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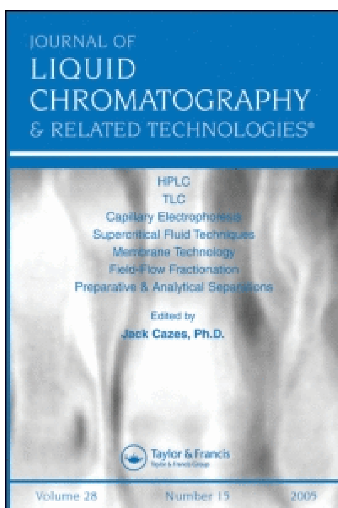

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High Performance Liquid Chromatography (HPLC): An Important Technique in the Studies of Lignocaine and its Metabolism

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): AN IMPORTANT TECHNIQUE IN THE STUDIES OF LIGNOCAINE AND ITS METABOLISM

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ABSTRACT

The high performance liquid chromatography (HPLC) assays which have been developed for the studies of lignocaine over the past 15 years have been reviewed. Studies of lignocaine and its metabolism have been benefited greatly by utilizing this modern technique. The sensitivity and process time of HPLC have been greatly improved by modifications including the use of solid-phase extraction for sample preparation. A further modification in the assay of hydroxylated derivatives is presented. In the study of lignocaine-monoethylglycine-xylidide (MEGX) as a dynamic test of liver function, HPLC assay possesses some advantages over fluorescence polarisation immunoassay (FPIA).

INTRODUCTION

Lignocaine was synthesised by Löfgren in 1946 [1], an important date in the development and field of local

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anesthetics. The chemical structure of lignocaine is an aromatic group, 2,6-xylidine, coupled to diethylglycine via an amide bond. In man, lignocaine mainly undergoes de-ethylation by the hepatic cytochrome P-450 IIIA4 to form monoethylglycine-xylidide (MEGX) and glycinexylidide (GX) [2]. However, in some animals like the rat, the main metabolic pathway for lignocaine is hydroxylation rather than de-ethylation, with 3- and 4-hydroxy (3- and 4-OH) lignocaine and 3- and 4-OH-MEGX being the main metabolites [3].

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Before the development of gas chromatography (GC), colorimetric (methyl orange) [4] and enzyme assay [5-6] were used for studies of lignocaine's metabolism. In the early 1960's, GC was established for the measurement of lignocaine [7-9], and it played a very important role in the initial studies of the metabolism of lignocaine [10-16].

High performance liquid chromatography (HPLC) was developed in the 1960's [17]. However, its application to lignocaine's metabolism and pharmacokinetics was delayed to the end of 1970's, becoming feasible with the development of so-called reversed-phase chromatography. Generally speaking, compared with GC, HPLC possesses many advantages in the measurement of drugs and their metabolite(s) in biological samples [18].

This review is intended to (1) describe generally the application of HPLC to the study of lignocaine metabolism with the establishment of various HPLC assays and (2) particularly discuss the use of lignocaine and the measurement of its main metabolite, MEGX, as a dynamic test of liver function in liver transplantation. The test is based on the conversion of lignocaine to MEGX, the latter concentration being used as an index of liver function. This test necessitates the use of a highly sensitive MEGX assay. The advantages and disadvantages comparing HPLC and another popular assay, fluorescence polarisation immunoassay (FPIA), used in this clinical assay role will also be discussed.

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THE DEVELOPMENT OF HPLC ASSAYS AND THEIR APPLICATION ON THE METABOLISM STUDIES OF LIGNOCAINE

De-ethylated Metabolites

The first published HPLC assay for the measurement of lignocaine was developed by Adams et al. in 1976 [19]. Two years later Narang et al. [20] established the first HPLC assay for the measurement of lignocaine and its two main metabolites, MEGX and GX. From that time HPLC gradually replaced GC and became the first choice for the simultaneous measurement of lignocaine and its metabolites in biological specimens [21-33].

Published HPLC assays for the determination of lignocaine and its metabolites utilize liquid-liquid or\and solid-phase extraction, ultraviolet (UV) or electrochemical detection, with bonded reversed-phase columns or a various bare (unbonded) silica gel column for separation and some chemicals or drugs have been nominated as the internal standard. Table 1. summarizes these major differences from the HPLC assays reported to the present time.

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Hydroxylated Metabolites

The first HPLC assay for the measurement of 3-OH- and 4-OH- lignocaine and 3-OH-MEGX was developed in 1984 by Tam et al. [37]. With modification, it was capable of the simultaneous determination of most hydroxylated and de-ethylated metabolites of lignocaine in plasma and urine [38]. A similar assay was reported by a Japanese group [39]. During our studies of lignocaine metabolism in the pig (submitted for publication), the latter assay [39] was substantially modified to allow the measurement of 3-OH-lignocaine and 3-OH-MEGX. In the reported procedure [39], the internal standard, procaine, was added after the first step of organic solvent extraction prior to the acid back-extraction. The neutralized acid phase then was evaporated to dryness with the residue reconstituted with mobile phase. In our experience, the

TABLE 1

Summary of Reported HPLC Assays for Lignocaine and Its Metabolites.

Item		Reference Number
Column	Reversed-phase	19-22,25,28-31, 32,34
	Silica gel	23,26
Detection	Ultraviolet (UV)	19-23, 25-35
	Electrochemical	24
Internal Standard	EMGX*	20-22, 25-27
	Trimethoprim	34-35
	p-Chlorodispyromide	29-30
	Bupivacaine	24
	Procaine	19,28,31-32
	Tocainide	33
Extraction	Liquid-liquid	
	Dichloromethane	19,22,26,30,34
	Ethyl acetate	21,25,28,31-32
	Chloroform	20
	Hexane	20
	Isopropanol	20
	Tert-butyl methyl ether	29
	Solid-phase	[24,27,33,35]

*: EMGX= Ethylmethylglycinexylidide

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evaporation procedure usually took more than five hours at 40 °C, and in addition required a vacuum facility. There were no validation data available in the reported assay. We therefore modified this procedure in order to improve assay precision and reduce the process time. The assay now utilizes trimethoprim as the internal standard which is added to the serum sample prior to the

organic solvent extraction. Following acid back extraction, the neutralized aqueous phase is blown with an air stream until the residues of organic solvent are completely driven off; this usually takes only two hours. The aqueous phase is then injected directly into the column. The standard curves of 3-OH-lignocaine between concentrations of 10 to 2000 μg/L and 3-OH-MEGX, 20 to 350 μg/L, were linear with correlation coefficients all more than 0.999. The coefficients of variation of 3-OH-lignocaine at concentrations of 15, 100, 1600 μg/L and 3-MEGX at 25 100, 300 μg/L were all less than 10%. Therefore, in our modified assay for the measurement of 3-OH-lignocaine and 3-OH-MEGX, the precision is ensured by adding internal standard before the extractions and the requirement of both vacuum evaporation and reconstitution become unnecessary.

Compared with alternative assays available for the de-ethylated metabolites of lignocaine (such as FPIA), HPLC assay has an obvious advantage for having the capacity to be modified as described for measurement of its hydroxylated metabolites. This unique suitability of the HPLC technique to be used for simultaneous assay of multiple metabolites will ensure it an important role in comprehensive studies on future lignocaine metabolism.

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HPLC ASSAY IN THE LIGNOCAINE-MEGX DYNAMIC LIVER FUNCTION TEST

Liver Function Test in Liver Transplantation

The rapid progress made in clinical liver transplantation has produced a climate conducive to the development of new and alternative tests of organ function other than those traditionally used. The predominant demands are that it be a sensitive and reproducible assay and that clinical relevance be demonstrated. Lignocaine and MEGX is being proposed in this major role. The first requirement of the MEGX assay, i.e the assay must be sensitive and reproducible, is established; the second, its clinical role, is currently under investigation.

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In the area of liver transplantation, three different clinical facets are being considered. First, there is assessment of potential liver donors [40-44]; in this case the test should be readily available at any hospital with a potential donor; turnaround time should be rapid (e.g. within one hour) and there should be good sensitivity as an indicator of current liver function. Secondly, this test may be used to assess the severity of chronic liver disease and may in the future, help to determine the appropriate time for transplantation [45].

Thirdly, once the liver has been transplanted, this test can be used to monitor the recipient with a good specificity and sensitivity. Clinical events which have been documented include primary non function [46], hepatic artery thrombosis [47], ischemia [47], rejection [42] and the general clinical course [48].

The ability of the liver to detoxify xenobiotics has been promoted previously as the basis of tests used to monitor liver function. Administration of particular drugs/compounds with subsequent monitoring of their elimination from serum has been studied for many years. Generally speaking, these tests have one or more deficiencies, notably a slow turnaround time, technical complexity, and poor specificity and sensitivity [36, 49].

In 1987, Oellerich and colleagues noticed that the rate of MEGX formation correlates with the rate of Lignocaine clearance [40] and for the first time, they suggested that the measurement of lignocaine metabolism can be used to assess liver function.

Measurement of MEGX Concentration

Sensitivity is essential for any assay in the measurement of MEGX concentration as an index of liver function in liver transplant recipients and end-stage

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liver disease patients awaiting for transplantation. In these patients, liver function is expected to be very poor and the production of MEGX can be anticipated to be very low. The detection limits of most published HPLC assays [19-23, 25-33] are more than 20 µg/L, which is obviously not sensitive enough [40-42, 45, 47]. Recently, we developed two HPLC assays [34-35] in which the sensitivity was increased to 10 µg/L. They have proved very useful for the measurement of MEGX concentration in dynamic testing in liver transplantation. The more recent method [35] utilized solid-phase extraction for sample preparation. Various packings were tested {C_{18}, C_8 and CN (1cc and 3cc) from Waters (Millipore, Bedford, MA, USA) and Analytichem Bond-Elut of C_8 (3cc), CN and phenyl (1cc) from Varian (Harbor City, CA, USA)}. The 1cc phenyl Bond-Elut was most appropriate for our needs. For optimum separation, it is important to select suitable packing, but at the same time attention must be paid to the volume of solid phase, for this is critical for the sensitivity of the assay, as it will determine the volume of final elution solution.

Comparison of Fluorescence Polarisation Immunoassay (FPIA) and HPLC

FPIA has been used for the measurement of lignocaine in serum samples for many years. However it

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was not until recently that a FPIA assay for the measurement of MEGX in serum specimens was developed (Abbott Tdx). According to the Production Information [50] this assay has many advantages such as good sensitivity and short process time which can meet the demand for a dynamic liver function test in liver transplantation.

However, FPIA has some obvious disadvantages. (1) The cost of an individual assay is expensive. (2) The claimed limits of detection in the assay is 10 ng/ml, however, this has not been supported by company CV data to show that if the measured concentration at this low level is reliable. (3) This assay is for MEGX only. For any metabolic and pharmacokinetic study, it is preferable if the concentration of parent drug is measured simultaneously. In the particular case of the MEGX test, the concentration of lignocaine provides useful information to check whether the required dose of lignocaine has been given. (4) FPIA is not suitable for those samples with high bilirubin levels. Raised bilirubin concentrations are common in liver transplantation recipients early after transplantation and in those end-stage liver disease patients who are awaiting transplantation. In the presence of raised bilirubin sample dilution is frequently needed and

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therefore the process time is prolonged and sensitivity decreased.

In the newly developed HPLC assays [34-35], MEGX sensitivity has been improved, the detection limit being 10 μg/L with a coefficient of variance (CV) < 4%. This is particularly useful for the measurement of low MEGX concentrations from patients with poor liver function. Additionally, trimethoprim was utilized as internal standard for the first time in a lignocaine-MEGX HPLC assay. Trimethoprim is more readily available than the previously most commonly-used internal standard, ethylmethylglycinexylidide (EMGX).

The claim for rapid assay of MEGX by FPIA is based on a total process time of approximately 40 minutes for 20 samples [50]. It was the only assay with which the MEGX results could be obtained within one hour. All previously reported HPLC assay using liquid-liquid extraction [19-22,25,28-32,34] have long process times involving time-consuming extraction and evaporation procedures and in a further three HPLC assays using solid-phase extraction [24,26,33], an evaporation step was still used. However, in our HPLC assay using solid-phase extraction [35], the final eluate can be directly injected onto the column. Total time for extraction of

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five serum/plasma samples is approximately 10 minutes and HPLC run time for individual sample is about 10 minutes. In practice, that means that in an assay-run incorporating a single point calibrator and two control samples, the results of MEGX and lignocaine concentrations from predose and 15-min samples from one liver transplantation patient could be obtained within one hour. This assay time is now comparable with what only FPIA offered previously. This HPLC assay offers not only an important alternative to the FPIA method with comparable process time but also better sensitivity. The limit of detection is 8 ng/ml. The CV's for MEGX concentrations between 10 and 200 μg/L are <9.5%. At the same time the simultaneous measurement of GX can be achieved through minor adjustment of mobile phase composition and flow rate. The limit of detection of GX is 10 μg/L in this assay, the lowest one reported to date.

In the initial study by Oellerich [40] , the comparison between HPLC and FPIA in the determination of MEGX concentrations in patient's samples was in good agreement with each other. Recently, another study [51] compared MEGX concentrations by HPLC and FPIA in 119 samples from 45 liver transplantation donors and recipients. The correlation coefficient between the two

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methods was r=0.89. By using the technique of Bland and Altman [52], the bias was found 12 μg/L towards FPIA over the MEGX concentration range 0-250 μg/L. The major difference between the two methods occurred in samples from four recipients and one donor. Significant cross reactivity was not found in FPIA between MEGX and lignocaine and two of its metabolites (GX and 2,6-xylidine). Elevated plasma cholesterol and triglyceride concentrations resulted in only relatively small increase in apparent MEGX concentrations and whilst there was an increase in apparent MEGX concentration, in some samples with raised bilirubin concentrations, the relationship was not constant.

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CONCLUSION

This review has clearly shown that HPLC has found broad and extensive application in studies of lignocaine metabolism over the past 15 years. Its further development is extending its utilization into other areas. In the particular practice of the dynamic testing of liver function in the field of liver transplantation, HPLC has demonstrated its unique advantages over FPIA. Undoubtedly, this modern technique will be further developed expanded in other areas in the future.

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