973) described a method for the assay of lignocaine, EGX and GX using gas chromatography with mass fragmentography. Few details of the method were given and the sensitivity was poor. GX could not be measured at all at concentrations below 1 $\mu g\ ml^{-1}$ and the standard curve for EGX was non-linear below about 0·4 $\mu g\ ml^{-1}$. Because of these disadvantages, existing methods are unsuitable for routine clinical application. We now describe g.l.c. methods for the simultaneous assay of lignocaine, EGX and GX in human plasma and urine down to concentrations of 10–30 ng ml^{-1} and for the estimation of conjugated 4-hydroxyxylidine in urine.

MATERIALS AND METHODS

Simultaneous assay of lignocaine, EGX and GX in plasma and urine

To plasma or urine samples (2 ml) in 15 ml glass stoppered round bottomed tubes was added 5N NaOH (0·5 ml) with mixing and then redistilled dichloromethane (5 ml) containing aceto-*p*-toluidide (APT, 0·125 to 2·5 μg ml^{-1} depending on the amount of drug present) as the internal standard. After being shaken mechanically for 20 min the tubes were centrifuged and the upper aqueous phase discarded. The dichloromethane extract was then decanted into a 15 ml tapered centrifuge tube. 10 μl of acetic anhydride and 2 μl of anhydrous pyridine were added and the contents of the tubes mixed using a vortex mixer. The tubes were incubated at 40° for 20 min in a water bath and the contents then evaporated to dryness with a rotary vacuum evaporator. The residue in each tube was dissolved in ethanol (20 μl) with the aid of a vortex mixer and 1 to 4 μl amounts were injected into the gas chromatograph.

Chromatography

A Hewlett Packard Model 402 chromatograph fitted with a Model 15161A nitrogen sensitive flame ionization detector was used with a Moseley model 7128A strip chart recorder. Column: 4 ft × $\frac{1}{4}$ inch o.d. U-shaped glass tube packed with 3% cyclohexane dimethanol succinate on 100/120 mesh Gas-chrom Q which had been conditioned at 245° for 72 h with a helium carrier gas flow or 20 ml min^{-1}. Before use, eight 10 μl injections of *N,O*-bis (trimethylsilyl) acetamide (BSA) were made at hourly intervals. Flash heater and detector temperatures: 320° and 350° respectively; oven temperature: 200° rising to 245° at 3° min^{-1} after an initial delay of 2 min. Helium carrier gas, hydrogen and air flow rates: 50, 30 and 210 ml min^{-1} respectively. The nitrogen detector was operated with the rubidium bromide crystal just below the position of maximum ionization.

The peak height ratios of lignocaine, EGX or GX to APT were plotted against known concentrations of the three compounds in plasma or urine. For the simultaneous estimation of lignocaine, EGX and GX, appropriate standards (0·5–2 μg ml^{-1} for plasma and 1–10 μg ml^{-1} for urine) were run under the same conditions as the unknowns. The concentrations in the unknown samples (U) were determined from the following formula: $U = SY/X$ where Y is the peak height ratio of lignocaine, EGX or GX to APT in the unknown, X the corresponding ratio for plasma or urine standards and S the concentration in μg ml^{-1} of lignocaine, EGX or GX in the standards.

Estimation of conjugated 4-hydroxyxylidine in urine

4-Hydroxyxylidine is unstable in aqueous solutions and must be freshly prepared. To urine (1 ml) containing 10–100 μg ml^{-1} of 4-hydroxyxylidine (conjugated) in 15 ml round bottomed stoppered test tubes was added sodium metabisulphite (1 mg) 0·2M sodium acetate buffer pH5 (1 ml) and glusulase (Endo Laboratories, Garden City, New York, U.S.A.) (0·05 ml). After mixing, the tube contents were incubated at 40° for 45 min and rapidly cooled to 0°.

To the hydrolysate (1 ml) in 15 ml round bottomed stoppered tubes was added 2M potassium hydrogen phosphate –NaOH buffer pH 10·7 (0·12 ml) and the pH of the solution was adjusted to 7·2 with NaOH. Freshly redistilled dichloromethane (5 ml) containing 5 μg ml^{-1} *N*-butyryl-*p*-aminophenol (NBA) as the internal standard

was added and the tubes were shaken for 15 min and centrifuged. The upper aqueous phase was transferred to another 15 ml stoppered tube and re-extracted with 3 ml of dichloromethane. After centrifugation the upper layer was discarded, the organic extracts were combined in a 15 ml centrifuge tube and acetic anhydride (60 μl) and anhydrous pyridine (5 μl) were added and mixed. The tubes were then stoppered loosely and incubated in a water bath at 45° for 40 min, their contents evaporated to dryness using a rotary vacuum evaporator and the residue dissolved in ethanol (20 μl); 1 to 4 μl samples were chromatographed.

Chromatography

A Hewlett Packard Model 402 chromatograph equipped with a standard flame ionization detector was used. Column: 4 ft × ¼ inch o.d. U-shaped glass tube packed with 1% Carbowax 20 M on 80/100 mesh Gas-chrom Q. Detector, flash heater and oven temperatures: 300, 280 and 205° respectively. Nitrogen carrier gas, hydrogen and air flow rates: 50, 25, and 200 ml min^{-1} respectively.

The peak height ratios of acetylated 4-hydroxyxylidine to that of NBA were plotted against 4-hydroxyxylidine concentrations in the range of 10 to 100 μg ml^{-1}. A urine standard containing 50 μl ml^{-1} 4-hydroxyxylidine was run through the whole procedure with the unknown samples and the 4-hydroxyxylidine concentrations in the unknowns (U) were determined from the following formula: $U = 50Y/X$ where Y equals the peak height ratio of the acetyl derivatives of 4-hydroxyxylidine to NBA in the unknown sample and X the peak height ratio of the urine standard to NBA.

RESULTS

Simultaneous assay of lignocaine, EGX and GX in plasma and urine

Symmetrical chromatographic peaks were obtained for all the compounds (Fig. 1) and the retention times of APT, lignocaine, EGX and GX were 2, 3·3, 11·8 and 17·3 min respectively. Linear calibration curves passing through the origin were obtained for plasma and urine standards over the concentration ranges, 0·05 to 10 μg ml^{-1} and 0·1 to 40 μg ml^{-1} respectively. The limits of detection for lignocaine, EGX and GX added to water, plasma or urine were about 0·01, 0·01 and 0·03 μg ml^{-1} respectively. The reproducibility of replicate analyses of samples containing different concentrations of the 3 compounds is shown in Table 1. Plasma and urine from 16 healthy volunteers not receiving drugs did not produce interfering peaks at the attenuations used for concentrations down to 0·05 μg ml^{-1} for all three compounds.

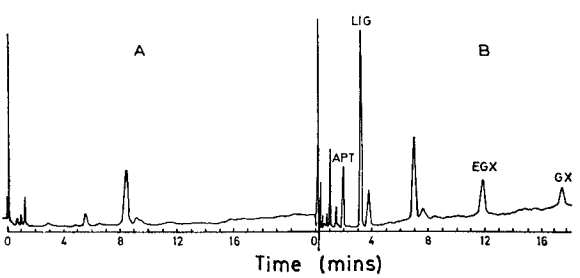

FIG. 1. Gas-liquid chromatogram of acetylated extracts of (A) blank plasma and (B) plasma from a patient receiving lignocaine. APT = aceto-*p*-toluidide, the internal standard, LIG = lignocaine (1·25 μg ml^{-1}), EGX = ethylglycylxylidide (0·39 μg ml^{-1}), GX = glycylxylidide (0·33 μg ml^{-1}).

Table 1. *Reproducibility of replicate simultaneous analyses (means from 6 samples at each concentration) of lignocaine, EGX and GX added to human plasma and urine.*

	Plasma concentration (μg ml^{-1})	± s.d. %	Urine concentration (μg ml^{-1})	± s.d. %
Lignocaine	0·1–2·0	3·4	1–40	3·1
EGX	0·1–2·0	6·0	1–40	3·6
GX	0·1–2·0	7·7	1–40	6·3

Table 2. *Mean recovery of lignocaine, EGX and GX from plasma and urine relative to aqueous standards.*

Compound	Mean relative recovery %*	± s.d. %
Lignocaine		
Plasma (0·1 to 2 μg ml^{-1})	96·6	6·4
Urine (0·5 to 40 μg ml^{-1})	99·4	4·5
EGX		
Plasma (0·1 to 2 μg ml^{-1})	86·3	6·1
Urine (0·5 to 40 μg ml^{-1})	101·9	6·1
GX		
Plasma (0·1 to 2 μg ml^{-1})	54·6	4·6
Urine (0·5 to 40 μg ml^{-1})	103·0	8·2

* Relative to recovery from aqueous solutions.

Comparisons were made with 34 plasma and 30 urine samples.

The recovery of lignocaine, EGX and GX from plasma and urine relative to water is shown in Table 2. The recovery of EGX and GX from plasma was significantly lower than from water or urine, but the recovery was constant.

Estimation of conjugated 4-hydroxyxylidine in urine

Symmetrical chromatographic peaks were obtained with acetylated 4-hydroxyxylidine and NBA and the respective retention times were 6·7 and 10 min. A linear calibration curve passing through the origin was obtained with 4-hydroxyxylidine concentrations between 5 and 100 μg ml^{-1} and the standard deviation of replicate analyses of urine samples containing added 4-hydroxyxylidine (10–100 μg ml^{-1}) was ± 6·4%. The sensitivity of the method was limited to about 3 μg ml^{-1} as some urine samples gave an interfering peak which was equivalent to about 0·5 μg ml^{-1} of 4-hydroxyxylidine.

The mean recovery of 4-hydroxyxylidine added to urine was significantly higher than that from aqueous solutions (mean 127 ± 11·2%).

DISCUSSION

The methods described have been used for several years for routine clinical studies of lignocaine metabolism. The combined assay of lignocaine, EGX and GX described is sensitive and has good reproducibility down to submicrogram concentrations in both plasma and urine. As little as 10–30 ng ml^{-1} can be detected. Analyses

should be carried out as soon as possible or samples acidified, since significant loss of EGX and GX was observed after storage for one month in neutral or alkaline solution at 4 to −20°. Accurate estimation of 4-hydroxyxylidine in biological fluids is difficult since the compound is unstable and the optimum pH for extraction is critical. For good reproducibility the extraction must be effected without delay once the pH of the hydrolysate has been adjusted to 7·2.

The nitrogen sensitive flame ionization detector has several advantages over conventional flame ionization detectors. The solvent front is small and provided the liquid phase does not contain nitrogen, single column temperature programming is possible at maximum sensitivity without appreciable base-line drift. Furthermore, elaborate 'clean up' procedures are usually unnecessary because of the selectivity of the detector.

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