LIQUID-PHASE MICROEXTRACTION FOR THE DETERMINATION OF ACIDIC DRUGS AND β-BLOCKERS IN WATER SAMPLES

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ABSTRACT

Liquid-phase microextraction (LPME) is a relatively simple and inexpensive sample preparation technique. Different LPME modes were designed in this work: two-phase LPME for extraction of hydrophobic acidic drugs, three-phase LPME for extraction of ionizable hydrophobic β-blockers, and carrier-mediated LPME for extraction of a highly hydrophilic β-blocker, atenolol (that was unable to be extracted by three-phase LPME). Under optimized conditions, two-phase LPME exhibited good linearity over four orders of magnitude in the concentration range, 0.2-200 ppb, with r^2 values >0.992 for most of the analytes. The RSD for these compounds were between 7.4-11.8%. The LODs for these drugs were in the range of 10^{-2} ppb with enrichment factor >74. Both three-phase and carrier-mediated LPME displayed good precision with less than 8 % RSD for selected β-blockers except for propanolol (18%). Both LPME modes also showed good linearity with r^2 values >0.996. Enrichment factors for various β-blockers were found to be around 50-fold in three-phase LPME, while the LODs were between 2-16 ppb. Conversely, carrier-mediated LPME provided 2.5-fold of enrichment with LOD of 62.5 ppb for atenolol. Both methods gave excellent extraction recovery with relative recovery in the range 85.7 to 108.2% for water samples.

Keywords: two-phase LPME, three-phase LPME, carrier-mediated LPME, acidic drugs, β-blockers

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SUMMARY

The development of fast, precise, accurate, sensitive and environmentally-friendlier methodologies is an important issue in chemical analysis. The introduction of liquid-phase microextraction (LPME) has opened a new chapter in solvent extraction techniques. With the combination of the liquid membrane and polymer technology, hollow fiber based LPME was developed and improvised. Hollow fiber with organic solvent impregnated within its wall pores serves as semi-permeable membrane to allow the target analytes but not extraneous matrix materials to pass through the membrane and be extracted. Two-phase LPME is designed to extract neutral or charged hydrophobic analytes and is compatible to GC analysis, while three-phase LPME is most suitable for moderately hydrophobic water-soluble charged analytes and is catered for HPLC and CE analysis. In order to extract highly hydrophilic compounds, carrier-mediated LPME is used instead. Different modes of LPME could also be used as complementary methods to analyze a wide range of compounds (neutral vs. charged, hydrophobic vs. hydrophilic, acidic vs. basic). Various experimental parameters as well as practical considerations for method optimization are discussed in detail in chapters 3 and 4. Without the complicated experimental set-up, the easy-to operate single-step procedure of LPME proves to be an attractive technique for sample clean up and preconcentration.

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ABBREVIATIONS

APIs	active pharmaceutical ingredients
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
LPME	liquid-phase microextraction
LOD	limit of detection
NSAIDs	Non-steroidal anti-inflammatory drugs
ppm	parts per million
rpm	round per min
RSD	relative standard deviation
SIM	selected-ion monitoring
TMSH	trimethylsulfonium hydroxide
TMPAH	trimethylphenylammonium hydroxide
UV	ultra violet

CHAPTER 1

Introduction

1.1 An overview of the development of solvent extraction

Nowadays, the development of fast, precise, accurate and sensitive methodologies has a significant impact in analytical science. Despite the great advancement in technology, most analytical instruments are unable to handle sample matrices directly. This incompatibility has made a sample preparation step compulsory prior to actual instrumental analysis. Sample preparations can be rather complex and time consuming, and thus require very careful manipulation. Moreover, multistep operations in the preliminary sample preparation are generally very critical because they could be the source of major errors that may hinder sample clean-up and analyte preconcentration that decisively influences the precision, sensitivity, selectivity, rapidity and cost.

One of the most frequently used sample pretreatment methods is solvent extraction. Solvent extraction has been used in analytical chemistry since the mid-1950s and its application as a powerful sample pretreatment in both trace and macro level of materials has steadily increased in the past twenty years due to its simplicity, reproducibility and versatility[1]. Solvent extraction is based on the distribution of a solute between two immiscible liquid phases, an aqueous phase and an organic phase. Most often, analytes that are dissolved in aqueous solution are extracted into an immiscible organic solvent in a separatory funnel. After the mixture is shaken, the phases are allowed to separate, analytes would distribute themselves between two phases according to a certain equilibrium ratio, and separation can be achieved. This

technique indeed gives good clean-up from the sample matrix simply by selection of a suitable organic solvent. Solvent extraction, however, has some drawbacks. It is laborious, time consuming and difficult to automate. In addition, large amounts of organic solvents pose both environmental and health hazards.

Given the disadvantages of solvent extraction, it is interesting and highly desirable to identify alternative methods for sample clean-up. In-line with the quest to pursue 'Green Chemistry' principles, evolution in solvent extraction has brought upon the introduction of miniaturized solvent extraction, better known as liquid-phase microextraction (LPME). Liquid-phase microextraction emphasizes minimal exposure to toxic organic solvents. Microdrop extraction was the first technique introduced in 1996 to reduce organic solvent usage[2]. In this simple technique, a microdrop of solvent was suspended directly at the tip of a microsyringe needle that was immersed in a stirred aqueous sample solution. After extraction, the microdrop was retracted into the microsyringe and was subjected to analysis[3]. One advantage of microdrop extraction over conventional extraction techniques is that only small volumes of organic solvent are required. One important feature of microdrop extraction is the simultaneous extraction as well as sample clean-up in a single operation. Apart from being inexpensive, microdrop extraction requires only common laboratory equipment and it does not suffer from carry-over between extractions which are encountered in conventional extraction techniques[3]. In addition, high preconcentration may be achieved for analytes with high partition coefficients as they are transferred from a relatively large sample volume (a few mililiters) into a microdroplet of typically a few microliters[4]. Unfortunately, microdrop extraction is not a very robust technique for routine analysis, as the droplet may be lost from the needle tip of the syringe while in the midst of extraction, especially when the stirring speed is high[4]. (Stirring facilitates

mass transfer of analytes). The viability of the drop also depends on the stability of the emulsion. Emulsion rupture is usually due to emulsion swelling caused by the transport of the external phase into the emulsion. Although emulsion rupture can be greatly decreased by including additives, it would slow down the rate of extraction, not to mention their solubility and the interaction with the bulk solution[5].

Efforts to circumvent the inconveniences in microdrop extraction have driven the research on supported liquid membrane as it combines the benefits from both liquid-phase microextraction and membrane technology. Apart from efficient cleanup, low organic solvent usage, low operating cost and elimination of emulsion formation, and the disposable nature of polymeric membrane also eliminates the possibility of carry-over between analytes. Two types of support configurations are used: flat sheet membrane modules or hollow fiber, but the techniques differ significantly in terms of instrumentation and operation. Flat sheet membrane is usually used in large-scale operation whereby a flowing system equipped with a pump is continuously feeding the membrane with fresh sample that is normally applied for a large number of extractions[4]. On the other hand, hollow fiber-based LPME is often applied when sample size is small. Hollow fiber provides large surface area to volume ratio (approximately 10^4 m^2/m^3)[5], thereby accelerating the extraction process. Besides, the hydrophobicity of polypropylene-based hollow fiber allows the organic solvent to wet the pores spontaneously, facilitating the immobilization of organic phase on the fiber. The inert nature of polypropylene fiber allows extraction to be carried out in corrosive condition (extreme pH) without sacrificing membrane integrity. Its low capital cost implies that the hollow fiber can be discarded after using it once only. Fouling is not an issue because each extraction takes place between 20 to 60 min only; there is insufficient time for contamination to occur.

The first hollow fiber-based LPME was introduced in 1999 by Pedersen-Bjergaard[6]. It can be carried out in a three-phase system where analytes in neutral form are extracted from aqueous samples, through a thin layer of organic solvent into an aqueous phase. Extraction can also take place in a two-phase system whereby the analytes are extracted from an aqueous phase directly into an organic phase. In the three-phase system, a liquid membrane consists of a water-immiscible organic solvent impregnated in the microporous hydrophobic polymeric support, and it is placed between the two aqueous phases (donor phase and acceptor phase). This allows organic phase to be thin, behaving like membrane. One of these aqueous phases (donor phase) contains the analytes to be transported through the membrane into the second phase (acceptor phase) that strips analytes from the liquid membrane. Furthermore, pH adjustment of acceptor phase in three-phase extraction ensures full ionization of extracted analytes and prevents back-extraction into the organic phase (liquid membrane). Thus, extraction and stripping take place at the same time and in the same extraction vessel, instead of multiple steps in the case of conventional solvent extraction. The two-phase system is one in which analytes are extracted into an organic phase in the wall pores as well as in the lumen of the hollow fiber. Hence, both two-phase and three-phase hollow fiber-based LPME is ideal for extraction of hydrophobic analytes with the latter providing higher selectivity towards those ionizable hydrophobic analytes. Overall, the two modes of liquid membrane is stabilized by capillary forces, making the addition of stabilizers to the liquid membrane unnecessary[5]. Unlike microdrop LPME, the sample may be stirred effectively without any loss of the extract back into the sample solution. Moreover, the solvent is effectively protected by the hollow fiber.

Similar to solvent extraction, hollow fiber based LPME exploits the

differences in the dissociation constants as well as the hydrophobicity of the extracted analytes. Organic compounds are readily distributed into the organic phase due to the "like dissolves like" principle. Therefore, partially ionized substances (e.g. acidic or basic drugs) can be deionized by suitable pH adjustment of the aqueous phase. However, this approach might not be sufficient to extract very hydrophilic compounds. It is necessary to introduce a carrier into the donor phase prior to the extraction. By incorporating different specific reagents, it allows improvement of the isolation of the analytes from the bulk sample and offers very selective extraction of analytes in very complex samples. These carriers bear a functional group with an opposite charge to the charge of transported molecules. In this way, the carrier would facilitate the analyte passing through the liquid membrane via a neutral, organic soluble ion-pair complex formation. A more detailed description of the characteristics of carrier is provided in section 2.1.3.

Hollow fiber based extraction can also be performed in either static mode or dynamic mode. In the static mode, the acceptor phase is stationary in the lumen of hollow fiber throughout the extraction process. On the other hand, in the dynamic mode, the plunger of the syringe is linked to, and its movement is controlled by, a syringe pump, where the acceptor phase is drawn in and out the lumen of hollow fiber during extraction to increase the mass transfer rate and to facilitate the possibility of automated interfacing to different analytical instruments. The principles of two-phase and three-phase LPME are further illustrated in Chapter 2 while two-phase and three-phase LPME-based experiments are demonstrated in Chapter 3 and Chapter 4 respectively.

1.2 Objectives of the project

In this study, optimization of various parameters involved in hollow fiber-based liquid phase microextraction was performed to investigate its applicability and versatility in trace analysis of active pharmaceutical ingredients in environmental waters. The following chapters will describe various LPME modes developed for applications to real aqueous samples.

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CHAPTER 2

Principles of Liquid-phase Microextraction

Liquid-phase microextraction has been used as a sample clean-up and preconcentration step in many analytical techniques and methods in response to the sample preparation problems posed in many fields such as environmental, forensic, life sciences etc. Among these areas, LPME has gained a notable momentum in trace analysis and this has motivated the development of different configurations of LPME catering to the extraction of different analytes, ranging from acidic to basic, hydrophobic to hydrophobic. These LPME set-ups are also rendered compatible to different analytical instruments so that extraction could be coupled directly to these systems.

2.1 Extraction principles

Despite the differences in dimensions, apparatus and implementation, LPME shares a similar working principle with solvent extraction. LPME also exploits the differential solubility of analytes in two immiscible solvent to achieve extraction and preconcentration. There are two main type of LPME, namely two-phase and three-phase LPME. More selective LPME, carrier-mediated LPME, is also being discussed in the later part of this chapter. Besides the equilibrium constants involved in LPME, some kinetic considerations are also included to provide a better understanding of hollow fiber-based LPME.

2.1.1 Two-phase liquid-phase microextraction

Analytes are extracted from the aqueous solution (donor phase) through a water-immiscible solvent impregnated in the pores of hollow fiber into the same organic solvent (acceptor phase) present in the lumen of hollow fiber, resulting in two-phase LPME where analytes are finally extracted into the organic phase. The extraction process of the two-phase LPME for analyte A may be illustrated as follows:

$$A_{(aq)} \leftrightarrow A_{(org)} \tag{2.1}$$

and is characterized by the distribution ratio D_A, defined as the ratio of the concentration of analyte A in the organic layer, $[A]_{org}$, to the concentration of analyte A in the aqueous solution, $[A]_{aq}$, at equilibrium. The mass balance relationship for analyte A at equilibrium can be expressed by

$$[A]_{aq,i}V_{aq} = [A]_{aq}V_{aq} + [A]_{org}V_{org} \tag{2.2}$$

where $[A]_{aq,\,i}$ is the initial concentration of analyte A in donor phase and V_{aq} ,V_{org} refer to volume of donor phase and acceptor phase respectively. By substituting D_A into the above equation, the equation can be rewritten as

$$[A]_{aq,i}V_{aq} = \frac{[A]_{org}V_{aq}}{D_A} + [A]_{org}V_{org} \tag{2.3}$$

or

$$[A]_{aq,i} = \frac{[A]_{org}}{D_A} + \frac{[A]_{org}V_{org}}{V_{aq}} \tag{2.4}$$

The enrichment factor, E, defined as the ratio of $[A]_{org}/[A]_{aq,i}$, may be derived as

$$E = \frac{1}{\left(\frac{1}{D_A} + \frac{V_{org}}{V_{aq}}\right)} \tag{2.5}$$

2.1.2 Three-phase liquid-phase microextraction

In three-phase LPME, the extraction process involves tandem reversible extractions. In the first step, the analytes are extracted from the donor phase (sample phase) into the organic phase immobilized within the pores of the hollow fiber. In the second step, the analytes are back-extracted into another aqueous phase held inside the lumen of the hollow fiber. For analyte A, the extraction process is illustrated as follows

$$A_{(aq1)} \leftrightarrow A_{(org)} \leftrightarrow A_{(aq2)} \tag{2.6}$$

where the subscript aq1 refers to the donor phase and aq2 refers to the acceptor phase; while org is the organic phase within the pores of the hollow fiber. At equilibrium, the distribution ratio for the analyte A, D_{A1}, between the organic and donor phase is given by

$$D_{A1} = \frac{[A]_{org}}{[A]_{aq1}} \tag{2.7}$$

and the distribution ratio for the analyte A, D_{A2}, between the organic and acceptor phase is given by

$$D_{A2} = \frac{[A]_{org}}{[A]_{aq2}} \tag{2.8}$$

where the concentration of analyte A in donor phase, organic phase and acceptor phase are denoted by $[A]_{aq1}$, $[A]_{org}$, $[A]_{aq2}$, respectively. Given that the volume of donor phase, organic phase and acceptor phase are V_{aq1}, V_{org} and V_{aq2}, and initial concentration of analyte is $[A]_{aq1,i}$, the mass balance relationship for analyte A at equilibrium can be expressed by

$$[A]_{aq1,i}V_{aq1} = [A]_{aq1}V_{aq1} + [A]_{org}V_{org} + [A]_{aq2}V_{aq2} \tag{2.9}$$

or

$$[A]_{aq1,i} = [A]_{aq1} + \frac{[A]_{org}V_{org}}{V_{aq1}} + \frac{[A]_{aq2}V_{aq2}}{V_{aq1}} \tag{2.10}$$

By substituting $[A]_{aq1}$ from (2.7) and $[A]_{org}$ from (2.8), and rearranging the above equation,

$$\begin{aligned}[A]_{aq1,i} &= \frac{[A]_{org}}{D_{A1}} + \frac{[A]_{org}V_{org}}{V_{aq1}} + \frac{[A]_{aq2}V_{aq2}}{V_{aq1}} \\ &= \frac{D_{A2}[A]_{aq2}}{D_{A1}} + \frac{D_{A2}[A]_{aq2}V_{org}}{V_{aq1}} + \frac{[A]_{aq2}V_{aq2}}{V_{aq1}} \\ &= [A]_{aq2}\left(\frac{D_{A2}}{D_{A1}} + \frac{D_{A2}V_{org}}{V_{aq1}} + \frac{V_{aq2}}{V_{aq1}}\right)\end{aligned} \tag{2.11}$$

The enrichment factor, E, defined as the ratio of $[A]_{aq2}/ [A]_{aq1,i}$, may be derived as

$$E = \frac{1}{\left(\frac{D_{A2}}{D_{A1}} + \frac{D_{A2}V_{org}}{V_{aq1}} + \frac{V_{aq2}}{V_{aq1}}\right)} \tag{2.12}$$

In LPME, the volume of organic phase immobilized in the pores of hollow fiber is small, and the enrichment factor, E, can be simplified to[1]

$$E = \frac{1}{\left(\frac{D_{A2}}{D_{A1}} + \frac{V_{aq2}}{V_{aq1}}\right)} \tag{2.13}$$

Thus, enrichment factor greatly depends on:

- phase ratio (volume of acceptor phase to volume of donor phase)
- distribution ratio between donor phase and organic phase as well as between organic phase and acceptor phase.

Equations 2.5 and 2.13 have clearly indicated that enrichment factors are greatly influenced by the ratio of acceptor phase to donor phase. By taking the distribution ratios as constant, the enrichment could be achieved by utilizing large volume of donor phase. However, this application limits the analysis to large sample

size subjects only and is impractical for biological and forensic samples. Nevertheless, the employment of hollow fiber in the extraction has allowed the use of microliters of acceptor phase and made it possible to preconcentrate samples that are present in minute amounts. A simple mathematical illustration of "Enrichment factor as a function of donor / acceptor volume ratio and the acceptor/ donor phase partition coefficient" can be found[1,4]. Equation 2.13 gives us some insight about how phase ratio has influence on enrichment factor. Nevertheless, enrichment would cease when the acceptor phase reaches saturation after prolonged extraction. In view of this limitation, a more comprehensive model of LPME that includes an even greater number of parameters is highly desirable; therefore further research is required to improve on the model. (On the other hand, having a more complex equation would be counter to the philosophy of LPME which embodies simplicity and ease of operation.)

Neutral analytes with high hydrophobicity can be extracted efficiently from aqueous solution to organic phase on the basis of "like dissolves like" principles. In addition, these compounds usually have high distribution ratio, D, which is indicated by their log P values in the literature. However, the analytes often carry charges or partially ionized in the aqueous solution, thus hindering their distribution into the organic phase. If the analytes are acidic or basic species, extraction can be carried out by pH adjustment. By considering extraction of an acidic analyte from aqueous solution, the analyte exists as a weak acid,

$$HA_{(aq)} \leftrightarrow H^{+}_{(aq)} + A^{-}_{(aq)} \tag{2.14}$$

with a particular dissociation constant, K_a,

$$K_a = \frac{[H^{+}_{(aq)}][A^{-}_{(aq)}]}{HA_{(aq)}} \tag{2.15}$$

According to Le Châtelier's principle, the extent of protonation of analytes tend to increase with increased concentration of H^+, thus pH adjustment of the donor phase with strong acid (e.g. HCl) will drive the equilibrium to shift in favor of the deionization of analytes and to facilitate their distribution to the organic phase. With the knowledge of the pK_a value(s) of analytes would allow us to manipulate the acidity of the aqueous solution in order to achieve higher extraction efficiency; in certain cases, manipulation of pH could improve selectivity by enabling only targeted analytes which are deinonized to be extracted into the organic phase. (Similarly, this principle can also be applied in the extraction of basic analytes, which is done under alkaline condition.) The magnitude of distribution ratio, D_{A1}, determines the feasibility of the extraction process; the higher D_{A1} the better the solute is being extracted into the organic phase.

On the other hand, stripping of analytes from the organic phase to acceptor phase in three-phase LPME requires analytes to be more soluble in aqueous phase. This is done by increasing the affinity of analytes towards acceptor phase to organic phase or the distribution ratio, D_{A2}. One way to increase the solubility of analytes and to prevent reentry of analytes back into the organic phase is to facilitate the ionization of the analytes in the acceptor phase. This could be done in a similar way by introducing OH^- to scavenge H^+, consequently, lowering the concentration of H^+ and leaving behind the ionized A^-. Consequently, those neutral compounds that are not or very poorly extracted into the acceptor phase in three-phase LPME would remain in the organic phase and thus provides higher selectivity for ionizable compounds in three-phase LPME. Thus, pH adjustment and organic phase selection play critical roles for successful extraction.

2.1.3 Carrier-mediated liquid-phase microextraction

The above mentioned two-phase and three-phase LPME modes are promoted by high partition of analytes to organic phase, yet, highly hydrophilic analytes or ionic species cannot be extracted successfully by using the same method. Hydrophilic analytes prefer water to organic solvent and they are insoluble in the membrane phase most of the time. Thus, they must be rendered hydrophobic in order to enter the organic phase. In these cases, a more selective extraction could be accomplished by carrier-mediated LPME, whereby the carrier used is a relatively hydrophobic ion-pairing reagent with acceptable water solubility, selectively forming ion-pairs with the target analytes and promoting extraction of these analytes into the organic phase. Considering that a charged hydrophilic analyte could become more hydrophobic by coupling to an oppositely charged water-soluble lipophilic molecule, they could ion-pair to form a complex that can be extracted into the organic layer. Usually, the sodium salts of organic acids would be a choice of an ion-pairing agent. Alternatively, the addition of ionizable organic extractant molecules into the organic phase could also aid the extraction process. Due to its simultaneous hydrophobic/ hydrophilic nature, the extracting reagent tends to orient itself at the interface with their polar or ionizable groups facing the aqueous side, while the rest of the molecule having a prevalent hydrophobic character will be directed instead towards the organic phase. Charged analytes in the aqueous phase could then complex with the ion-pairing reagent and increase its affinity to the organic phase. For example, during the extraction of basic analytes, the pH of the sample solution is adjusted to ionize the basic analytes; while a carrier that carries an opposite charge with the appropriate hydrophobic moiety under that particular pH is added to ion-pair with the ionized analytes. The ion-pair then diffuses across the membrane. In three-phase LPME, at the

interface of the organic phase and the acceptor phase, the carrier reacts with the counter ion added to the acceptor phase so that stripping takes place. The analytes are released from the ion-pair complex and collected in the acceptor phase while the carrier recovers from the stripping process and is transferred back to the extraction interface to begin another extraction cycle. This is usually called the carrier shuttle mechanism[5].

A typical application of carrier mediated transfer is the recovery of metal cations from aqueous phases. The overall reactions involved in the extraction and stripping stages can be represented by the following reversible reaction:

$$M^{+}_{(aq)} + RH_{(org)} \leftrightarrow RM_{(org)} + H^{+}_{(aq)} \tag{2.16}$$

where M^+ is a metal cation, RH is an oil-soluble liquid ion-exchange reagent, and RM is the metal complex[2]. The forward reaction takes place at the interface between donor phase and the membrane, and the reverse reaction at the other membrane interface that is in contact with the acceptor phase. For a given concentration of metal ion, a high concentration of extractant favors the forward reaction, whereas a low pH facilitates the reverse reaction. In the entire extraction process, the ion-exchange reagent shuttles between two interfaces to extract metal cation from the sample solution into the acceptor phase resulting in the preconcentration of the metal cation.

2.2 Parameters that affect liquid-phase microextraction

There are several parameters that affect the performance of LPME, namely the pH of the aqueous solution, the type of the polymer-based hollow fiber and the type of organic phase immobilized on the hollow fiber's pores, etc. Besides that, the kinetics of the microextraction plays an important role. The factors are discussed below.

2.2.1 Hollow fiber selection

Besides those chemical parameters, selection of the appropriate hollow fiber exerts a great influence on the success of LPME. Polypropylene fiber has been widely used in hollow fiber-based LPME, although the use of polyvinyldene difluoride has also been documented[3]. Polypropylene is more prominent in LPME because it has higher compatibility with many organic solvents. Polypropylene can also easily be moulded to hollow fiber configuration with high mechanical strength that can withstand vigorous agitation throughout the extraction process. The hollow fiber configuration also provides high surface area to volume ratio that facilitates the mass transfer rate during extraction. The hollow fiber is a highly porous material with a suitable pore size that serves as a semi-permeable membrane to allow the target analytes but not extraneous matrix materials to pass through. This hydrophobic polymer also plays an important role in maintaining the integrity of the extraction system by ensuring proper organic solvent immobilization and preventing direct mixing of donor phase with acceptor phase in three-phase LPME. Due to affordability of the hollow fiber, it is economically affordable to have a "one time usage" of fiber for each extraction and thus eliminates the possibility of sample carries over.

2.2.2 Organic solvent selection

Similar to conventional solvent extraction techniques, the organic solvent immobilized in the pores of hollow fiber should be immiscible with aqueous solution. In addition, the selected organic solvent should be chemically inert to the polymeric hollow fiber and yet have a polarity that matches the fiber to ensure strong impregnation in the pores of the hollow fiber. It should also possess appropriate

volatility to prevent premature evaporation during extraction, yet the volatility should not be too high that could hinder the mass transfer. An organic solvent with inherent specific chemical nature (e.g. hydrogen bonding) that is able to help in the improvement of extraction selectivity should also be considered to achieve higher extraction recoveries. If the extract is meant for GC analysis as in the case of two-phase LPME (Chapter 3), the organic solvent should soluble in derivatization agent (if derivatization is required) and display excellent GC behavior.

2.2.3 Kinetics of liquid-phase microextraction

Most hollow fiber-based LPME procedures are described in terms of the equilibrium constant. Yet, the equilibrium constant does not reveal the kinetics of the extraction process. In most cases, equilibrium would only be attained after an hour or so, and this is too long to be considered as an effective extraction method when the chromatographic or electrophoresis separation processes could be completed in less than half an hour. Thus, another factor that must be considered when evaluating an extraction process' performance is the kinetics of mass transfer. The extraction rate depends on the rate of interfacial transfer of analyte A, i.e., the interfacial flux, J, and the interfacial area between the two liquid phases, Q. These are linked by the equation[2]:

$$\frac{d[A]}{dt} = \frac{JQ}{V} \tag{2.17}$$

where V is the total volume of the phase, and the subscript t indicates the contact time. By introducing the definition of specific interfacial area, a_s:

$$a_s = \frac{Q}{V} \tag{2.18}$$

Eq. (2.17) becomes:

$$\frac{d[A]}{dt} = Ja_s \tag{2.19}$$

This equation indicates that the transfer rate increases with both the interfacial flux and the specific interfacial area. The value of J will depend on the mass transfer coefficients or the degree of turbulence in the phases. Most often, LPME takes place in static mode, in which extraction kinetics is enhanced by extensive stirring of the sample solution. Additionally, LPME may also be carried out in a dynamic mode, whereby the acceptor phase is withdrawn or dispensed repeatedly through the hollow fiber using a pump system. By doing so, the concentration of analytes would not build up at the interface and this facilitates transfer of analytes more effectively into the acceptor phase. Furthermore, the usage of a pump (e.g. syringe pump) can facilitate the automation of extraction process and make it feasible to have an on-line LPME coupled to instrument analysis. A more in-depth experimental aspect of various parameters mentioned above are demonstrated in Chapter 3 and Chapter 4 respectively for two-phase, three-phase and carrier-mediated LPME.

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CHAPTER 3

Application of two-phase liquid phase microextraction and on-column derivatization combined with GC-MS to determine acidic drugs in water samples

3.1 Introduction

With the government's plan to transform Singapore into a knowledge-based economy, it has declared making the life sciences industry the economy's "fourth pillar". This decision has successfully attracted some new investment in areas such as pharmaceutical manufacturing. These investors include several major pharmaceutical companies: Pfizer, GlaxoSmithKline, Merck Sharp & Dohme, Schering-Plough, Aventis, Wyeth-Ayerst, Baxter and BD[1].

With the rapid expansion of the pharmaceutical industry, it is important to have a better understanding of pharmaceutical products and their impact on the environment. One emerging area of interest across the scientific community is the issue of active pharmaceutical ingredients (APIs) that are present at very low levels in some wastewater and surface waters. APIs can be released into the environment through human and animal use and, to a lesser extent, from the manufacturing site (in countries where industrial discharge is not carefully monitored).

Non-steroidal anti-inflammatory drugs (NSAIDs) have come into spotlight as they can enter the drinking water source if waste water treatment is incomplete[2]. NSAIDs are commonly prescribed to relieve inflammation and pain, and they include ibuprofen, diclofenac, naproxen, ketoprofen, celecoxib and rofecoxib. Ibuprofen and other similar pain-relieving drugs are used frequently in Singapore for treatments such as headaches and arthritis[3]. Ibuprofen and other commonly used painkillers for

treating inflammation may increase the risk of heart attack[4]. In most countries where ibuprofen is made available without prescription, some patients purchase it over the counter without any difficulty. Given the high prevalence of use of these drugs in the general population, their potential widespread occurrence and environmental accumulation could have profound implications for public health. In view of these problems, focus on the development of analytical methods on APIs detection in the environment is undoubtedly important. In this chapter, two-phase LPME coupled with gas chromatography/mass spectrometry (GC-MS) has been selected to quantitatively evaluate the presence of acidic NSAIDs (ibuprofen, naproxen and ketoprofen), and another acidic API (clofibric acid) in aqueous matrices.

3.2 Experimental

3.2.1 Chemicals and materials

Trimethylphenylammonium hydroxide (TMPAH) was purchased from Supelco (Deisenhofen, Germany). *n*-Octanol was obtained from Riedel de Haën (Seelze, Germany). Sodium chloride was bought from GCE (Chula Vista, CA, USA). Hydrochloric acid was purchased from J.T. Baker (Philipsburg, NJ, USA).

Pharmaceutical drugs (clofibric acid, ibuprofen, naproxen, ketoprofen) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Stock solutions of 1mg/ml (1000ppm) were prepared in methanol, stored in the dark at 4^{o}C, and diluted to the desired concentration with ultrapure water. HPLC-grade methanol was obtained from Fisher (Loughborough, UK). Ultrapure water was prepared on a water purification system supplied by Nanopure (Barnstead, Dubuque, IA, USA).

Tap water was collected in the author's laboratory after having allowed the water to run for 5 min, while the drain water was collected from a drain situated in front of the National University Hospital (NUH). Drain water samples were stored at 4°C after collection.

3.2.2 Apparatus

A 10-μl microsyringe with a cone needle tip (SGE, Sydney, Australia) was used to introduce the acceptor phase (organic phase), to support the hollow fiber and to act as the injection syringe for instrumental analysis.

The Accurel Q3/2 polypropylene hollow fiber membrane was purchased from Membrana GmbH (Wuppertal, Germany). Its dimensions are 600 μm inner diameter, 200 μm wall thickness, and 0.2 μm pore size.

The hollow fiber was manually cut into a predetermined length so as to hold a certain capacity of acceptor phase. The hollow fiber was ultrasonically cleaned in methanol to remove impurities and was dried before use. Each fiber was discarded after each usage to avoid sample carry over.

3.2.3 Instrumentation

The GC-MS analysis was carried out with a Hewlett-Packard (HP) (San José, CA, USA) 6890 Series GC system equipped with 5973 mass selective detector. The column was Valco Bond-1 column (with dimensions 30 m x 25 mm I.D. x 0.25 μm film thickness) from Valco Bond, (J&W Scientific, Folsom, CA, USA). The injection was carried out in splitless mode (purge time 60s, 270°C) and the injection volume

was 2 μl (1 μl of acceptor phase and 1 μl of derivatization reagent). The carrier gas was helium which flowed at 2.0mL/min at a pressure of 17.7psi. The temperature was programmed to 60°C isothermal for 2 min before it was ramped to 270ºC at 10ºC/min and then held isothermal at 270°C for 2 min. The GC−MS interface temperature was set at 270°C. The MS ion source was set at 230°C and MS quadrupole at 150°C. The mass spectra were obtained with electron impact ionization at 70eV. A mass range of m/z 50–500 was scanned to confirm the retention times of the analytes. Retention times and m/z ratios used for quantification by selected-ion monitoring (SIM) are shown in Table 3.1. Data acquisition was performed by ChemStation from Agilent Technologies (Palo Alto, CA, USA).

3.2.4 Two-phase LPME

Extractions were performed according to the following procedure. The 10-μl microsyringe was prefilled with 6.0 μl acceptor phase. The needle tip of the microsyringe was inserted into the hollow fiber and the assembly was immersed into the organic solvent for ~ 10 sec in order to impregnate the pores of hollow fiber with the organic solvent. After the impregnation, the acceptor phase was dispensed to fill the lumen of the hollow fiber.

Then, the fiber/needle assembly was removed from the organic solvent and placed into a sample vial containing a 4mL aliquot of sample solution equipped with a magnetic stirring bar (Figure 3.1). The sample solution contained 50 ppb of spiked analytes and the extraction was carried out on a stirring plate (Heidolph, Kelheim, Germany) at room temperature for 20 min at 1000 rpm stirring rate. After extraction, the acceptor phase was drawn into the syringe; the hollow fiber was then removed.

The acceptor phase volume was adjusted to 1μl, followed by 1μl of the derivatization reagent and introduced into the heated GC injection port.

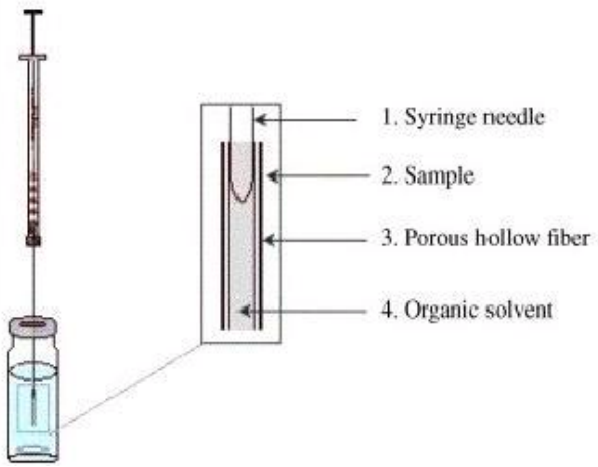

Figure 3.1 Schematic representation of two-phase LPME

3.3 Results and discussion

3.3.1 Derivatization

A derivatization reagent is usually applied to polar analytes to improve their chromatographic properties as well as to increase their volatility for GC analysis. Different types of derivatization reagents (namely bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylsulfonium hydroxide (TMSH) and trimethylphenylammonium hydroxide (TMPAH)) are used to derivatize the four pharmaceutical drugs in this work. Among these derivatization reagents, as we discovered in preliminary experiments, TMPAH was the best reagent as it provided convenient, efficient and quantitative derivatization. Analytes went through "on-column" derivatization in the hot injection port of the GC at 270^{o}C.

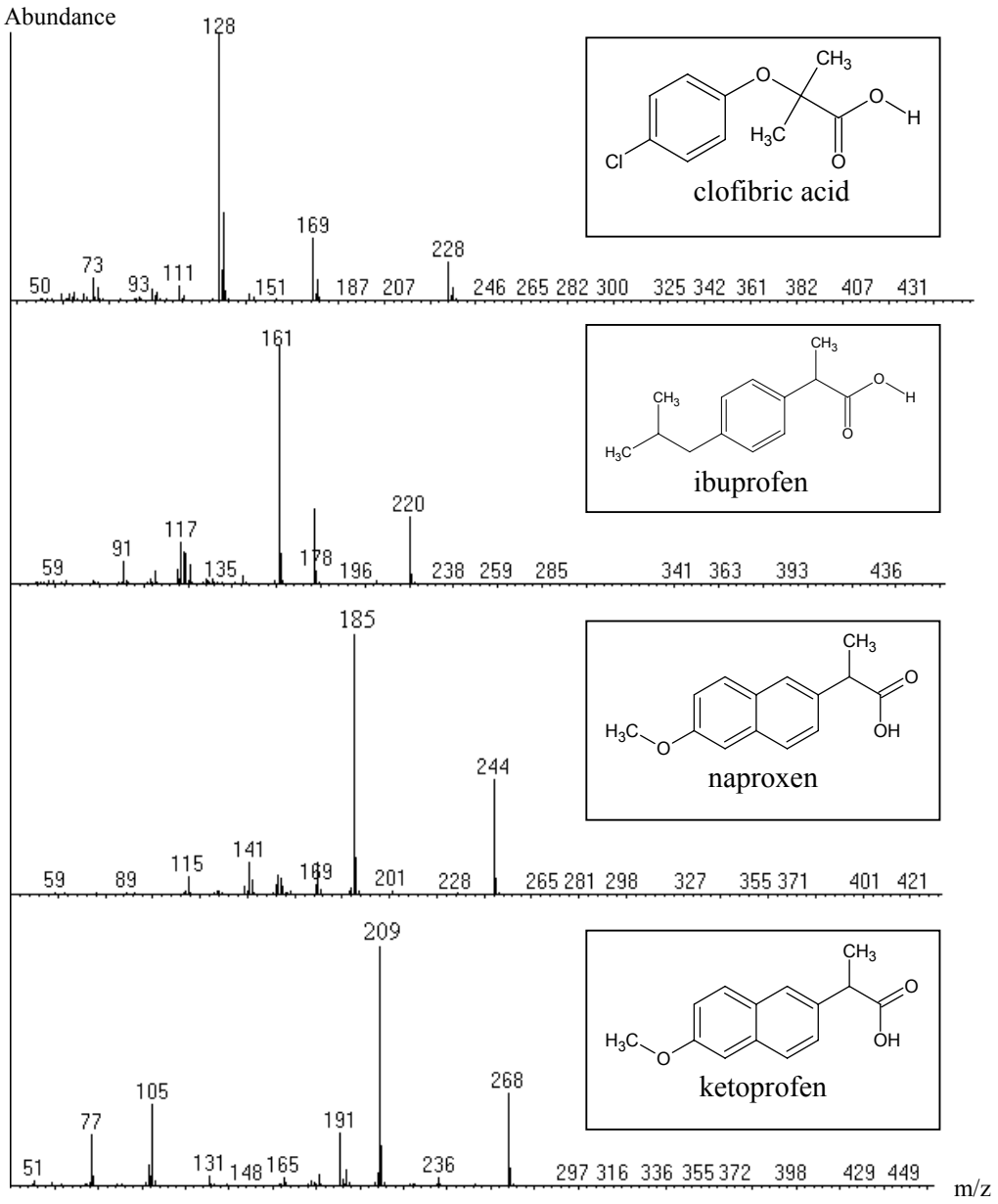

Figure 3.2 Structure of the acidic drugs and their respective mass spectra.

Compounds	Formular weight	pK_a	LogP(octanol/water)	Retention time (min)	m/z for quantification
clofibric acid	214.65	2.57	3.18	12.73	128,169
ibuprofen	206.3	3.97	4.91	13.24	161, 220
naproxen	230.3	3.18	4.15	18.23	185,244
ketoprofen	254.3	3.12	4.45	19.26	209,268

Table 3.1 Physical properties and chromatographic information of the acidic drugs.

Derivatization of TMPAH was performed via thermal decomposition of the reagent, and subsequently transesterification reaction of analytes to form methyl derivatives. Thus, either the methylated parent ions or daughter ions were used for m/z quantification of the four acidic drugs (Figure 3.2). Different concentrations of TMPAH were investigated to optimize the derivatization process. It was found that derivatization was incomplete when the concentration of TMPAH was lower than 0.005M (data not shown). Thus, undiluted TMPAH (0.2M in methanol) was utilized for the following experiments to ensure complete derivatization.

3.3.2 Comparison of extraction solvents

Organic solvent plays a critical role in LPME as illustrated in Section 2.1.5. Various organic solvents that are immiscible with water were tested in two-phase LPME to evaluate their suitability in the extraction. Among these solvents, *n*-octanol displayed better extraction efficiency in two-phase LPME. Polar analytes, such as NSAIDs and clofibric acid, are more soluble in polar solvents; hence *n*-octane that possesses low polarity was least favorable in the extraction of these drugs (Table 3.2). On the other hand, toluene and *n*-butyl acetate were not suitable for extraction due to their volatility at room temperature, whereas the low viscosity of chloroform impeded the stability of the organic phase immobilized in the hollow fiber pores due to dissolution of chloroform in the midst of extraction. *n*-Octanol was the only solvent that offered satisfactory extraction results as a consequence of its appropriate viscosity and its compatibility with the hollow fiber material. The Hansen solubility parameter also indicated a favorable feature of *n*-octanol as an extraction solvent owing to its ability to form hydrogen bonding with the analytes. It was possible that formation of hydrogen bonds with the polar drugs, making them more soluble in the organic phase,

facilitate the extraction. Another important factor for the success of *n*-octanol to be used as an extraction solvent was its compatibility with TMPAH (dissolved in methanol). Thus, *n*-octanol was chosen as the organic phase as well as the acceptor phase for the subsequent extractions.

Organic solvent	chloroform	toluene	*n*-octane	*n*-octanol	*n*-butyl acetate
Absolute viscosity (@25°C cP)	0.57	0.59	0.5	7.5	0.73
Solubility in H_2O (25°C %w/w)	0.82	0.052	0.000063	0.6	0.7
Hansen Solubility parameter					
~ Nonpolar	17.8	18	15.6	17	15.8
~ Polar	3.1	1.4	0	3.3	3.7
~ Hydrogen bonding	5.7	2	0	11.9	6.3
Evaporation rate (n-butyl acetate =1)	n.a	2	1.23	0.007	1

Table 3.2 Physical properties of the organic solvents (adapted from [5] and [6]).

3.3.3 Acceptor phase volume

After deciding on the type of organic solvent for immobilization of the hollow fiber pores, experiments were carried out to determine a suitable volume of organic solvent that served as the acceptor phase. By fixing the sample volume, different volume of n-octanol (acceptor phase) in the range of 1-5 μl was attempted for extraction. According to equation (2.5) in Section 2.1.1, enrichment factor was greatly influenced by the ratio of acceptor phase to donor phase. The larger the difference in the phase ratio, the greater the enrichment factor. Thus, 1 μl acceptor phase would be expected to display higher extraction efficiency. However, Figure 3.3 showed that 2 μl of acceptor phase exhibited a better result.

It may be that solvent loss arising from evaporation and dissolution of *n*-octanol during extraction significantly affected the final acceptor phase volume and recovery when the acceptor phase was 1 μl. On the other hand, a higher acceptor

volume can lead to dilution of the extract. A compromise appeared to be necessary to address these conflicting phenomena. In order to obtain quantitative results, therefore, 2 µl of acceptor phase was used, although only 1 µl acceptor phase extract was eventually injected into the GC-MS.

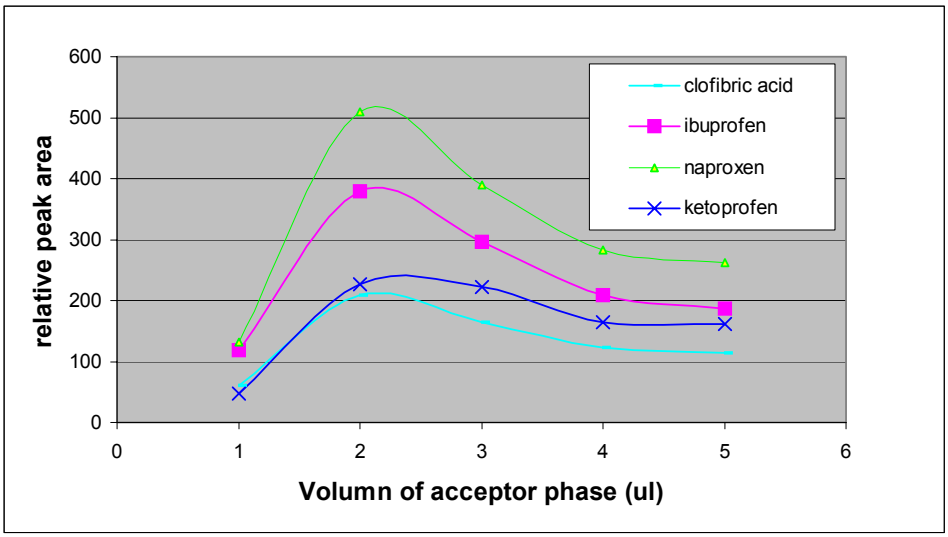

Figure 3.3 Effect of acceptor phase volume on extraction.

3.3.4 pH of sample solution

In order to promote the distribution of charged analytes into the organic phase, the pH of the sample solution (donor phase) should be adjusted to ensure deionization of the analytes. In this study, an acidic pH maintained the NSAIDs and clofibric acid in their extractable molecular forms. Various concentrations of HCl were used instead of varying the pH value because the sample solution was prepared without using any buffer. By varying the concentration of HCl in the sample solution, better extraction efficiency for all the analytes was observed at 0.001M HCl (Figure 3.4) where the pH value is approximately 3, slightly lower than the pK_a values for most of the analytes (Table 3.1). A higher HCl concentration could have induced hydrolysis of the analytes while a lower HCl concentration might lack the acidic strength to deionize the

analytes. Hence, 0.001M HCl was used to decrease the water solubility of analytes, which in turn elevated their extractability into the organic phase.

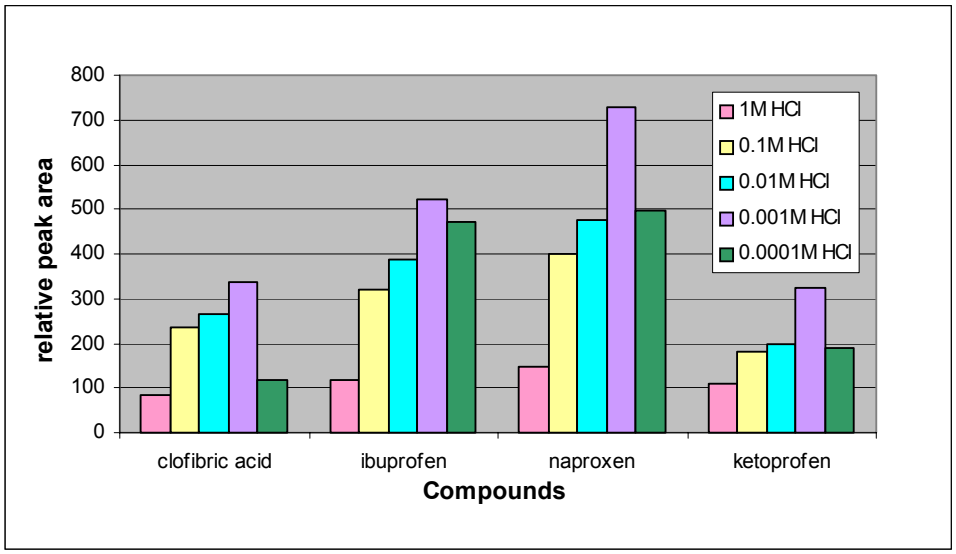

Figure 3.4 Effect of different HCl concentrations in sample solution on extraction efficiency

3.3.5 Salting out effect

Addition of a salt such as sodium chloride (NaCl) into the sample solution is known to have a "salting out" effect on some analytes by the formation of hydrated salt ions so that less free water is available for solvation of analytes[7]. This means that extraction of these analytes into the organic solvent is enhanced. Thus, the effect of salt addition on the extraction efficiency of these acidic drugs was determined by adding separately, 0, 2.5, 5, 7.5, 10, 15% (w/v) of NaCl into the sample solution. In general, the addition of NaCl increased the extraction efficiency for the four drugs, but each analyte reacted differently to the salt concentration. Upon addition of 2.5% (w/v) of salt, the extraction efficiency increased as shown in Figure 3.5. Further addition of salt beyond 2.5% did not improve the extractability significantly for clofibic acid and ketoprofen, instead it has a negative effect on ibuprofen and naproxen. Further increment in salt concentration elevates the viscosity of the sample solution which in

turn results in decline of mass transfer rate of analytes to the organic phase and hence lowers the extraction efficiency. As a result, 2.5 % (w/v) of NaCl was added for the subsequent analysis. This conflicting observation is not unusual in LPME and has reported been previously[9].

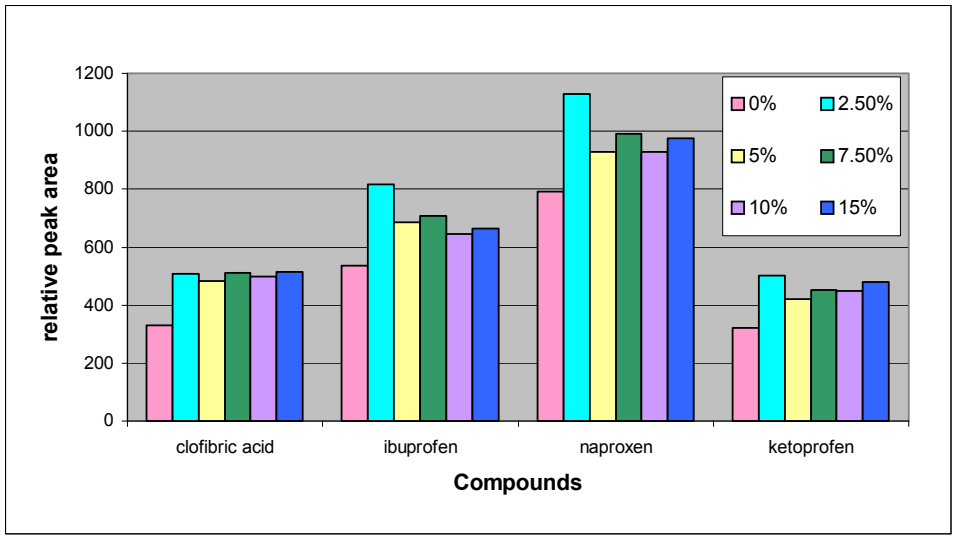

Figure 3.5 Salting out effect on extraction efficiency for acidic APIs

3.3.6 Stirring rate

The extraction in two-phase LPME can be further enhanced by stirring the sample solution. By increasing the stirring speed, the thickness of the boundary layer at the outer membrane surface would be reduced[8] and this increases the mass transfer rate of acidic drugs to the acceptor phase, thus an equilibrium is achieved in a shorter period of time. As depicted in Figure 3.6, the partition of analytes into the acceptor phase increased with the stirring speed until 1000 rpm. Stirring rates above 1000 rpm resulted in dislodgement of the acceptor phase from hollow fiber and caused instability of the liquid membrane that in turn contributed to poorer precision in extraction. Hence, the stirring speed for all subsequent experiments was standardized at 1000 rpm.

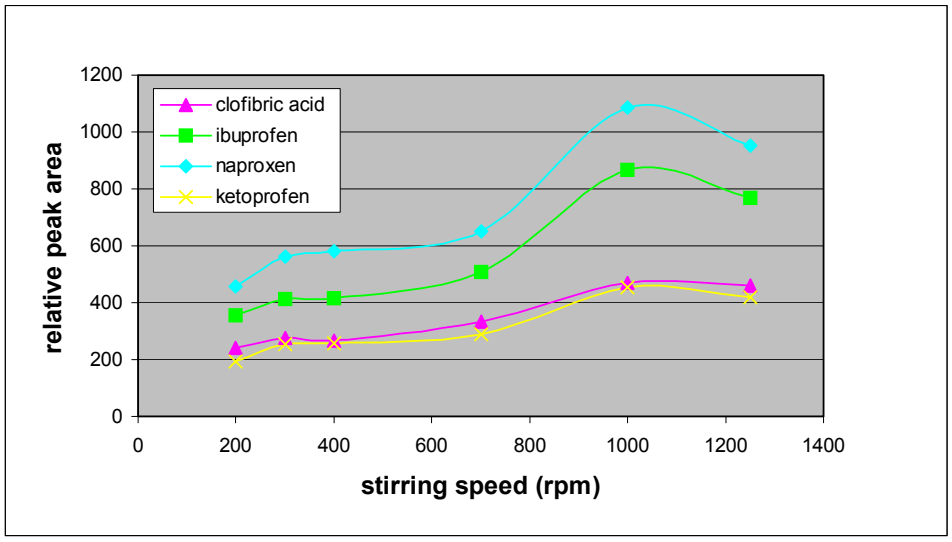

Figure 3.6 Extraction yield vs. stirring speed of NSAIDs and clofibric acid

3.3.7 Extraction time

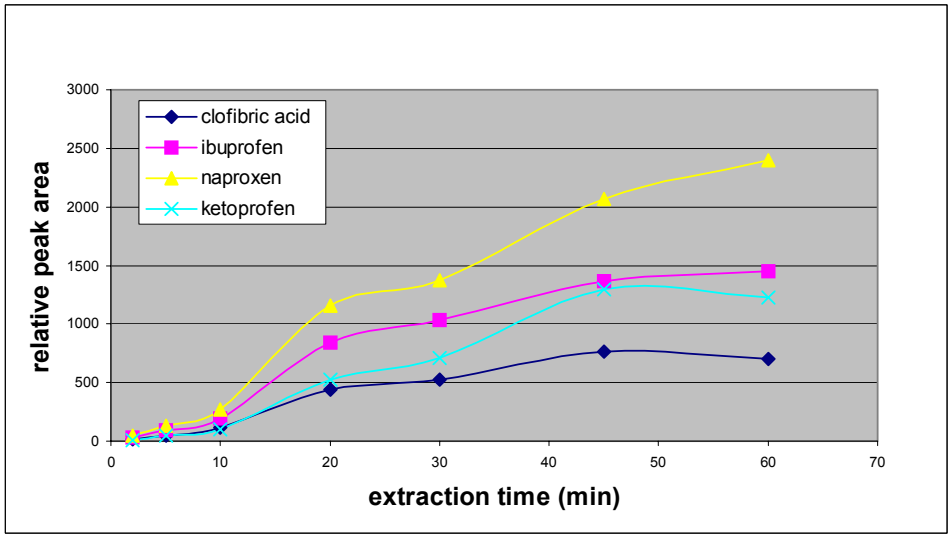

Figure 3.7 Two-phase LPME extraction profile vs. extraction time of NSAIDs and clofibric acid

Extractions were conducted respectively at 2, 5, 10, 20, 30, 45 and 60 min. For all compounds, the extraction efficiency increases with time and most of the analytes achieves equilibrium only after 45 min (Figure 3.7). For clofibric acid, the increase in extraction was more moderate after 20 min mainly due to its log P value (Table 1) while ketoprofen reached equilibrium after 45 min. On the other hand, naproxen and

ibuprofen did not attain equilibrium within the experimental period. Although the extraction recovery was higher with longer exposure time, it should be emphasized that reaching equilibria for analytes is not essential. The recovery obtained upon 20 min of stirring exhibited sufficient extraction. However, extraction parameters must be kept consistent, particularly extraction time, for each experiment to ensure quantitative analytical reliability. Hence, 20 min extraction time was preferred instead in view of the short GC analysis time that is more suitable for high throughput analysis. Thus, the optimum extraction condition was done at 0.001M HCl with 2.5% NaCl in the sample solution and n-octanol as the acceptor phase at 20 min extraction time with stirring at 1000 rpm.

3.3.8 Enrichment factor, linearity and precision

The enrichment factors for the four acidic drugs are shown in Table 3.3. These enrichment factors refer to the ratio of extract concentration to sample concentration. The optimized conditions were employed to investigate the enrichment factors of two-phase LPME: 2 µl of *n*-octanol, 0.2 M of TMPAH, 0.001 M HCl, 2.5% (w/v) of NaCl, 20 min of extraction time at 1000 rpm stirring rate. The enrichment factor ranged from 74.6 for ibuprofen to 153.6 for ketoprofen. These results indicated that the enrichment factor of two-phase LPME extraction could achieve around 100-fold for compounds having log P values of 3-4 under optimized conditions. Nevertheless, the exceptionally high enrichment factor for ketoprofen might be due to erroneous chromatographic integration (please see below). In general, a much higher enrichment factor could be obtained if larger sample volume is permitted due to large difference in the sample and acceptor phase ratio.

By using the optimized conditions in ultrapure water sample, LPME exhibited a good linearity for over 4 orders of magnitude for most of the analytes except ketoprofen. Ketoprofen has a higher limit of detection (LOD) and higher relative standard deviation (RSD) mainly due to difficulty in peak integration resulted from noisy baseline towards the later part of the chromatographic analysis (data not shown). Nevertheless, the coefficient of estimation value, r^2, was acceptable (0.9975). The RSD, LOD and r^2 values for naproxen, ibuprofen and clofibric acid showed reasonable performance and was comparable or even better than other conventional extraction methods.[9]

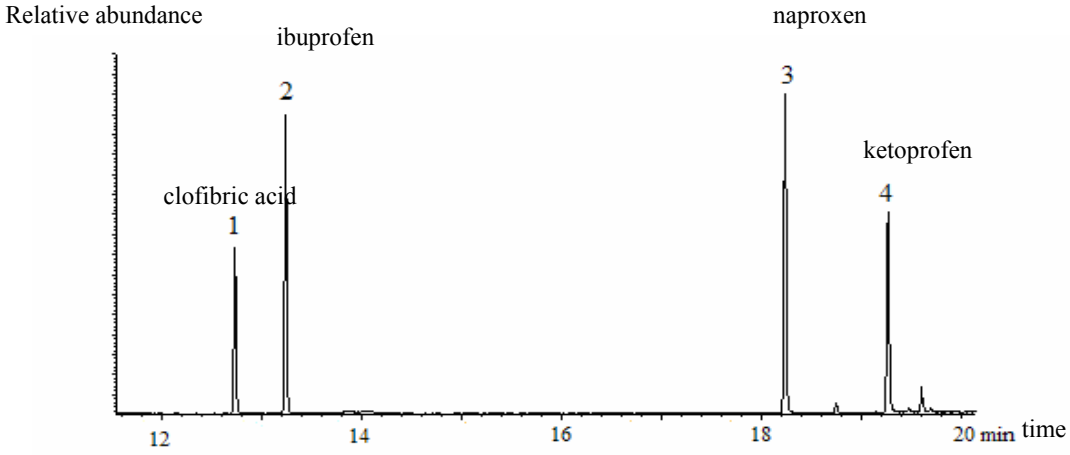

Figure 3.8 Chromatograms of NSAIDs and clofibric acid (at 10ppb) in spiked ultrapure water

Further improvements of the LPME method include the addition of internal standard in the analysis and automation by employment of pump system and robotic fiber preparation which may provide a solution to the current low precision problem. Large volume injection for GC analysis might also lower the LOD in LPME.

Compound	%RSD	Linearity range (ppb)	r^2	LOD (S/N=3) (ppb)	Enrichment	Relative recovery (%)	
						tap water	drain water
clofibric acid	8.9	0.2~200	0.9984	0.02	95.6	101.6	0
ibuprofen	8.7	0.2~200	0.9922	0.01	74.6	109.8	60.0
naproxen	7.4	0.2~200	0.9988	0.05	99.6	113.4	23.8
ketoprofen	11.8	1~200	0.9975	1	153.6	173.3	16.2

Table 3.3 Analytical performance of two-phase LPME on selected acidic drugs

3.3.9 Application of two-phase LPME to real samples

Two-phase LPME was used for extracting NSAIDs and clofibric acid from tap water and drain water and both sources showed the absence of these drugs. Despite the low LODs, this is not surprising since Singapore has implemented the highest possible standards for tap water quality, and it is unlikely for such drugs to be disposed of through the drainage system (domestic wastewater effluent is channeled separately to countrywide sewage collection system that leads to wastewater treatment plants).

Further investigation was done by spiking 10 ppb into the water sources to assess the matrix effects on extraction recovery. Relative recoveries obtained from both water samples varied significantly, it ranged from 101.6 to 113.4% for tap water (except for ketoprofen). The result obtained for ketoprofen deviates significantly from the others in tap water, probably due to hydrolysis of ketoprofen in the standard prepared overnight. The deterioration of ketoprofen has caused the calculation of relative recovery obtained "appeared" to be higher (false positive result), thus it should not be regarded as a representative result. Relative recovery is defined as the ratio of the GC peak areas of spiked real water extracts over that of spiked ultrapure water extracts (standard). On the other hand, relative recoveries for analytes obtained in spiked drain water were 0 to 60.0%. This variation could be explained in terms of their sample nature and treatment received. Tap water being a treated water sample has most of its solid particles removed. On the contrary, drain water contained high level of suspended particles beside some domestic waste. The complex sample matrix in drain water might have hampered analyte diffusion to the liquid membrane due to non-specific adsorptions of analytes to the suspended particles. Ultrasonication of water sample, with high level of suspended particles, before extraction might be useful

to dislodge analytes from the particles. It is possible the presence of small amount of detergent may provoke a loss of the organic phase immobilized within the pores of the membrane. This means that the matrix had different effect on the extraction recoveries depending on the nature of the water samples.

3.4 Conclusions

A simple yet sensitive two-phase LPME has been successfully employed to determine residues of acidic drugs in water samples. Most importantly, it could reduce the organic solvent waste significantly through out the extraction process by utilizing only microliters of organic solvent. After optimization of the extraction conditions, ~ 100-folds of enrichment factors and detection limits of 0.01-1 ppb were achieved. The RSD values were found to be in the range of 7.4-11.8%. Linearity of this method includes the concentration range expected in environmental samples (0.2-200ppb) with r^2 of 0.992 or above. Despite its drawbacks in more complicated matrix, two-phase LPME is nevertheless a reliable method for the examination of tap water quality. In order to circumvent the drawbacks, some improvements on two-phase LPME include: large volume injection for GC analysis, ultrasonication of water sample prior to the extraction process (for water sample with high level of suspended particles), the use of internal standard, automation by employment of pump system and robotic fiber preparation. Further effort in seeking a more “rugged” organic phase immobilized in the hollow fiber pores is mostly desired.

3.5 References

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CHAPTER 4

Application of three-phase microextraction and carrier-mediated microextraction coupled to HPLC in the determination of β-blockers in water samples

4.1 Introduction

β-blockers belong to a group of drugs known as β-adrenergic blocking agents, which are commonly used to treat patients with high blood pressure (hypertension). They are used to relieve angina (chest pain), to prevent heart attacks among heart attack patients, to correct irregular heartbeat, to prevent migraine etc. β-blockers work by affecting the response to some nerve impulses in the body, which in turn reduce cardiac oxygen demand by lowering heart rate and blood pressure[1].

With the increasing occurrence of cardiovascular diseases worldwide, the demand for these drugs for treatment has also elevated. The growing production of these drugs from production sites, direct disposal of surplus drugs in household waste, excretion after administration to patients in hospitals result in increasing amounts of highly biological active material being discharged into the environment. Due to their polarity, persistence and water solubility, these drugs may be able to pass through the water treatment plants. Pharmaceuticals that are not removed by the sewage treatment process may reach the population via the drinking water supply. Such bioactive substances and their metabolites would then tend to accumulate in our body. Although detailed knowledge about the ecotoxicological effects of these compounds is still lacking, these contaminants must be classified as environmentally relevant. Assessment of the occurrence of pharmaceuticals in the environment is therefore in demand and it greatly relies on sensitive analytical

procedures. In this chapter, three-phase LPME coupled with HPLC is developed to quantitatively evaluate the presence of basic β-blockers (atenolol, acebutolol, pindolol, oxprenolol and propranolol).

4.2 Experimental

4.2.1 Chemicals and materials

Sodium octanoate and octanoic acid were bought from Fluka (Buchs, Switzerland). Sodium heptanoate and sodium nonanoate were purchased from TCI (Tokyo, Japan). Sodium monohydrogen phosphate and sodium dihydrogen phosphate were supplied by Merck (Darmstadt, Germany). Sodium hydroxide was bought from GCE (Chula Vista, CA, USA). Ammonium bicarbonate and pharmaceutical drugs (atenolol, acebutolol, pindolol, oxprenolol and propranolol) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Stock solutions of 1mg/ml (1000ppm) were prepared in methanol, stored in the dark at 4^{o}C, and diluted to the desired concentration with ultrapure water. HPLC-grade acetonitrile was obtained from Fisher (Loughborough, UK). Hydrochloric acid, *n*-Octanol, sodium chloride, methanol and water purification system were supplied by the same sources given in section 3.2.1.

Tap water was collected in the author's laboratory after allowing the water to run for 5 min. Meanwhile the drain water was collected from the drain situated in front of National University Hospital (NUH). Drain water samples were stored at 4^{o}C after collection.

4.2.2 Apparatus

A 10-µl microsyringe with a flat needle tip (SGE, Sydney, Australia) was used to introduce the acceptor phase, to support the hollow fiber and to act as the injection syringe for instrumental analysis.

The Accurel Q3/2 polypropylene hollow fiber membrane was obtained from the same source as mentioned in section 3.2.2. The hollow fiber was manually cut into 2.5cm to hold 6.5 µl of acceptor phase and was treated in the same way as described in section 3.2.2.

4.2.3 Instrumentation

Instrumentation analysis of β-blockers was performed on a Waters (Milford, MA, USA) HPLC system which consisted of a Rheodyne (Cotati, CA, USA) 77251 injector with a 5 µl sample loop, a Waters 1525EF binary pump and a Waters 2487 UV-visible spectrophotometric detector. Data was collected and analysed using Waters Empower version 5.0 data analysis software. HPLC column Zorbax Eclipse XDB- C_8 (4.6 x 150mm, 5 µm) from Agilent Technologies (Palo Alto, USA) was utilized. The mobile phase consisted of acetonitrile-10mM ammonium bicarbonate (pH 10) in 70: 30 ratio. The isocratic elution was maintained at a flow rate of 0.4mL/min. The detection wavelength was set at 220nm.

4.2.4 Three-phase and carrier-mediated LPME

Extractions were performed using hollow fiber supported in the U-shaped configuration by two microsyringes (Figure 4.1). 6.5 µl of acceptor phase (hydrochloric acid) was drawn into the 10 µl microsyringe. A fiber length of 2.5 cm was selected to provide an inner volume of 6.5µl for the acceptor phase solution. The needles tip of both microsyringe were inserted into the respective opposite ends of the hollow fiber and the assembly was immersed in the organic solvent for ~ 10 sec in order to impregnate the pores of the hollow fiber with the organic solvent. After the impregnation, the acceptor phase was dispensed to fill the lumen of the hollow fiber.

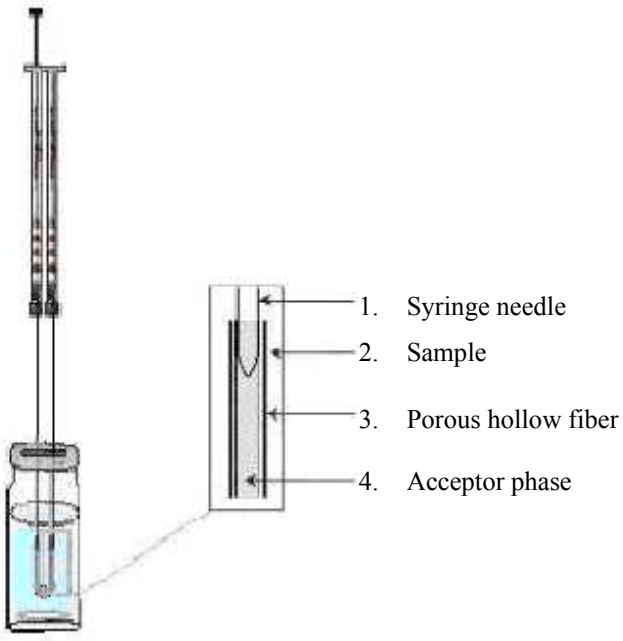


Figure 4.1 Schematic representation of three-phase LPME

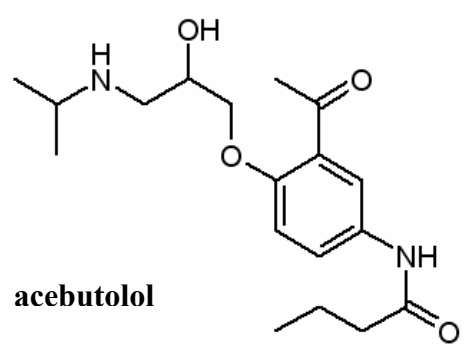
After that, the assembly was removed from the organic solvent and placed into a sample vial containing a 4mL aliquot of sample solution equipped with a magnetic stirring bar. The sample solution was continuously stirred at room temperature with a magnetic stirring bar at 1000 rpm (or otherwise stated) using a stirring.

After extraction, 5 μl of the acceptor phase was drawn into the syringe; the hollow fiber was removed and the acceptor phase was then injected into the HPLC for analysis.

4.3 Results and discussion

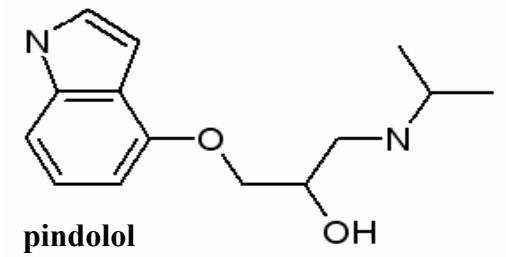
4.3.1 Organic solvent selection

In three-phase LPME, the organic solvent selection is more stringent than in two-phase LPME as the analytes need to be only moderately soluble in it. High solubility of analytes in the organic phase might hinder back-extraction of analytes into the acceptor phase, while low analytes solubility contributes to poor extraction of analytes from sample solution into the organic solvent. Dihexyl ether and *n*-octanol were considered as the organic solvents immobilized in the pores of the fiber as previous studies have proven their suitability for three-phase LPME[2,3]. In preliminary studies, it was found that *n*-octanol could provide higher recoveries than dihexyl ether for three out of five of the analytes (acebutolol, pindolol and oxprenolol), while more propranolol could be extracted by dihexyl ether (data not shown).



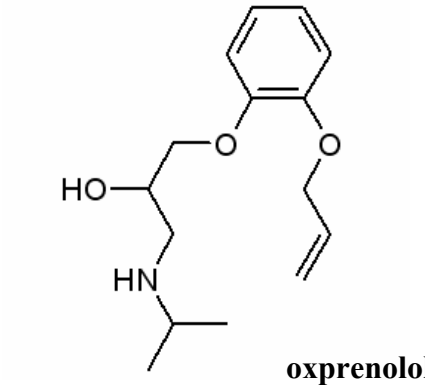
acebutolol

logP	2.593±0.274
Molecular weight	336.43
pKa	13.78±0.20
	9.11±0.38



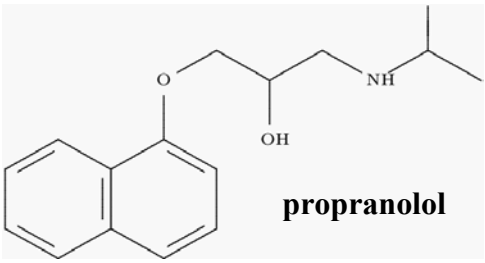
pindolol

logP	1.970±0.219
Molecular weight	248.32
pKa	13.94±0.20
	9.21±0.38



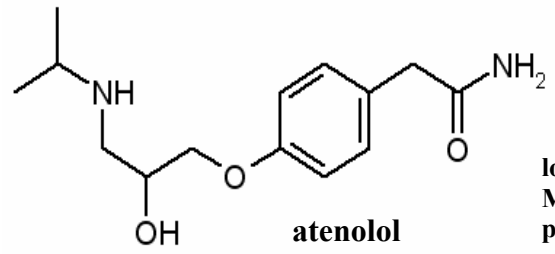
oxprenolol

logP	2.291±0.243
Molecular weight	265.35
pKa	13.82±0.20
	9.13±0.20



propranolol

logP	3.097±0.193
Molecular weight	259.34
pKa	13.84±0.20
	9.14±0.20



atenolol

logP	0.097±0.246
Molecular weight	266.34
pKa	13.88±0.20
	9.17±0.38

Figure 4.2 Structures of β-blockers considered and their physical properties[7, 9]

Further investigations on solvent mixed (for example, 80% *n*-octanol: 20% dihexyl ether) did not improve the extraction efficiency. However, both organic solvents failed to extract atenolol. This was not unexpected as atenolol has a much lower log P value as compared to other β-blockers and it is ionized in ultrapure water. The hydrophilic nature of atenolol has rendered it more soluble in water and less soluble in organic solvent. In view of this problem, the pH of the donor phase needed to be investigated to adjust the ionization status of the analytes. For the following experiments, *n*-octanol was selected to be the organic solvent impregnated on the hollow fiber because it showed a higher ability to extract most of the targeted analytes.

4.3.2 pH of sample solution

The pH of sample solution was of high importance as it affected the ionization state of analytes which in turn influenced the extractability of analytes into the organic phase. Various concentrations of NaOH (10^{-5} M to 1 M) were tested in the extraction of basic amino alcohols. In order to keep the ionic strength constant, lower concentrations of NaOH were topped up with adequate salt solution. Figure 4.3 exhibited that the extraction recoveries were almost similar from 1 M to 10^{-3} M NaOH, but they declined dramatically for four of the amino alcohols (acebutolol, pindolol, oxprenolol and propranolol) when the NaOH concentration was lower than 10^{-3} M. Meanwhile, hydrophilic atenolol showed limited extractability. Based on the pK_a values displayed in Figure 4.2, all of the analytes were protonated when the pH of the solution was lower than 9 and were negatively charged when the pH was almost 14. This explained why these analytes were insensitive to alkaline pH because they existed in molecular form. Nevertheless, atenolol with a log P

value of 0.097 was regarded as too hydrophilic, thus was unable to be extracted even in its molecular state. Further investigation on acceptor phase was necessary to explore the possibility of shifting the equilibrium to favor extraction of amino alcohols into the organic phase. Due to slightly higher recovery obtained at 10^{-1} M NaOH, this concentration was selected for the following experiments.

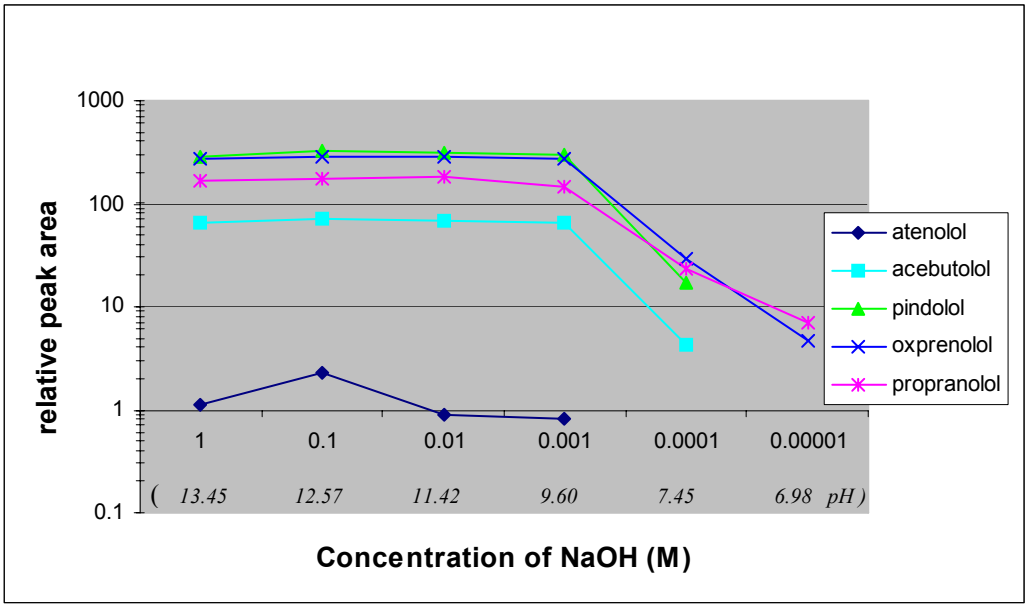


Figure 4.3 Effect of NaoH concentrations on extraction efficiency. The sample was spiked with 250ppb of acebutolol, pindolol, propranolol, 500ppb of atenolol and 1000ppb of oxprenolol. The acceptor phase was 0.1 M HCl. Extraction time was 30min.

4.3.3 pH of acceptor phase

The function of the acceptor phase in three-phase LPME is to aid in the ionization of alkaline analytes and hence prevent the reentry of analytes back into the organic phase. By doing so, it enables more analytes to be extracted from the organic membrane into the acceptor phase. In this case, HCl was introduced as the acceptor phase, and its concentration varied from 0.0005 M to 1 M. From Figure 4.4, acebutolol was least sensitive to changes when the HCl concentration was $\geq$ 0.005 M. Extraction of oxprenolol

and pindolol increased moderately when the concentration was reduced from 1 to 0.005 M. On the other hand, propranolol showed drastic augment when the concentration was reduced from 1 to 0.005 M. For the four compounds, extraction was the most favorable at 0.005 M HCl but diminished at a lower HCl concentration. HCl concentrations lower than 0.005 M have low acidic strength to accomplish the stripping process, while high acidic concentration might have induced hydrolysis of the extracted analytes. Nevertheless, atenolol failed to show significant recovery in all the above conditions. Even though addition of salt (NaCl) was thought to have a salting-out effect on certain compounds as demonstrated in Chapter 3, it had no effect on the extraction efficiency of these β-blockers. Thus, the three-phase LPME could not be applied successfully in extracting hydrophilic analytes such as atenolol. (Atenolol was isolated from the mixture and was further considered separately -see section 4.3.4). Further optimization on the other four compounds was conducted with 0.005M HCl as acceptor phase.

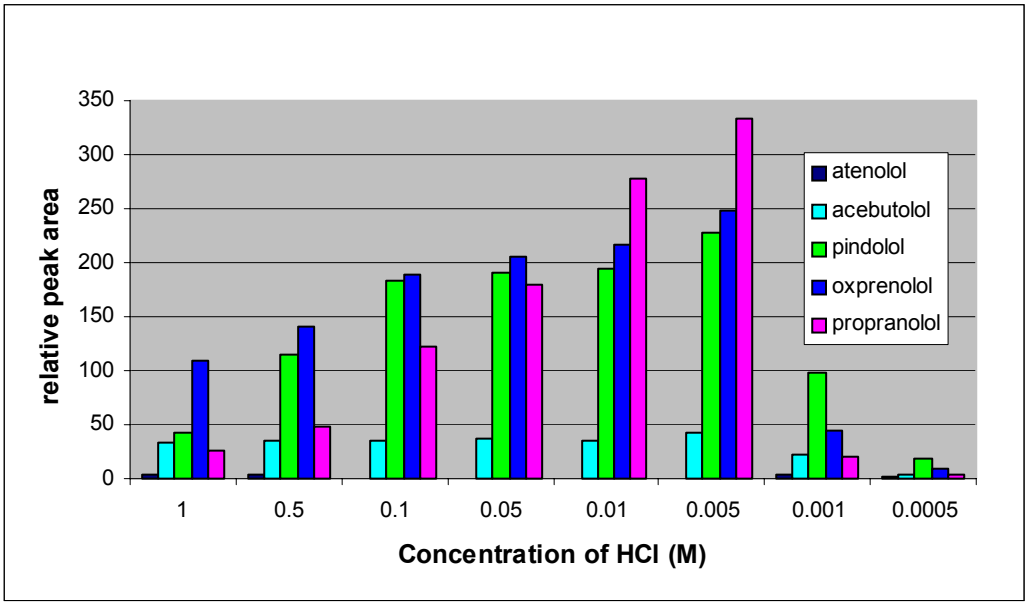


Figure 4.4 Effect of HCl concentrations on extraction efficiency. The sample was basified to 0.1 M NaOH and spiked with 250ppb of acebutolol, pindolol, propranolol, 500ppb of atenolol and 1000ppb of oxprenolol. Extraction time was 30min.

4.3.4 Composition of donor phase and acceptor phase in carrier-mediated LPME

Since atenolol could not be extracted as a neutral molecule, carrier-mediated LPME was utilized instead. In this method, the analyte was ionized to form a charged species and then ion-paired with an oppositely charged carrier to form a hydrophobic ion complex. Atenolol is a basic compound and it is easier to protonate it. Sodium octanoate was selected as the ion-pairing reagent as reported by *Ho et al*[4]. Sodium octanoate is a sodium salt of aliphatic carboxylic acid, with hydrophobic characteristic and some water solubility. Since sodium octanoate is a weak acid, it is able to ionize in aqueous solution forming a free $-COO^-$ group to ion-pair with the protonated atenolol. In this case, the pH of the sample solution is an important parameter to ensure both the analyte and the ion-pairing reagent were ionized but they carried opposite charges to form an ion-pair that was soluble in the organic layer.

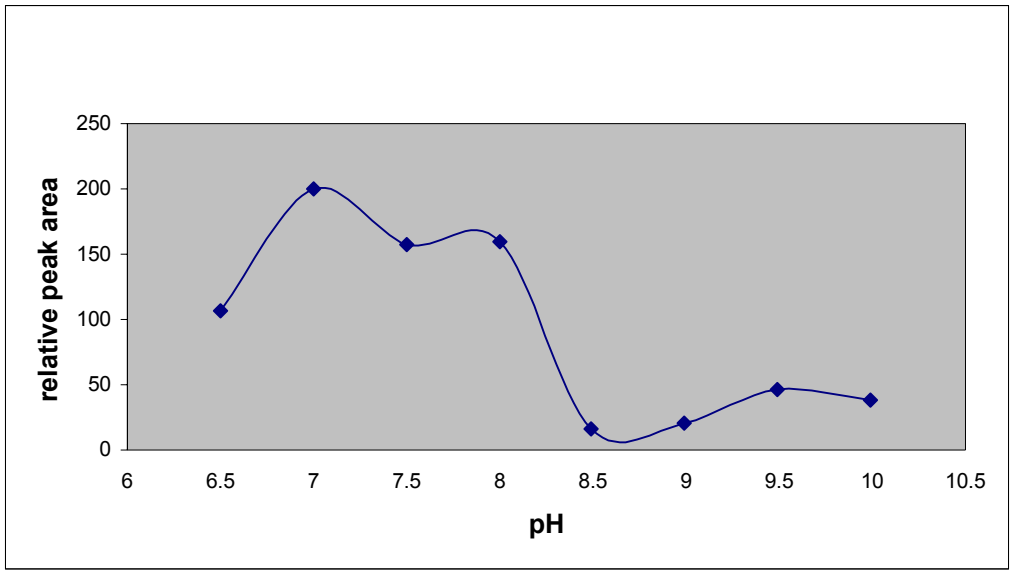


Figure 4.5 Effect of pH in sample solution. The pH of sample was adjusted with 100 mM sodium phosphate (6.5-8) or 100 mM ammonium acetate (8.5-10) and spiked with 10ppm of atenolol and 25mM sodium octanoate. The acceptor phase was 0.1 M HCl. Extraction time was 40min.

In the absence of an ion-pairing reagent, there was no extraction of atenolol in sample solution with buffer (data not shown). However, upon the addition of sodium octanoate, atenolol was extracted into the acceptor phase. Although the extraction efficiency was not significant, it prompted us to further optimization of the LPME conditions. The sample solution was adjusted with sodium phosphate to cover pH ranging from 6.5 to 8.0 while ammonium acetate was used to cover pH ranging from 8.5 to 10 (Figure 4.5). It was found that sodium phosphate was a better choice of buffer as it coincided with the pH where atenolol was protonated. The optimum extraction result was achieved at pH 7. At this pH, octanoic acid having a pK_a value of 4.78 [3] would deprotonate to form an anion. Hence, the two species could ion-pair and enter the organic phase as a complex.

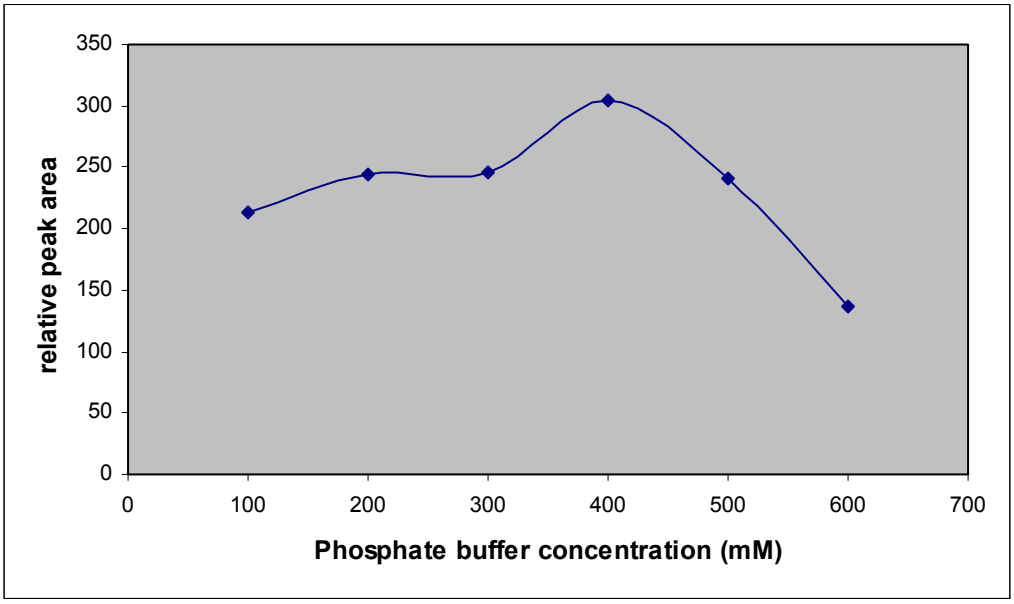


Figure 4.6 Concentrations of phosphate buffer on extraction. The pH of sample was adjusted to 7 and spiked with 10ppm of atenolol and 25mM sodium octanoate. The acceptor phase was 0.1 M HCl. Extraction time was 40min.

The effect of sodium phosphate buffer concentration was then examined to enhance the extractability of atenolol. Higher recovery was observed when concentration

of sodium phosphate was elevated from 100 to 400mM probably due to the salting-out effect (Figure 4.6). At a higher concentration of phosphate buffer, the extraction efficiency declined as the solubility of sodium octanoate was seriously affected. 400mM of sodium phosphate was thus chosen for the following experiments. Different types of carboxylic acid salts with varying concentrations were dissolved in 400mM phosphate buffer, pH 7. Due to differences in their chain length, different carboxylic acids have different degree of solubility in sample solution. Sodium heptanoate is the most water-soluble ion-pairing reagent among these carboxylic acid salts; thus, the hydophobicity is probably too low to promote effective extraction. On the other hand, sodium nonanoate has limited solubility in the phosphate buffer; hence, precipitation of carrier during extraction complicated the recovery. Sodium octanoate displayed a transitional characteristic between these two salts. At 25mM sodium octanoate, the extraction recovery was the highest (Figure 4.7). Undoubtedly, it was the most suitable candidate among these carriers as it had sufficient solubility in phosphate buffer and was able to form a hydrophobic ion-pair with atenolol.

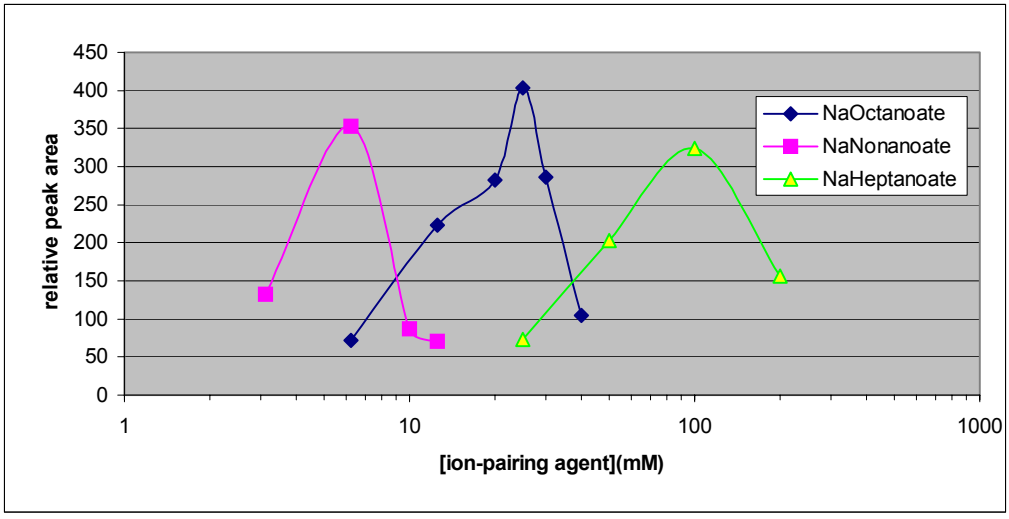


Figure 4.7 Types and concentrations of ion-pairing reagent on extraction. The sample solution was adjusted to 400mM phosphate buffer, pH 7 and spiked with 10ppm of atenolol. The acceptor phase was 0.1 M HCl. Extraction time was 40min

Instead of adding the carrier to the sample solution, the effect of adding octanoic acid as a carrier in the organic phase (from 0 –100%) was investigated. However, the recovery was not satisfactory due to the small contact area between atenolol and the carrier. In addition, dilution of liquid membrane (organic phase) affected the stability of the membrane. Further optimization was therefore performed with 25mM sodium octanoate as ion-pairing reagent in the sample solution. Sodium chloride was also included into the sample solution to reduce the solubility of atenolol in sample solution by the salting out effect, unfortunately, precipitation of sodium octanoate but not enrichment of atenolol has occurred. Thus, only 400mM phosphate buffer and 25mM of sodium octanoate were added into the sample solution.

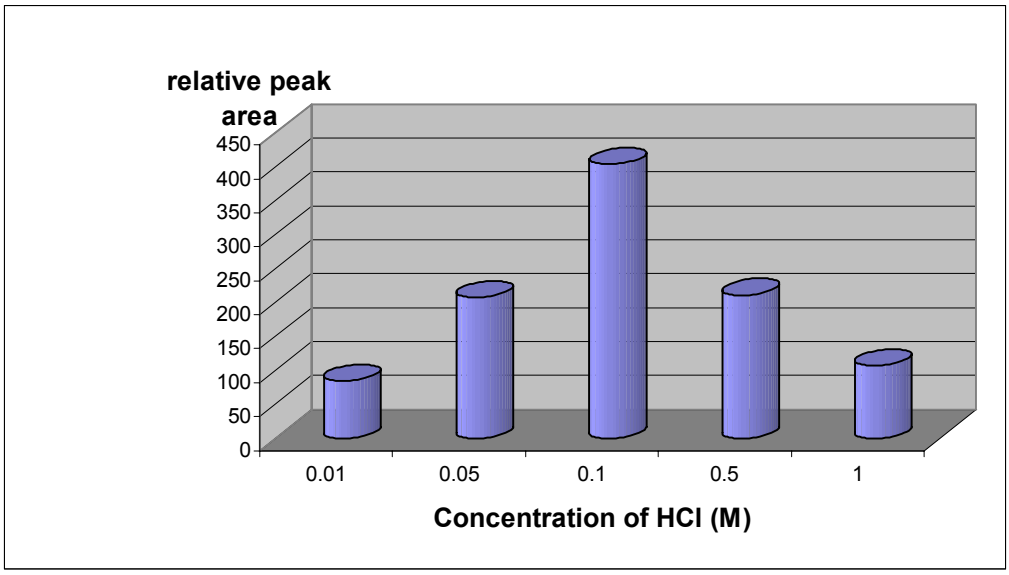


Figure 4.8 Concentration of HCl on extraction recovery. The sample solution was adjusted to 400mM phosphate buffer, pH 7 and spiked with 10ppm of atenolol and 25 mM sodium octanoate. Extraction time was 40min

Various concentrations of HCl, ranging from 0.01 M to 1 M, were tested as acceptor phase. The extraction efficiency increased with the elevated HCl concentration and extraction achieved the highest recovery at 0.1 M HCl. Further increment of HCl did

not improve the result. Moreover, it was acknowledged that the high acidity could damage the chromatographic column. So, the optimum HCl concentration was set at 0.1 M.

A proposed mechanism for carrier mediated LPME[4] was that the ion-pairing reagent (sodium octanoate) carrying a negative charge was able to couple with the oppositely charged analytes (atenolol) and bring it into the organic phase. Unpaired analyte ion was barred from entering the organic layer due to its solubility problems. After being distributed into the organic phase, the ion-complex would travel to the organic-acceptor interface where pH-induced dissociation occurred.

At highly acidic pH, octanoate would gain a proton from the acceptor phase (HCl) and become protonated. Atenolol became a charged species after losing octanoate ion and has no affinity for the organic phase. Thus, it enters the acceptor phase. Due to the low solubility of the carboxylic acid in an acidic medium, the majority of the octanoic acid molecules would distribute into the organic phase, travel out into the sample-organic interface and deprotonate at neutral pH. The deprotonated octanoate ion would then be able to couple with new analyte ion whereby a new cycle of shuffling analyte ion from sample solution to acceptor phase continues. Atenolol was concentrated in the acceptor phase, against its concentration gradient, due to a gradient of the counter ion (proton) between the sample solution and acceptor phase. This explained why a high concentration of HCl was needed to drive the coupling process. At lower concentration of HCl, the ion-complex could have a stronger affinity towards the organic phase due to hydrophobic interaction and reluctance to dissolve in the acceptor phase. Alternatively, if the stripping

process was successful, the octanoic acid would have a higher solubility in the acceptor phase and cause a futile shuffling mechanism. Nevertheless, a sufficient amount of sodium octanoate was needed in the sample solution to drive the complex formation which in turn elevated the distribution of the ion-pairs into the organic phase. Based on the above observations, the sample solution was adjusted to 400mM phosphate buffer, pH 7 with the addition of 25mM sodium octanoate. In addition, 0.1 M HCl was selected as the acceptor phase.

4.3.5 Stirring rate

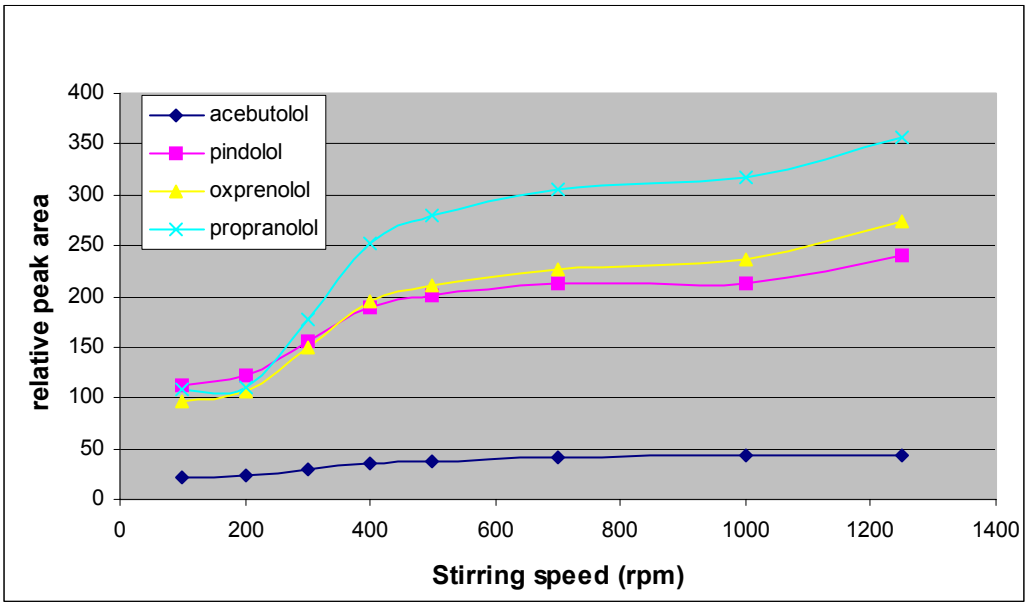


Figure 4.9 Effect of stirring speed on extraction efficiency. The sample was basified to 0.1 M NaOH and spiked with 250ppb of acebutolol, pindolol, propranolol and 1000ppb of oxprenolol. The acceptor phase was 0.005 M HCl. Extraction time was 30min

Generally, an increase in stirring speed increases the speed of extraction by reducing the thickness of the boundary layer at the outer membrane surface. As shown in Figure 4.9, the extraction recovery increased dramatically from 200 to 400rpm. The increment was more moderate after 700rpm for pindolol, oxprenolol and propranolol. As

for acebutolol, the augmentation was trivial, attributed to its molecular weight. The fact that acebutolol has a higher molecular weight compared to the other three analytes can be disadvantageous because of its poorer mass transfer kinetics, resulting in a worse extraction efficiency.

On the other hand, atenolol displayed a similar extraction curve as oxprenolol (data not shown) but with a lower extraction recovery. The extraction recovery increased drastically with increasing stirring speed until 700 rpm and the increment was more gradually at a higher speed. In a stirred sample solution, the analyte ions and carrier ions could be brought together to form ion-pairs more effectively and also this increased their distribution into the organic phase. Similar to the case of acebutolol, the ion complex, which was bulkier, had a lower mass transfer rate and this reduced the diffusion rate of analytes to the organic phase as well as to the acceptor phase. As a result, the extraction recovery of atenolol was low. The poor mass transfer rate was probably not limited to the sample-organic interface because the increase in stirring speed after 700 rpm did not significantly overcome the problem.

An attempt to use dynamic LPME as described by *Wu et al.*[5] was made in this study, whereby the acceptor phase was withdrawn or dispensed repeatedly through the hollow fiber using a syringe pump. By doing so, the concentration of analytes would not build up at the organic-acceptor interface and this facilitates transfer of analytes more effectively into the acceptor phase. However, the movement of plunger that was supposed to improve the transfer rate at the interface did not increase the extraction recovery. This was because dislodgement of organic phase during extraction seriously affected the

stability of the organic membrane. Thus, dynamic LPME was not applied for the rest of the experiments.

In conclusion, the extraction speed of 1250rpm was selected for the extraction of both atenolol as well as the other four amino alcohols by using static mode.

4.3.6 Extraction time profile

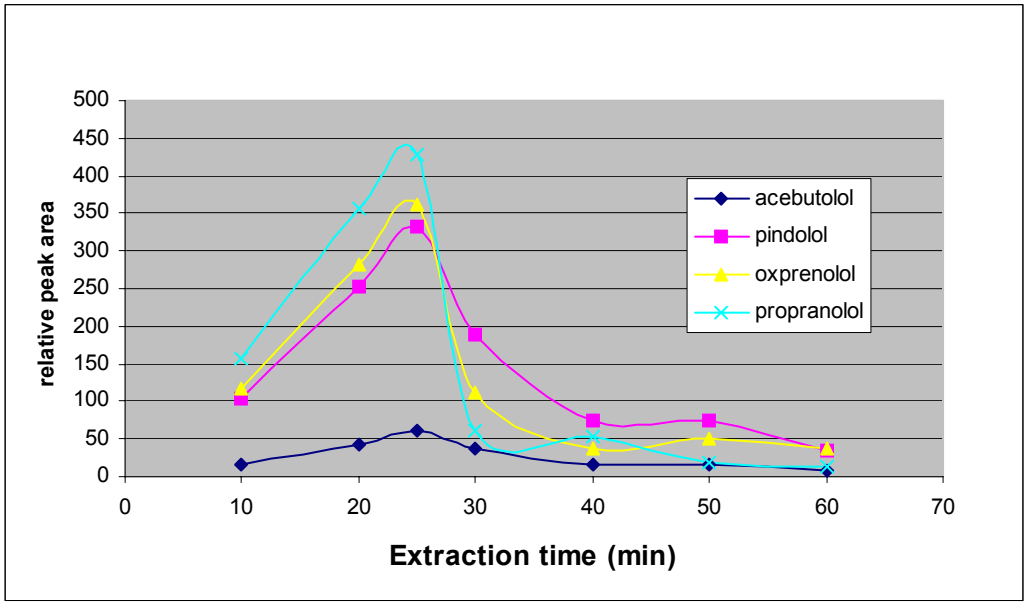


Figure 4.10 Effect of extraction time on extraction efficiency. The sample was basified to 0.1 M NaOH and spiked with 250ppb of acebutolol, pindolol, propranolol and 1000ppb of oxprenolol. The acceptor phase was 0.005 M HCl. Extraction speed was 1250rpm

With the hollow fiber impregnated with *n*-octanol, 10^{-1} M NaOH in the sample solution and 0.005 M HCl as the acceptor phase, the extraction time was optimized for the four amino alcohols with the stirring rate at 1250 rpm (Figure 4.10). The amount of analytes extracted increased with extraction time until 25 minutes; higher exposure time diminished the extraction efficiency. The sudden drop in extraction recovery was probably due to dislodgement of organic phase after prolonged exposure at a high stirring speed.

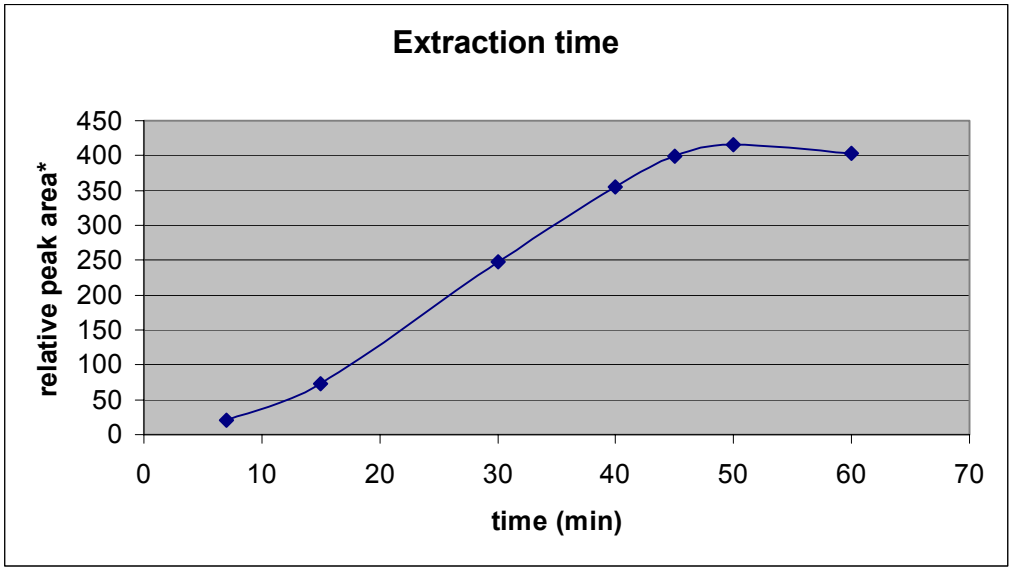


Figure 4.11 Extraction yield vs. extraction time. The sample solution was adjusted to 400mM phosphate buffer, pH 7 and spiked with 10ppm of atenolol and 25 mM sodium octanoate. Extraction speed was 1250rpm. * The relative peak area for Figure 4.11 and 4.10 are not drawn to the same scale.

On the contrary, the extraction of atenolol exhibited different phenomenon (Figure 4.11). The extraction recovery increased with increasing extraction time and reached a plateau at around 50 min. At the initial state, there was a slight time lag in the extraction due to the ion-pairing process. As the extraction time increased, the ion complex entering the organic phase also increased. The increase in the number of carrier molecules increased the flux of atenolol entering the organic phase, and this was represented by a remarkable increment of extractability from 15 to 40 minutes (Figure 4.11). Concurrently, the viscosity of liquid membrane also rose significantly due to the accumulation of the carrier in the organic phase. In addition, the octanoate ion presented at the sample-organic interface with their ionizable $–COO^-$ group facing the aqueous side could have interacted with the water molecules via hydrogen-bonding. As a consequence, interfacial water also became more viscous. This helped to promote the stability of the organic membrane during prolonged extraction at a high stirring speed. Nevertheless, the high viscosity of both the sample–organic interface and liquid membrane may impose a higher barrier to

the diffusing ion-pairs. These would eventually slow down the coupling reaction occurring at the interface (after 40 min), thus allowing equilibrium to be attained. A similar behavior to this has been reported for extraction of glyphosate by a supported liquid membrane technique.[8]

4.3.7 Quantitative analysis

Compound	Precision (% R.S.D.)		Linearity		LOD (ppb)[c]	Enrichment Factor[d]	Relative recovery(%)[e]	
	Intra-day[a]	Inter-day[b]	(r^2)	Range (ppb)			Tap water	Drain water
atenolol	4.3	6.8	0.9996	62.5-20,000	62.5	2.5	108.2	107.2
acebutolol	2.9	2.9	0.9991	8-500	8	47.4	90.2	95.4
pindolol	3.3	3.6	0.9996	4-500	2	55.6	91.6	97.6
oxprenolol	2.7	7.2	0.9986	31-1000	16	52.1	85.7	96.8
propranolol	18.4	22.0	0.9962	8-500	4	26.3	72.2	90.6

[a] Ultrapure water spiked with 1ppm of atenolol, 50ppb of acebutolol, pindolol, propranolol and 200ppb of oxprenolol (n=4)
[b] Ultrapure water spiked with 1ppm of atenolol, 50ppb of acebutolol, pindolol, propranolol and 200ppb of oxprenolol (n=12)
[c] (S/N=3)
[d] (n=4)
[e] Water samples spiked with 1ppm of atenolol, 50ppb of acebutolol, pindolol, propranolol and 200ppb of oxprenolol (n=3)

Table 4.1 Validation data of the three-phase and carrier-mediated LPME method and relative recoveries of the tested compounds in tap water and drain water

To evaluate the practical applicability of the proposed LPME technique, precision, linearity, limit of detection and enrichment factor were investigated by spiking standards in ultrapure water. The result of Table 4.1 indicated that the enrichment factor for pindolol was the highest followed by oxprenolol, acebutolol, and propranolol. Since atenolol was extracted by a different LPME technique, it was excluded for the comparison. It was found that the enrichment factor was higher for β-blockers with lower log P values. These results indicated that analytes with higher hydrophobicity would have higher retention in the organic phase and thus had lower recovery in the acceptor phase.

This explain why for propranolol, which has the highest log P value (Figure 4.2), only an enrichment factor of 26.3 was obtained.

Intra-day precision was carried out on the same day with four replications, while inter-day precision was done on three alternate days with four replications each day. The intra-day and inter-day precision was in the range 2.7-4.3 % R.S.D. and 2.9-7.2 % R.S.D., respectively, with the exception of propranolol which has 18.4 % R.S.D. and 22 % R.S.D. for intra-day and inter-day precision. The poor precision was probably due to manual injection and manual fiber manipulation. An autosampler device and robotic fiber manipulation would give more reproducible results.

The exceptional high R.S.D. value for propranolol suggested that the extraction was not very reproducible. *Müller et al*. has demonstrated that in the case of analytes with a very high log P value, adsorption within the hydrophobic polypropylene membrane could occur[6]. Some propranolol molecules that were extracted into the organic phase could have adsorbed on the hollow fiber membrane instead of entering the acceptor phase. This indicates that not only the distribution equilibrium of the analytes between water and liquid membrane, but also the adsorption of the compounds within the microporous hollow fiber membrane have to be taken into account.

Overall, the linearity of all β-blockers was satisfactory with r^2 of at least 0.996 being obtained. The LODs for the amino alcohols were in the range of 2 to 62.5 ppb. Among the five compounds, oxprenolol is a weak chromophore, therefore, a higher concentration need to be introduced to obtain a UV response. This was also the reason for it having a higher LOD value as compared to the other three β-blockers extracted by the

three-phase LPME. Derivatization of oxprenolol prior to extraction may be able to improve the LOD.

From Table 4.1, it may be seen that atenolol has a lower enrichment factor and a higher LOD than the other four analytes. This means that although carrier-mediated LPME could be applied to extract a hydrophilic analyte, it was still less powerful than the three-phase LPME. The carrier-mediated LPME was closely linked to three processes: the chemical interaction (ion-pairing), distribution into the organic phase and shuffling mechanism of the carrier. It depends highly on a suitable carrier to ion-pair with the analyte before the analyte could distribute itself into the organic layer. More importantly, it requires a counter-ion to drive the extraction process. In order to improve the enrichment factor and LOD, the identification for a more compatible carrier was definitely required.

On the whole, the LODs for these analytes are one or two magnitude higher than other detectors (e.g. mass spectrometer, fluorescence) due to the limitations of the UV-visible detector and no additional preconcentration method (such as 'stacking' method in capillary electrophoresis) was employed after the extraction[2,3,4]. Lower detection limits could be achieved by using of a more sensitive detector with some minor modification of acceptor phase.

4.3.8 Application of three-phase and carrier-mediated LPME to real samples

The previous experiments were based on extraction of the standard drugs in ultrapure water and it was finally applied to the water samples collected from different

sources. In order to be a robust extraction method, the extraction recovery is an important parameter in method development and it should not be affected significantly by matrix effects. Extraction was first done in tap water and drain water without spiking, and there was no detection of analytes within the effective concentration as determined in Table 4.1 (please refer to section 3.3.9). Then water samples were then spiked with the analytes and extracted. The relative recoveries are shown in Table 4.1. They ranged from 72 to 108 %, and within the uncertainties of the experimental set-up. The extraction of water sample also displayed a clean chromatogram with base line separation for these amino alcohols. The results showed that both three-phase LPME and carrier-mediated LPME were insensitive to matrix effects. In three-phase LPME, only ionizable hydrophobic analytes were extracted into the acceptor phase. Hydrophilic analytes have limited solubility in the liquid membrane (such as atenolol), while non-ionizable hydrophobic compounds would be retained in the liquid membrane. The carrier-mediated LPME was a more selective method by allowing only the targeted analytes that was able to ion-pair with the carrier to be extracted into the acceptor phase. Thus, just by introducing a third phase in the LPME system (aqueous acceptor phase), a more selective extraction could be performed.

4.4 Conclusions

Three-phase LPME and carrier-mediated LPME were able to combine extraction and preconcentration as well as sample cleanup in a single step operation. They provided new alternatives of sample preparation for being a simple, fast and effective analytical technique although they have some limitations (see below). These techniques are also highly compatible with HPLC analysis. Moreover, reduced usage of organic solvent has

also minimized the exposure of operator as well as the environment to toxic solvent. More importantly, both three-phase LPME and carrier-mediated LPME provided high selectivity in extraction. In fact, these two methods were more selective than two-phase LPME due to additional pH adjustment at the organic-acceptor interface. Only analytes which have penetrated the organic layer and have ionized at the organic-acceptor interface would be able to be extracted into the acceptor phase. This explains why these methods were insensitive to matrix effects.

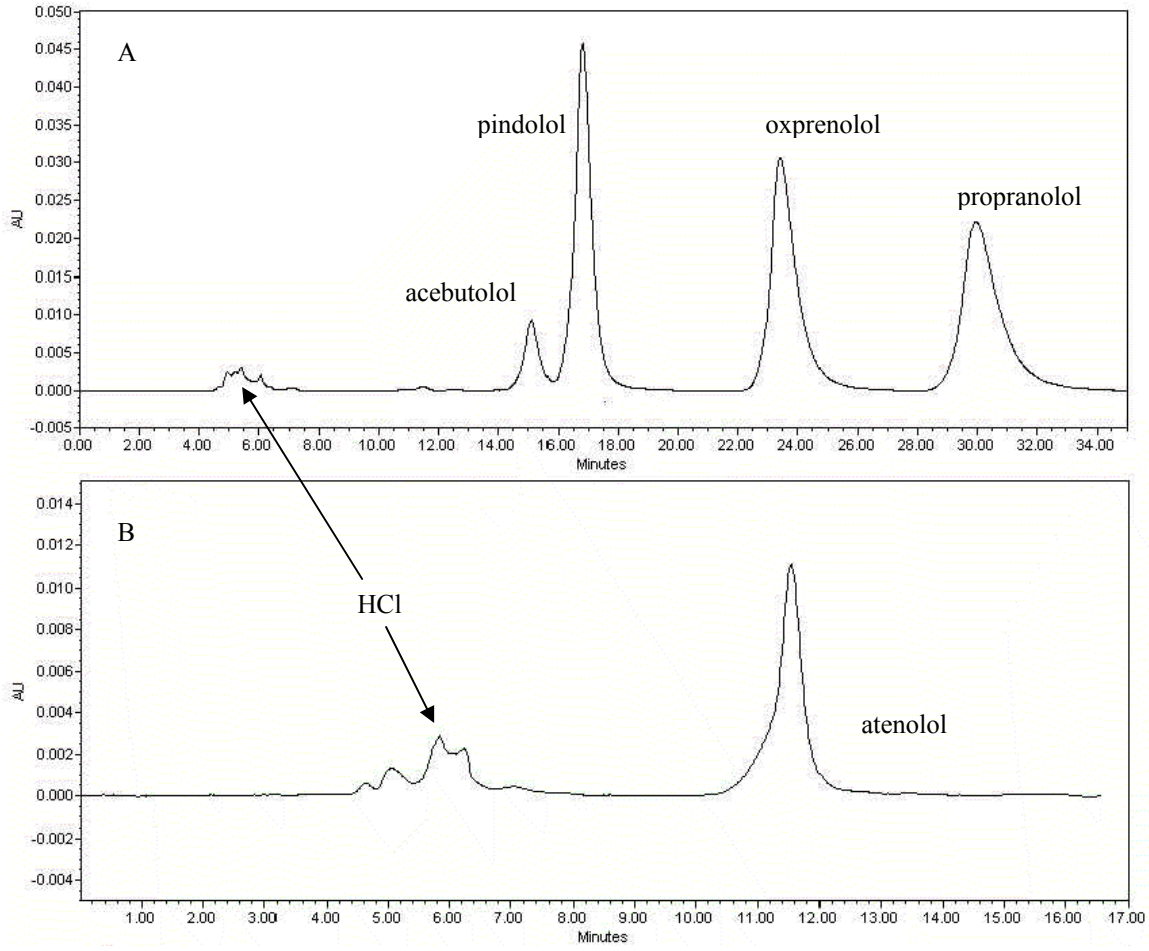


Figure 4.12 Matrix effects on extraction performance.
A The drain water was spiked with 50ppb of acebutolol, pindolol, propranolol and 200ppb of oxprenolol
B The drain water was spiked with 1ppm of atenolol

The major differences in three-phase LPME and carrier-mediated LPME are as follows: Application of the three-phase LPME is limited to moderately hydrophobic ionizable analytes, while the latter is designed to extract hydrophilic analytes by special chemical interaction. In terms of transportation process, three-phase LPME is based on passive diffusion while carrier-mediated LPME is an active transport that depends heavily on the chemical gradient across the membrane (in this case, the proton gradient). In carrier-mediated LPME, more parameters are also required to be optimized, especially the selection of a suitable carrier. Nevertheless, these two LPME methods should be regarded as complementary techniques in sample pretreatment steps instead of as two mutually exclusive techniques.

In conclusion, three-phase LPME and carrier-mediated LPME represent new alternatives to extract amino alcohols from environmental samples. Further improvement on fiber preparation and organic solvent impregnation process or automation or semi-automation of the LPME process would increase the precision of the techniques. However, some fundamental problems remain to be solved in order to improve the LPME performance. These include maintaining the integrity of liquid membrane even with high stirring speed and prolong extraction time in three-phase LPME and eliminating the adsorption of highly hydrophobic compounds on the membrane. Thus, more research has to be done to develop them into more robust and "rugged" methods.

4.5 References

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Chapter 5

Conclusions

The main purpose of the study was to promote more environmental friendly analytical technique by minimizing the usage of toxic organic solvents in sample preparation. The prospect of making such technique routine approaches is also an objective, at least ultimately. In this study, the possibility of using liquid-phase microextraction (LPME) as an emerging methodology on top of the conventional solvent extraction for analyzing trace amount of active pharmaceutical ingredients in water samples was described. Different modes of LPME were introduced to cover the extraction of diverse analytes, ranging from acidic to basic, hydrophobic to hydrophilic.

In the analysis of acidic or basic drugs, two major concepts that govern the success of LPME are the equilibrium constants (log P) and the dissociation constants (pK_a) of the analytes. Thus, selection of the organic solvent and pH adjustment of sample solution are very important for high recovery extraction. The experimental results have indicated that organic solvent immobilized in the hollow fiber pores was the most critical parameter in LPME. Solubility, polarity, volatility and additional chemical properties of the organic solvent had great influence on the extraction efficiency of analytes.

For extraction of acidic or basic drugs, pH adjustment is also crucial for all modes of LPME, as dissociation equilibria are strongly associated with the solubility of the acidic or basic analytes. In this work, the pH of the donor phase was adjusted to deionise the target compounds, reduce their solubility in the sample solution and ensure efficient transfer into

the organic phase. Furthermore, pH adjustment of the acceptor phase in three-phase LPME was to promote stripping of analytes from organic phase and to drive the carrier-shuffling mechanism in carrier-mediated LPME. Thus, three-phase and carrier-mediated LPMEs were shown to be only suitable for ionizable analytes. Conversely, two-phase LPME catered to highly to moderately hydrophobic analytes.

Similar to solvent extraction, mass transfer in LPME is a time-dependent process and equilibrium is only attained after exposure of the solvent to the sample solution for a period of time. Although extraction efficiency generally increased with extraction time in most cases, shorter extraction time comparable with total chromatographic time was employed to ensure high sample throughput. Stirring was an important parameter often applied to accelerate the extraction kinetics. Other factors such as volume of acceptor phase and salt addition were also investigated in this study.

Two-phase and three-phase LPME modes are both based on passive diffusion where extraction requires high partition coefficients from the sample (aqueous phase) into the acceptor (organic) phase. However, for highly hydrophilic analytes, partition coefficient into the organic solvent is suppressed, and thus their extractability into the final extracting phase for two-phase and three-phase LPME is very poor. With the introduction of carrier-mediated LPME, hydrophilic compounds could be extracted by ion-pairing with a suitable carrier. The carrier has to be relatively hydrophobic with acceptable water solubility, and it must be able to ion-pair with the targeted analyte so that extraction into the organic phase could be accomplished. A suitable donor phase (sample solution) pH is vital to keep both the carrier and analyte in ionization state to keep them in ion-pair complex, transportable form.

Moreover, it is necessary to mention that the transport of hydrophilic analyte is based on the counter-coupled transport mechanism; the analyte is released from the ion-pair complex by counter ion-exchange at the liquid membrane- acceptor phase interface. Thus, the counter ion gradient is essential. In addition, the carrier should have limited solubility in the acceptor phase to ensure the free carrier is available for the shuffling of analytes in the sample solution into the acceptor phase.

Owing to low cost and the disposable nature of hollow fiber, the extraction device was utilized only for single extraction, thus eliminating cross-contamination problems. Different modes of LPME are also made to be compatible to most of the current analytical instruments. Although the fundamental principles between LPME and conventional solvent extraction are similar, the success of LPME relies virtually on the large phase-ratio differences. LPME significantly reduces solvent waste and simplifies the sample preparation procedure; typically extraction is completed in a single step. Three-phase and carrier mediated LPMEs are very good techniques in extracting hydrophobic or hydrophilic analytes as they provide satisfactory extraction recoveries and sample clean up from environmental sample. Two-phase LPME is more prone to matrix effects as shown in Section 3.3.9 and the use of an internal standard is strongly recommended. Both two-phase and three-phase LPME provide excellent quantification limits, good enrichment factor and good linearity with low sample consumption (4 mL). However, the limit of detection and enrichment factor were less satisfactory in carrier-mediated LPME.

Automation of both the fiber preparation and the LPME operation could improve the precision of the technique and is highly desirable for high throughput analysis. In order to

improve the current performance of LPME, more research in membrane technology and organic solvent are required. Even though the use of hydrophobic polypropylene membrane was ideal for organic solvent immobilization, adsorption of highly hydrophobic analytes within the micro pores of hollow fiber might affect the reproducibility of experiment and a more inert polymeric material may be necessary.

On the other hand, membrane stability is the primary problem associated with the use of hollow fiber based LPME. Solvent loss is most often the causative factor for membrane stability, especially after prolonged exposure at high stirring speeds during extraction. Such solvent loss arises from evaporation and dissolution as well as from excessive pressure differential applied across the membrane during dynamic LPME (which forces solvent out of the pores of the membrane due to the pumping motion of a syringe pump). The use of new organic solvent with low mutual solubility in water yet possessing high dissolving power, high polarity, low volatility and having special chemical properties is highly desirable.

In conclusion, LPME combines extraction, preconcentration and sample cleanup in a single step operation. Different modes of LPME can be used as a complementary technique for rapid screening tool to yield detailed information on the behavior and fate of the active pharmaceutical ingredients in the environment. In addition, with minor modification on the extraction unit, different mode of LPME could be performed, hence offering a high degree of flexibility. With the inherent advantages and limitations of different modes of LPME in mind, further investigations to improve the approaches described in this work to provide a strong platform for future analytical microextractions would be necessary.

5.1 Future research

The current LPME model is limited to extraction under equilibrium condition. Therefore a more in-depth study should be carried out to incorporate those experimental parameters to illustrate their influences on enrichment factor at any time-point.

Apart from that, LPME is limited by the creativity of the chemist preparing suitable polymeric hollow fibers, ion-pairing reagents as well as alternative solvents (e.g. ionic liquids). Further research could possibly include the consideration of the above materials for LPME.