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Automated liquid membrane extraction for high-performance liquid chromatography of Ropivacaine metabolites in urine

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Abstract

An automatic method for the determination of metabolites of Ropivacaine in urine was set up. It utilizes supported liquid membrane extraction for sample clean-up and enrichment, followed by ion-pair chromatography determination using UV detection. The extraction was very selective with no observed interfering compounds from the urine matrix, permitting simple isocratic chromatographic analysis. The detection limits for spiked urine samples were 2–18 n*M* for the different compounds. The repeatability was 1–3% (RSD) with an internal standard that was also extracted, and about twice without this standard. A throughput of 3.3 samples per hour was achieved and the liquid membrane was stable for more than a week.

Keywords: Pharmaceutical analysis; Membranes; Extraction methods; Ropivacaine

1. Introduction

The determination of polar compounds such as drugs and their metabolites in biological matrices is an important task in pharmaceutical and biomedical analysis. These compounds often occur at low concentrations together with a large excess of other, similar compounds. It is of great importance that the methods used are selective enough to ascertain the correct identity of the analytes determined and to avoid quantification errors due to insufficient resolution from disturbing compounds.

The methods used often involve tedious manual procedures for derivatization, clean-up, concentration and reconstitution before a final determination, usually by liquid chromatography or, in some cases, by gas chromatography. A common technique for clean-up and enrichment in this context is solid-phase extraction (SPE), which is amenable to automation using several commercially available instruments. This technique has been amply reviewed [1]. However, it is clear that it works best for relatively hydrophobic compounds, although polar hydrophilic compounds sometimes can be extracted using ion-exchange SPE material. The classical alternative to SPE is liquid–liquid extraction (LLE), but this technique has a number of drawbacks, such as the need for substantial amounts of organic solvents (often chlorinated and environmentally unfriendly), the difficulty to automate the procedures and the formation of troublesome emulsions.

Ropivacaine (Astra Pain Control, Sweden) is a local anaesthetic drug mainly used in surgery and for post-operative pain relief. It is extensively metabolized before being excreted, mainly in the urine [2],

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and the main pathways involved are aromatic hydroxylation and N-dealkylation [3].

Recently, a method for the determination of Ropivacaine and its major metabolites in urine and blood plasma was presented [4]. In that procedure, samples were extracted using SPE with cation extraction columns followed by evaporation and reconstitution, and analyzed by ion-pair liquid chromatography with gradient elution. The application of gradient elution was necessary to obtain sufficient separation of the analytes from remaining matrix components. The total chromatographic analysis time was about 1 h and the process was partly manual. The concentrations found in actual samples are not exceedingly low and the stated detection limits of 1 μM in urine and 0.1 μM in blood plasma were sufficient for the purpose.

In another recent paper [5], a method for the determination of Ropivacaine and three of its metabolites in blood plasma was presented. It is based on LLE with an organic solvent and a subsequent back-extraction into an acidified aqueous solution, followed by ion-pair liquid chromatography. Also, this procedure, although successful, was largely manual and time-consuming.

The technique of supported liquid membrane extraction (SLM) [6–8] has the potential to efficiently extract polar compounds from different matrices and can easily be automated, especially when combined with HPLC. It can be seen as a combination of dialysis and LLE and provides selective extraction for selected classes of analytes, as well as efficient rejection of matrix constituents in biological samples [9–11].

In this work, an automated SLM method has been set up and validated for determination of Ropivacaine metabolites in urine. The aim was to extract the compounds of interest more selectively than in Ref. [4], thereby permitting the application of an isocratic HPLC method and increasing the sample throughput.

2. Experimental

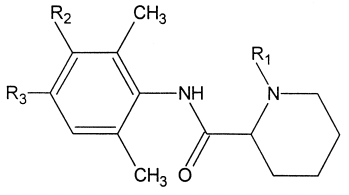
2.1. Chemicals and membranes

Ropivacaine and its metabolites (see Table 1) were obtained as hydrochlorides from Astra Pain Control and used as received. In Table 1 is also listed the pK_a and log P (octanol–water partition coefficient) values, as calculated by the computer programs ACD/pK_a DB and ACD/Log P DB, respectively (Advanced Chemistry Development, Toronto, Canada).

1-Octanesulfonic acid and tri-*n*-octyl phosphine oxide (TOPO) were obtained from Sigma (St. Louis, MO, USA) and di-*n*-hexyl ether from Sigma–Aldrich (Steinheim, Germany). Other chemicals were of analysis quality and obtained from Merck (Darmstadt, Germany).

Porous PTFE membranes were used, Fluoropore FG (Millipore, Bedford, MA, USA) (pores size 0.2

Table 1



Compounds studied[a]

Name	R_1	R_2	R_3	pK_a	Log P
Ropivacaine	*n*-pr	H	H	8.2±0.4	3.1±0.3
PPX (pipecoloxylidide)	H	H	H	9.4±0.4	2.3±0.3
3-OH-PPX	H	OH	H	9.4±0.4	1.9±0.4
3-OH-Ropivacaine	*n*-pr	OH	H	8.1±0.4	2.8±0.4
4-OH-Ropivacaine	*n*-pr	H	OH	8.2±0.4	2.4±0.4
Isopropyl-PPX	*i*-pr	H	H	8.3±0.4	2.9±0.3

[a] Dissociation constants (pK_a) and octanol–water partition coefficients (log P) are calculated using the programs ACD/pK_a DB and ACD/Log$P$ DB, respectively (Advanced Chemistry Development).

μm, porosity 0.7, total thickness 175 μm, of which 60 μm is PTFE and the rest a netlike polyethene backing). The membranes were soaked for 30 min in the membrane liquid, which was 10% TOPO in di-*n*-hexyl ether.

2.2. Instrumentation

The essential part of the instrumentation is a Gilson Model 232 sample processor (A) (Gilson, Villiers-le-bel, France); see Fig. 1. It contains an autosampler tray and a robotic arm to transfer liquid between the vials and an injection port (B). It is further equipped with an integral six-port injection valve (C; Model 7010, Rheodyne, Cotati, CA, USA) and two syringe pumps (D, E; Model 401, Gilson) with syringes of 10 ml and 1 ml capacities, respectively. The instrument is controlled by an internal computer and programmed using a terminal pad. The membrane unit (F) consisted of two blocks made by polyvinylidenedifluoride (PVDF), each with a machined groove (2.5×0.1×40 mm). When the blocks are clamped together with the membrane between using 6 bolts, a 10 μl channel is formed on each side of the membrane. Polyether ether ketone (PEEK) tubes fitted with low-pressure connectors (Upchurch Scientific, Oak Harbor, WA, USA) were used to assemble the flow system as shown in the figure.

The HPLC setup consisted of an isocratic pump (G; Model 422, Kontron, Milan, Italy) and a variable-wavelength UV detector (H; Lambda-Max Model 480, Waters Assoc., Milford, MA, USA). The column (I) was 150×4.6 mm, packed with Nucleosil C_{18} 5 μm. The mobile phase consisted of 30% acetonitrile and 30 m*M* phosphate buffer at pH 2.5, containing 10 m*M* 1-octanesulfonic acid. It was pumped at a flow rate of 0.9 ml/min.

The chromatographic data were collected using a JCL6000 for Windows Chromatographic Data System (Jones Chromatography, Hengoed, UK), which also provided signals for the coordination of the sample processor operation and the chromatography.

2.3. Procedure

Spiked urine samples (1.1 ml each) are loaded in vials in the autosampler. By means of the first syringe pump (D), 1.1 ml of carbonate buffer, containing 40 m*M* of EDTA, is added to the sample to be analyzed in order to adjust the pH to approximately 9.5, at which value the analytes (which all are bases with pK_a around 8–9; see Table 1) are

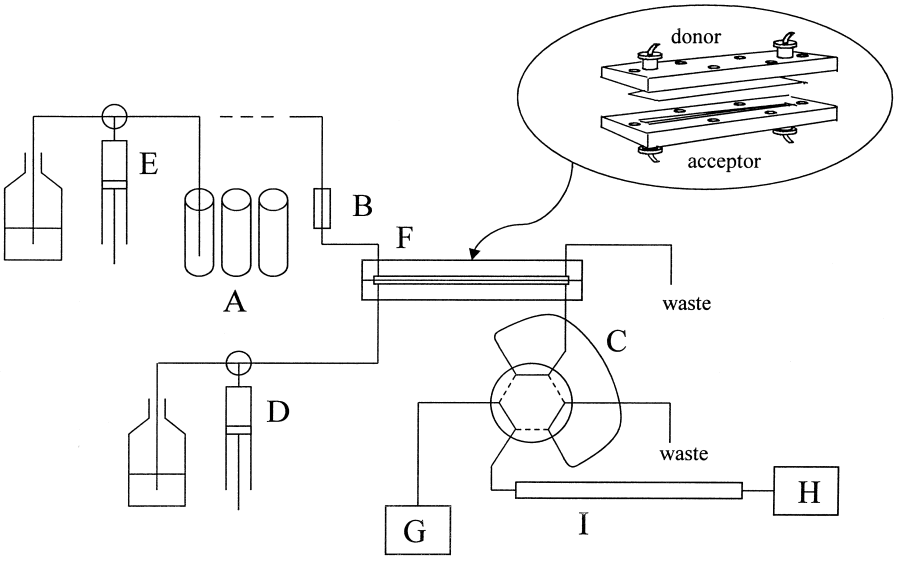


Fig. 1. Schematic diagram of experimental setup, for details see the text. A: Sample vials; B: Injection port; C: Injection valve; D,E: Syringe pumps (dilutors); F: Membrane unit (see also insert); G: HPLC pump; H: UV detector; I: HPLC column.

uncharged. The addition of EDTA prevents precipitation of metals at the high pH. After mixing by aspirating the sample three times and dispensing it again, an aliquot of 1.87 ml is withdrawn and transferred to the injection port B. With a flow-rate of 0.18 ml/min, the sample is dispensed through the donor (upper) channel and the analytes are extracted through the organic liquid membrane and trapped in the stagnant acidic buffer in the acceptor channel (24 m*M* phosphate buffer at pH 2.5). After the sample has passed through the membrane extraction unit (which takes 10.55 min), the contents of the acceptor channel are transported to the injection loop by pumping 80 μl with the second syringe pump (E). The volume of the loop is 100 μl, so the entire extract from the acceptor is accommodated and injected into the chromatographic column when the valve is switched and the collection of the chromatography data is started. Before a new sample is extracted, the donor and acceptor channels are rinsed with 1.0 ml each of the respective buffers.

The total extraction cycle including pH adjustment and rinsing takes 18 min, which is the same as the chromatographic run. To optimize sample throughput, one sample is extracted while the previous one is chromatographed, leading to a throughput of 3.3 analyses per hour.

3. Results and discussion

3.1. Selectivity

Fig. 2a shows a chromatogram after extraction of an aqueous solution of the analytes at concentrations around 1 μ*M*. As seen, the ion-pair chromatography procedure leads to a complete separation of the compounds of interest within 18 min using an isocratic eluent. The analytes were detected at 210 nm.

In Fig. 2b is shown a chromatogram of an identically spiked urine sample extracted in the same way. It can be observed that these chromatograms are virtually indistinguishable, demonstrating the high degree of selectivity that can be obtained with SLM extraction. Similar results have been shown in earlier papers [10,12].

3.2. Optimization of the extraction procedure

The compositions of the three phases (donor, membrane and acceptor), as well as dimensions, flowrates, etc. were optimized according to previous experience and theory. Thus, the membrane unit dimensions (giving channel volumes of 10 μl each) were the same as previously used [10] in a similar setup. This permits the injection of the entire extract collected in the acceptor channel into the column via an injection loop of appropriate dimensions (here 100 μl). A larger membrane unit would necessitate larger sample volumes without other advantages. A smaller membrane unit leads to technical problems.

The donor flowrate and sample volume are related. A low flowrate increases the extraction efficiency but also extraction time. Extracting a smaller volume could counteract this. Given the time of the chromatographic separation and the limited choices of flowrates possible with the syringe pumps used, the selected compromise conditions provide optimal enrichment with a reasonable sample volume.

According to the requirements arising from the chemistry of the SLM process [8], the pH of the donor phase should be higher than the pK_a of the analytes, so they are largely protonated and therefore extractable. Also, the pH of the acceptor phase must be at least 3.3 pH units lower than the pK_a in order to obtain complete trapping. It is important that the buffer capacity and initial pH of the acceptor phase ensure that the pH requirements are met during the entire extraction. With biological samples, especially urine, non-analyte basic substances might also be extracted, leading to partial neutralization of the acceptor. From these considerations, suitable conditions as described above were selected and evaluated in preliminary experiments.

The composition of the membrane phase was selected from a previous work [12] on similar compounds, where 5% TOPO in di-*n*-hexyl ether was selected. In the present work, including some additional compounds, the concentration of TOPO was increased to 10%, leading to better extraction of the most polar compounds. This was evaluated in preliminary experiments.

3.3. Extraction efficiency and enrichment

The extraction is characterized by the extraction

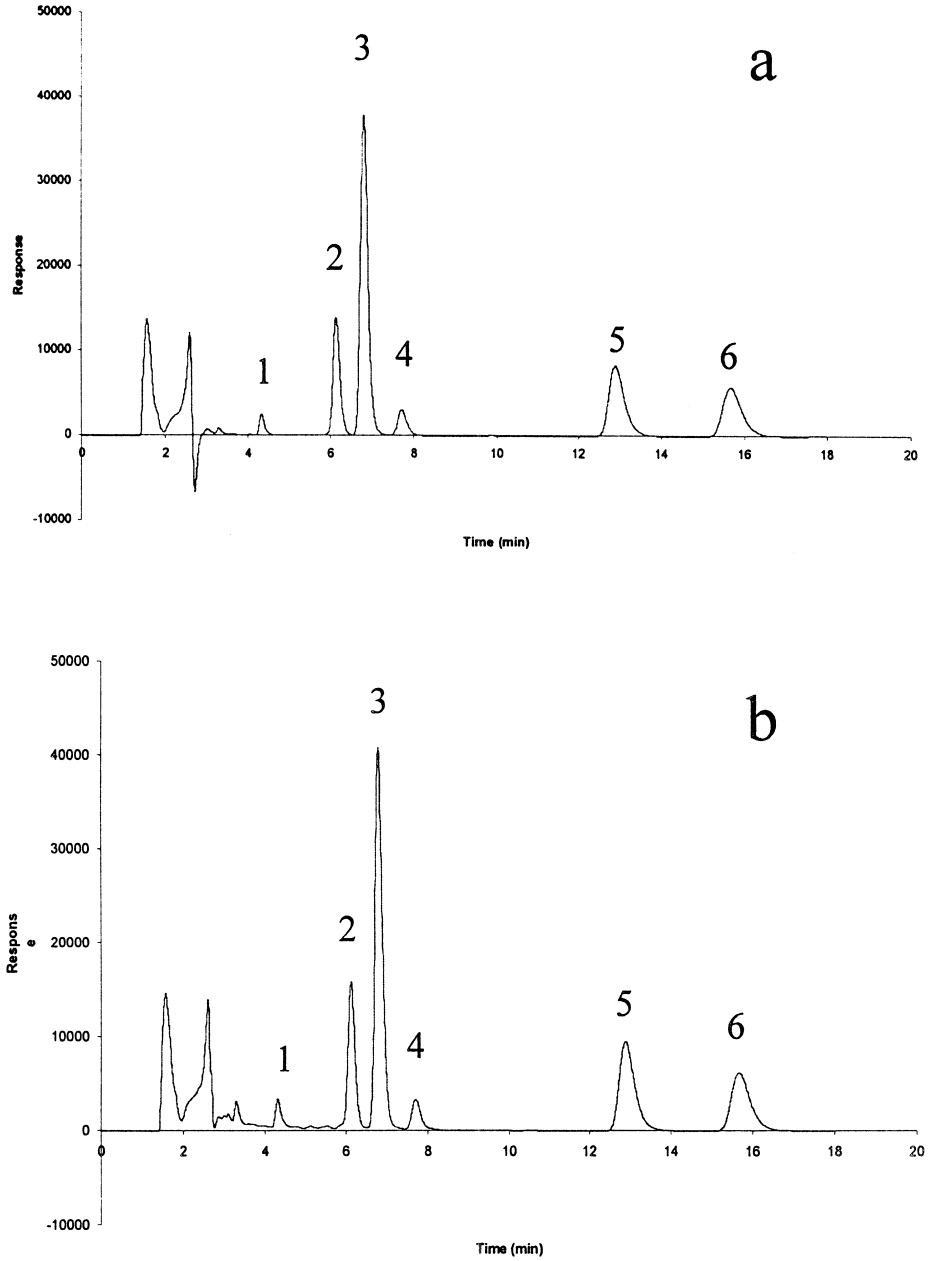


Fig. 2. Chromatograms of a water solution (a) and a urine sample (b), both spiked with 3-OH-PPX (1; 1.0 μ*M*), 4-OH-Ropivacaine (2; 0.80 μ*M*), 3-OH-Ropivacaine (3; 0.83 μ*M*), PPX (4; 1.0 μ*M*), Iso-PPX (5; 0.84 μ*M*) and Ropivacaine (6; 0.90 μ*M*). Chromatographic conditions were as described in the text.

efficiency, i.e. the fraction of analyte in the extracted sample that is found in the acceptor channel:

$$E = n_A / n_I \quad (1)$$

where n_I and n_A are the number of moles input to the system during the extraction time and collected in the acceptor, respectively. For a complete treatment of the theory for SLM extraction, see Refs. [6–8].

Table 2 presents the extraction efficiency values measured for the analytes under the selected con-

Table 2
Extraction parameters[a]

Name	E	E_e
Ropivacaine	37%	69
PPX (pipecoloxylidide)	9%	17
3-OH-PPX	3%	6
3-OH-Ropivacaine	73%	136
4-OH-Ropivacaine	23%	45
Isopropyl-PPX	53%	99

[a] Extraction efficiency (E) and enrichment factors (E_e) after the extraction procedure described in the text.

ditions. It is seen that for the compounds PPX and 3-OH-PPX, the extraction efficiency is smallest due to the higher polarity of these compounds (cf. Table 1). However, the extraction efficiency is sufficient for determination of these compounds in the desired concentration range with adequate precision. This illustrates that quantitative measurements can be made with extraction efficiencies well below 100% [9].

The enrichment factor E_e signifies the number of times that the extraction process increases the analyte concentration. It is defined as:

$$E_e = c_A / c_D \quad (2)$$

where c_D and c_A are the concentrations in the donor and acceptor phase, respectively. Also this factor is listed in Table 2, and its relation to the extraction efficiency is simply:

$$E_e = E \cdot V_I / V_A \quad (3)$$

where V_I and V_A are the volumes of sample input to the system and that of the acceptor, respectively. In calculation of the values in Table 2, V_A is taken as 10 μl. As explained above, the entire contents of acceptor is transferred to the 100 μl injection loop. Therefore, in a sense, the enrichment factors in the table are not fully utilized when compared with injections of 100 μl samples. For 3-OH-PPX, there is even a 'dilution', as direct injection of a solution of that compound with the injection loop would give larger peaks than after extraction. Even so, the enrichment is enough for the purpose. Obviously, with the relatively moderate demands on detection limits, the rationale for the extraction is mainly clean-up of the urine matrix.

3.4. Repeatability

Four different urine samples were spiked with about 1 μ*M* of each of the model compounds, extracted and analyzed as described above. The results were evaluated by single factor Anova analysis (using Microsoft Excel 97), thereby separating the within-sample variation from that between the different urine samples. The within-sample RSD was found to be between 1.7 and 3.6%. The variation between the samples was significant ($p = 0.05$), and this probably reflects uncertainties in the spiking. The between sample variation was between 2.3 and 5.5% for the different compounds.

Iso-PPX can be considered as an intern standard, which is the use of this compound in practice. Thus, the area of a certain peak is divided by that of Iso-PPX. This resulted in lower within-sample RSD values (0.9–2.6%). The change was significant ($p = 0.05$) for three of the compounds. The between-sample variation was unchanged (2.3–4.9%). Thus, the use of internal standard corrected for some of the variation in the combined extraction–HPLC procedure.

3.5. Linearity

Linear calibration curves were obtained with concentrations in the range 1–2.4 μ*M*. As seen in Table 3, the curves are linear with insignificant intercept and satisfactory correlation coefficients.

Table 3
Quantitative results[a]

Name	Intercept (a)	Slope (b)	r	LOD (μ*M*)
Ropivacaine	−6±54	340	0.9975	0.015
PPX (pipecoloxylidide)	−2±10	120	0.9995	0.018
3-OH-PPX	−2±5	75	0.9996	0.017
3-OH-Ropivacaine	−1±127	940	0.9979	0.002
4-OH-Ropivacaine	33±45	350	0.9979	0.005
Isopropyl-PPX	3±69	470	0.9975	0.009

[a] Parameters (with 95% confidence intervals) of calibration curves ($y = a + b \cdot x$) in arbitrary units from spiked urine samples (approx. 1–2.4 u*M*), correlation coefficients (r) and detection limits (LODs) in urine with signal-to-noise ratio = 3.

3.6. Detection limits

The detection limits were determined from the chromatograms by determining the smallest concentration that gives a signal that is three times the baseline noise. Obviously, the detection limit is influenced by the enrichment factor.

3.7. Membrane stability

The membrane was usually exchanged once a week, as a precaution. No changes in extraction properties were observed during a week, but the interval was selected from previous experience. There are no obvious visual changes after a week of extracting a large number of urine samples.

4. Conclusions

It is demonstrated that the SLM extraction technique can be used for extraction of the polar metabolites studied. This extraction is more selective than previously presented SPE and LLE procedures for these compounds [4,5]. This permits a simple isocratic separation, as there are no interferences from the urine matrix at the concentration levels studied. Also, the procedure is completely automatic and can run unattended. Although the procedure is not thoroughly validated, detection limits and repeatabilities are appropriate.

In fact, the detection limits are considerably lower than needed for the application (1 μM in urine [5]), and therefore the extraction time might be reduced in order to further increase the sample throughput. Optimally, the extraction time (plus a washing cycle) is equal to the chromatographic run time. As described here, the run time is 18 min, which gives full separation of all compounds. If this time is shortened by using a more efficient column or by partly sacrificing resolution, the extraction time can be shortened accordingly. This would increase throughput while increasing the detection limits. The change should also reduce the sample volume needed, which might be of minor importance for urine samples, but significant for samples of blood and other biological fluids.

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