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Effective on-line purification for cationic compounds in rat bile using a column-switching LC technique

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Abstract

An on-line purification method for cationic compounds and their metabolites in rat bile was investigated using a column-switching technique. 8-Hydroxyquinoline and its glucuronide were used as test compounds. Bile samples were injected directly into the system and successful on-line extraction with high purification efficiency for analytes was achieved using two-dimensional extraction LC; that is, reversed-phase chromatography followed by cation-exchange chromatography. After removal of the endogenous component by extraction LC, chromatographic separation of the target analyte was performed on an analytical ODS column, followed by identification using UV detection. The quantitative ability of the method was evaluated on the basis of injection repeatability, linearity and accuracy. This novel method was also applied to LC/MS analysis in order to characterise the pharmacokinetics of propranolol in rats, and the metabolites were successfully identified.

Keywords: 8-Hydroxyquinoline; Cation-exchange; Column-switching; Pharmacokinetics; Propranolol; Rat bile

1. Introduction

During pharmaceutical research and development, it is important to clarify the drug metabolism in order to provide the information of efficacy and safety of drug candidate and their metabolites for both pre-clinical and clinical studies. Hence, the knowledge of drug metabolism obtained in early drug discovery stage contributed to the efficient drug development.

To obtain the information of drug metabolism in vivo system and their metabolites of candidate drug, it is essential to analyse the various biological matrices; e.g. serum, plasma, bile and urine. Especially, evaluation of the metabolites in bile offers us numerous information. However, the analysis of bile sample has some subject matter in sample preparation in comparison with other biological matrices. Actually, existence of endogenous components such as bile acid, cholesterol, bilirubin and phospholipids in bile [1], sometimes makes us utilise radio-labelled tracer technology [2–4].

On the other hand, several studies have addressed the analysis of drugs in bile by using liquid chromatography-inductively coupled argon plasma mass spectrometry (LC-ICP/MS) [5–7], LC–nuclear magnetic resonance (LC/NMR) [8] and LC–mass spectrometry (LC/MS) [9,10]. These study evaluated the stability of a target drug and structural analysis of metabolites in bile without radioactive samples. In all cases, analysis of drugs and metabolites in bile normally requires tedious off-line sample preparation procedure, such as filtration, solid phase extraction and concentration in order to eliminate endogenous components.

Therefore, we attempted a development of a simple and reproducible on-line sample preparation procedure for drugs and their metabolites in bile using column-switching LC. In this evaluation, we employed two-dimensional extraction columns (reversed-phase column and cation-exchange

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column) to achieve high purification of target analytes. Furthermore, the quantification ability of the method had also been examined.

2. Experimental

2.1. Chemicals and regents

Acetic acid was purchased from Kanto Chemicals (Tokyo, Japan). Acetonitrile was purchased from Wako Pure Chemicals (Osaka, Japan). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). All other reagents were analytical grade and were used without further purification. Isotonic sodium chloride solution was purchased from HIKARI pharmaceutical (Tokyo, Japan). Water was purified using a Milli-Q® water-purification system (Millipore, Bedford, MA, USA). 8-Hydroxyquinoline, 8-hydroxyquinoline glucuronide and propranolol were purchased from Wako Pure Chemicals. The structures, dissociation constants (pK_a) [11] and log octanol–water partition coefficients (log P_{ow}) [12] of the drugs used in this study are shown in Fig. 1.

2.2. Animals

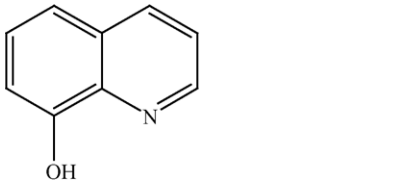
Male Sprague-Dawley (SD) rats aged 7–8 weeks were purchased from Charles River Japan (Yokohama, Japan).

2.3. Sample preparation

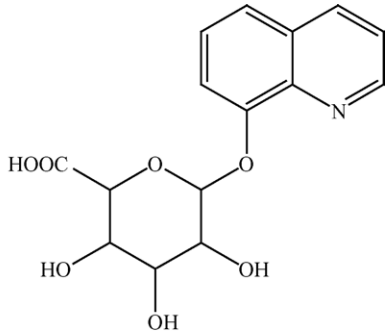
8-Hydroxyquinoline and its glucuronide were dissolved in a mixture of water and acetonitrile (1:1 v/v) to a concentration of 10 μg/mL and used as a stock solution. This was diluted with water to 1 μg/mL for use as a standard solution. SD rat bile was obtained after anti-coagulation treatment and used as a blank bile sample. The stock solution was also diluted 10-fold with rat bile at a concentration of 1 μg/mL for use as a spiked bile sample. Propranolol was dissolved in water to a concentration of 10 μg/mL and used as a standard solution. Bile samples were collected at 30 min intervals after the intravenous administration of 2.5 mg/kg of propranolol to the rats. These samples were segregated by centrifugation at 2000 × g for 5 min and the supernatant was stored at −20 °C until analysis. The drug-containing rat bile samples were prepared by adding 8-hydroxyquinoline and its glucuronide at concentrations of 0.3, 0.5, 1.0, 3.0, 5.0 and 10.0 μg/mL. The calibration standard samples for 8-hydroxyquinoline and its glucuronide were dissolved in 5% acetonitrile aqueous solution at the same concentration as the spiked bile samples. Similarly, the drug-containing rat bile samples were prepared by adding propranolol at concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/mL. The calibration standard samples for propranolol were dissolved in water at the same concentration as the spiked bile samples.

2.4. Chromatographic system

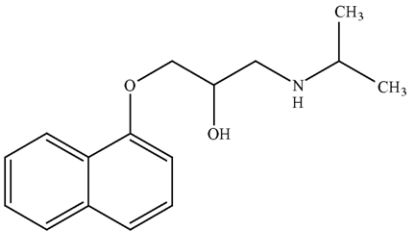
A schematic diagram of the column-switching LC–LC–UV system is presented in Fig. 2. The system consisted of four LC-10AD pumps (Shimadzu, Kyoto, Japan), an auto-sampler, a vacuum degasser, a column oven and UV detector, and three six-port switching valves (V1, V2 and V3), which were used for the on-line pre-treatment and analysis of samples. A bypass line was used for a



8-Hydroxyquinoline
pKa: 6.10(basic), 5.46(acidic)
ClogP: 2.08



8-Hydroxyquinoline Glucuronide
pKa: 4.56 (basic), 2.50 (acidic)
ClogP: -0.24



Propranolol
pKa: 10.08(basic)
ClogP: 2.75

Fig. 1. The structures, pK_a values and calculated partition coefficients (ClogP) of 8-hydroxyquinoline, 8-hydroxyquinoline glucuronide and propranolol.

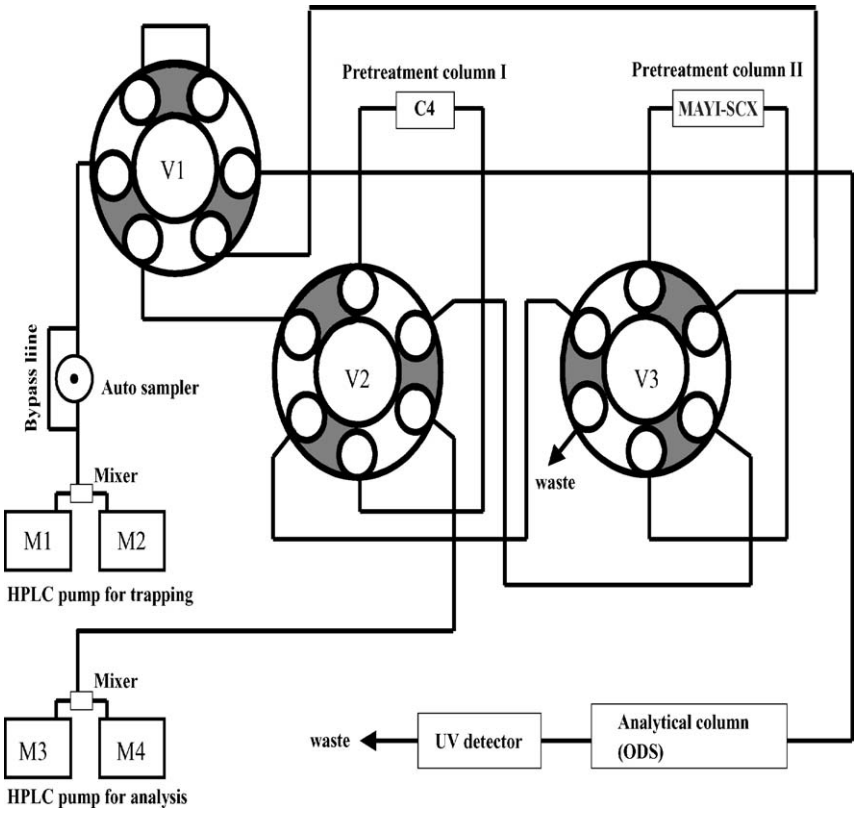


Fig. 2. Schematic diagram of the LC–LC–UV system.

one-ninth dilution of the sample solution with an extraction mobile phase. The mobile phases M1 and M2 were used for the pre-treatment, whereas M3 and M4 were used for the analysis. This system allows several column-switching procedures to occur by altering the three six-port valves. The valve-switching procedure is illustrated in Fig. 3.

2.5. Mass spectrometry

Analyses were performed on a Shimadzu LC/MS-2010A single-stage quadrupole mass-spectrometer equipped with an electrospray ionization interface. The scan range was between 100 and 500 *m/z*. The probe voltage was set at 4.5 kV. The nebuliser gas flow was set at 1.5 L/min. Data processing was carried out using LC/MS Solution software version 2.05 (Shimadzu).

2.6. Chromatographic conditions for the analysis of 8-hydroxyquinoline and its glucuronide

The following columns were used: pre-treatment column I, which was a C4 column (4.6 mm × 35 mm i.d.; 5 μm) (GL Sciences, Tokyo, Japan); pre-treatment column II, which was a MAYI-SCX column (4.0 mm × 30 mm i.d.; 50 μm) (Shimadzu) [13]; and a reversed-phase analytical L-column ODS (4.6 mm × 150 mm i.d.; 5 μm) (CERI, Tokyo, Japan). The mobile phases M1 and M2 comprised 0.05% acetic acid aqueous solution and acetonitrile containing 0.1% acetic acid, respectively. Each phase was pumped at a flow rate of 1 mL/min for the on-line pre-treatment.

The mobile phases M3 and M4 comprised water containing 0.6% TFA and acetonitrile containing 0.6% TFA, respectively. Chromatographic separation on the analytical ODS column was carried out at a flow rate of 1 mL/min. The column temperature was held constant at 40 °C and the samples were stored at 6 °C during the analysis. A 100 μL sample of standard solution and bile spiked with 8-hydroxyquinoline and its glucuronide was injected into the LC–LC–UV system. The column-switching system was operated according to the time programme shown in Fig. 4 and the column-switching procedure illustrated in Fig. 3.

Initially, the bile samples were injected directly into a pre-treatment C4 column connected in-line to a MAYI-SCX column (Fig. 3b) using an auto-sampler equipped with a diluting bypass line. From 0 to 3 min after the sample injection, the hydrophilic endogenous components were eliminated from the bile (Fig. 4a). From 3 to 18 min after the elution of the hydrophilic endogenous components, the basic compounds, bile acid and other hydrophobic components were transferred from the C4 column onto the MAYI-SCX column (Fig. 4b). The MAYI-SCX column retained the cationic compounds using electrostatic interactions, whereas anionic compounds, such as bile acid, and neutral compounds were eliminated. The valve then switched automatically (Fig. 4c) to connect

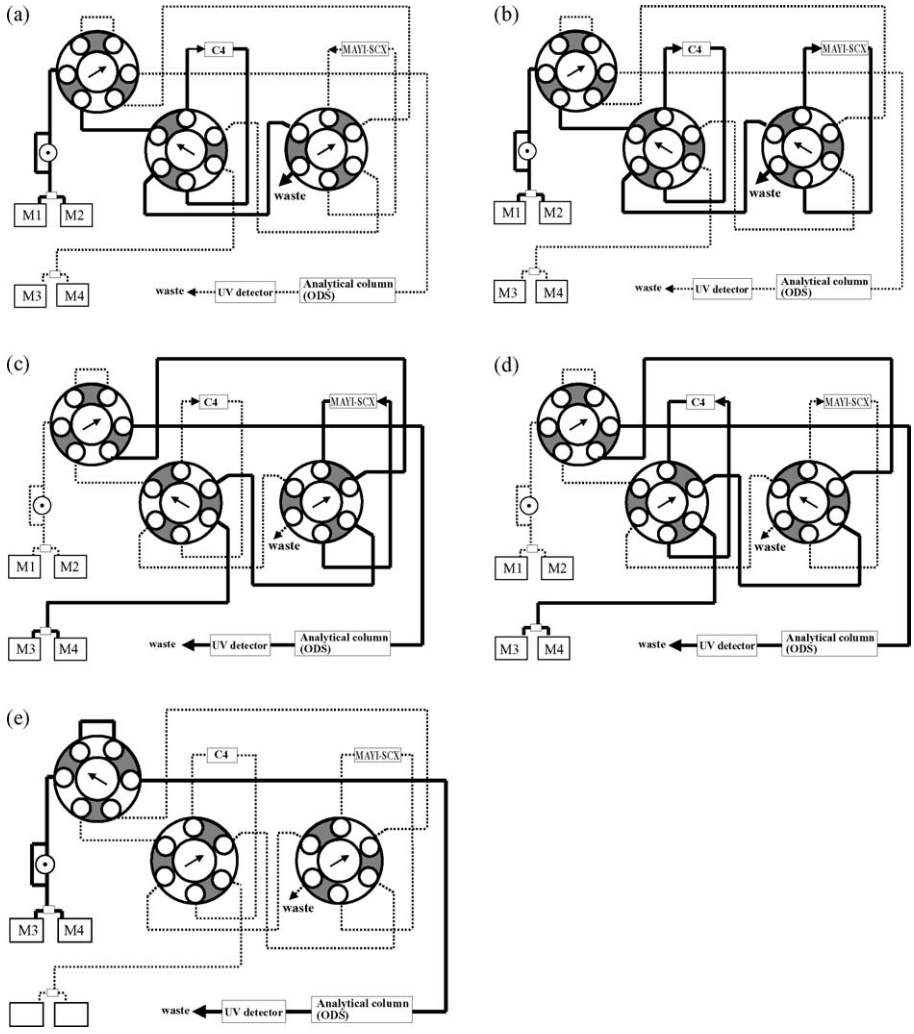


Fig. 3. Column-switching procedure of the LC–LC–UV system. (a) Trapping on the C4 pre-treatment column and elimination of the hydrophilic components. (b) Transfer to the MAYI-SCX column from the C4 pre-treatment column and trapping on the MAYI-SCX column using electrostatic interactions. (c) Transfer to the analytical ODS column from the MAYI-SCX column, elution and separation through UV analysis. (d) Analysis of the C4 column by connection of the C4 and analytical ODS columns. (e) Direct ODS analysis without the pre-treatment process.

the MAYI-SCX column to the analytical column (Fig. 3c). During the analysis, the pre-treatment C4 column was washed by a gradient that reached 100% acetonitrile in the mobile phase M2 for 3 min (Fig. 4d). Between 18 and 55 min, 8-hydroxyquinoline and its glucuronide were transferred in the backflush to the analytical column and the chromatographic separation was performed (Fig. 4e). A chromatogram was obtained at UV 254 nm on CR-4A. The valve then switched automatically (Fig. 4f) to connect the pre-treatment C4 column to the MAYI-SCX column (Fig. 3b).

2.7. Influence of pH and ionic strength in the mobile phase M1 in extraction LC

The influence of pH and ionic strength in the mobile phase M1 in extraction LC was investigated with respect to the recoveries of test compounds using an aqueous solution of acetic acid and an acetate (Na) buffer solution. The recovery of test compounds from spiked bile samples was investigated at a pH range of 3.3–4.1 with an ionic strength of 17.3×10^{-3} and at pH 3.5 with a range of ionic strengths from 8.7×10^{-3} to 120×10^{-3}. As predicted, increased ionic strength and pH in the mobile phase M1 resulted in decreased recovery of the test compounds. A 0.05% acetic acid aqueous solution (pH

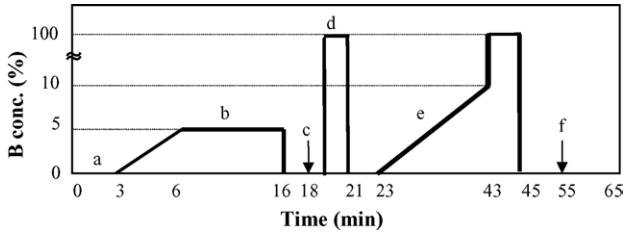


Fig. 4. Time programmes of the gradient curve. (a) Trapping on the C4 column. (b) Trapping on the MAYI-SCX column. (c) Changing the valve. (d) Washing the C4 column. (e) Analysis. (f) Changing the valve.

Table 1
The recovery of 8-hydroxyquinoline and its glucuronide from a spiked bile sample using several pre-treatment columns

Pre-treatment column	8-Hydroxyquinoline glucuronide		8-Hydroxyquinoline	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
MAYI-SCX	7.6	2.6	93.4	0.4
C4 + MAYI-SCX	101.0	0.3	103.8	1.2
C8 + MAYI-SCX	86.0	0.3	41.8	1.4
C18 + MAYI-SCX	83.8	0.8	5.7	3.3

Recovery (%) for an average of three determinations. CV (%) for peak area ($n = 3$). The concentration of each compound was 1 μg/mL. The injection volume of each sample was 100 μL.

3.5; ionic strength = 8.7×10^{-3}) was therefore used for the mobile phase M1 in extraction LC.

2.8. Evaluation of the pre-treatment column in extraction LC

The effect of the hydrophobicity of the reversed-phase column (C4, C8, C18: 4.6 mm × 35 mm i.d.; 5 μm) (GL Sciences) on the recoveries of 8-hydroxyquinoline and its glucuronide from a spiked bile sample was evaluated using a 0.05% acetic acid aqueous solution for the mobile phase M1. Table 1 shows that the C4 column gave the highest recovery for the two compounds; the higher the hydrophobicity of the pre-treatment column, the greater the amount of organic solvent required to transfer the test compounds from the pre-treatment column to the MAYI-SCX column. However, increasing the amount of organic solvent reduced the electrostatic interactions between the two compounds and the MAYI-SCX column in extraction LC. Thus, a C4 column with a low hydrophobicity was used as the pre-treatment column. The use of a tandem pre-treatment column, on which the sample was desalted and purified before elution onto the analytical column, provided a simple and effective means of sample introduction, and allowed reliable quantitation without the loss of target compounds.

Next, the effect of the C4 column was evaluated on the recovery of test compounds from various co-ion (sodium ion) concentrations in the sample solution. Fig. 5 shows a comparison of the recovery of test compounds at different co-ion concentrations (0–1.0 mol/L) in the sample solutions using the MAYI-SCX column with or without the C4 column. As predicted, the test compound recoveries were significantly reduced when the sample solution was injected directly into the MAYI-SCX column in extraction LC. However, by connecting the MAYI-SCX column to the C4 column, the test-compound recoveries were raised to levels of around 100%. This suggested that the influence of the ionic strength of the sample solution on the electrostatic interactions of the MAYI-SCX column was minimised by trapping the test compounds on the pre-treatment C4 column and by desalting the samples before elution onto the MAYI-SCX column.

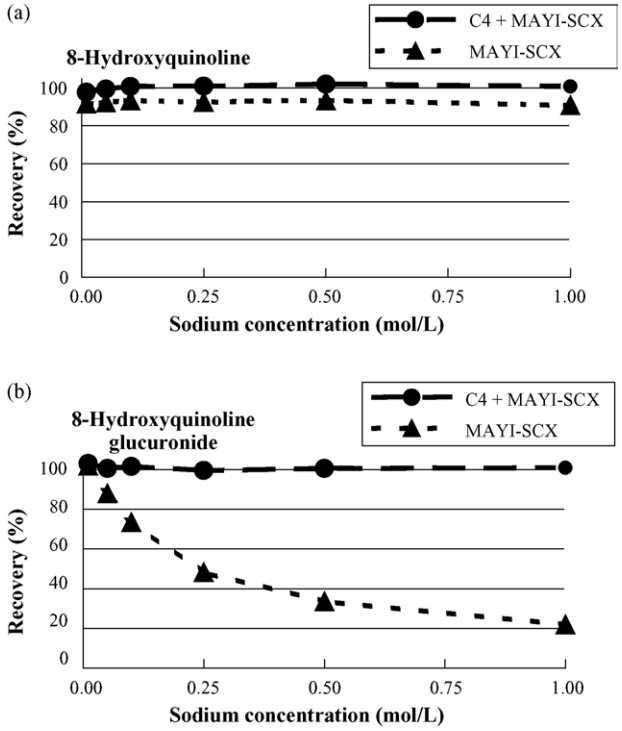


Fig. 5. Comparison of the recoveries of 8-hydroxyquinoline and its glucuronide at different co-ion (sodium ion) concentrations in the sample solution using the MAYI-SCX column with or without the C4 column.

2.9. Chromatographic conditions for the analysis of propranolol

The protocol for the analysis of propranolol was similar to that used for 8-hydroxyquinoline and its glucuronide. The mobile phases M1 and M2 comprised 0.05% acetic acid aqueous solution and 0.1% acetic acid in acetonitrile, respectively. Each phase was pumped at a flow rate of 1 mL/min for the on-line pre-treatment. The mobile phases M3 and M4 comprised 0.05% TFA in water and 0.05% TFA in acetonitrile, respectively. Each phase was pumped at a flow rate of 0.4 mL/min for the analysis. An analytical ODS column (2.0 mm × 150 mm i.d.; 3 μm) (Imtakt, Japan) was used and the column-switching system was operated according to the time programme shown in Fig. 6. Initially, the injected sample passed through the pre-treatment C4 column connected to the

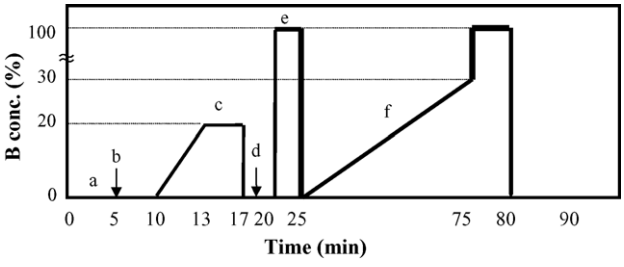


Fig. 6. Time programmes of the gradient curve. (a) Trapping on the C4 column. (b) Changing the valve. (c) Trapping on the MAYI-SCX column. (d) Changing the valve. (e) Washing the C4 column. (f) Analysis.

waste line (Fig. 3a). From 0 to 5 min after the sample injection, most of the hydrophilic salts were eliminated from the bile (Fig. 6a). Propranolol was retained in the pre-treatment column. The valve then switched automatically (Fig. 6b) to connect the pre-treatment C4 column to the MAYI-SCX column (Fig. 3b). Between 5 and 20 min, propranolol was eluted onto the MAYI-SCX column (Fig. 6c). The valve then switched automatically (Fig. 6d) to connect the MAYI-SCX to the analytical column (Fig. 3c). Concurrently, the pre-treatment C4 column was washed by a gradient that reached 100% acetonitrile in the mobile phase M2 for 5 min (Fig. 6e). Between 25 and 90 min, the propranolol was transferred to backflush the analyte from the MAYI-SCX column into the analytical column, and the chromatographic separation was performed (Fig. 6f). A chromatogram was obtained at UV 288 nm using LC/MS Solution software version 2.05.

3. Results and discussion

3.1. LC–LC–UV analysis of 8-hydroxyquinoline and its glucuronide in bile

The majority of new candidate drugs currently developed are cationic compounds and the major issue of drug analysis in bile by using reversed-phase column is the polarity of metabolites. Hence, 8-hydroxyquinoline and its glucuronide were used as test compounds of the target cationic compound and polar metabolite, respectively.

The typical LC–LC–UV chromatograms obtained for 8-hydroxyquinoline and its glucuronide in standard solution, spiked bile and blank bile samples are shown in Fig. 7.

Sufficient separation of the 8-hydroxyquinoline and its glucuronide was observed in the spiked bile samples. The

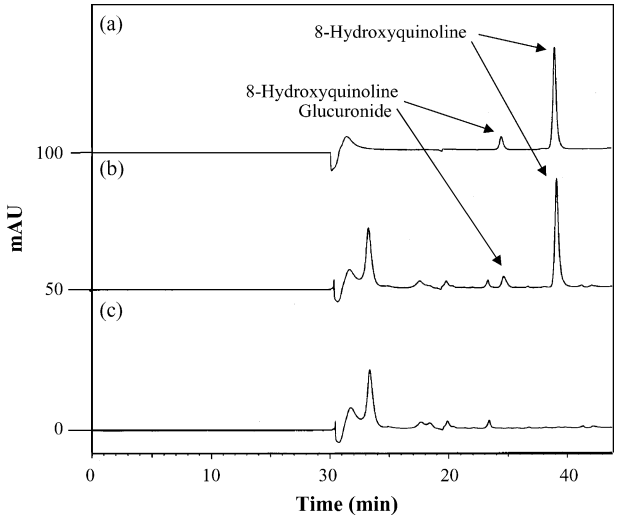


Fig. 7. LC–LC–UV chromatograms of 8-hydroxyquinoline and its glucuronide. (a) Standard solution. (b) Spiked bile sample. (c) Blank bile sample. The concentration of each compound was 1 μg/mL. The injection volume of each sample was 100 μL.

Table 2
The recovery of 8-hydroxyquinoline and its glucuronide from a spiked bile sample

Concentration (μg/mL)	8-Hydroxyquinoline glucuronide		8-Hydroxyquinoline	
	Recovery (%, mean)	CV (%)	Recovery (%, mean)	CV (%)
0.3	105.2	4.9	91.2	0.8
0.5	101.4	1.8	95.8	0.3
1.0	107.4	0.6	96.5	0.2
3.0	101.6	0.5	99.1	0.1
5.0	101.9	0.3	102.3	0.1
10.0	101.2	0.5	99.9	0.1

Recovery (%) for an average of three determinations. CV (%) for peak area ($n = 3$). The injection volume of each sample was 100 μL.

UV chromatograms revealed similar elution profiles for the compounds; the peak shape and retention time of the compounds were similar to those of the standard solution. No matrix interference was observed in the blank bile samples.

The coefficient of variation (CV) values of the retention time for each compound were between 0.01 and 0.17% ($n = 3$ for each compound), even when the standard solution and the spiked bile samples were injected (concentration: 1 μg/mL; injection volume: 100 μL). A linear relationship was observed for both the standard solution and the spiked bile samples at concentrations ranging from 0.3 to 10 μg/mL. The peak areas of each compound were linearly related to the six different concentrations. Although no internal standard was used, there was a strong correlation between the peak area of 8-hydroxyquinoline (y) and its concentration (x) ($y = 628{,}326x - 14{,}975$, $r = 1.000$ for the standard solution; $y = 624{,}273x - 730.5$, $r = 1.000$ for the spiked bile samples), and between the peak area of 8-hydroxyquinoline glucuronide (y) and its concentration (x) ($y = 99{,}663x + 7379.2$, $r = 1.000$ for the standard solution; $y = 98{,}618x + 4789.1$, $r = 1.000$ for the spiked bile samples). Recoveries from the spiked bile samples were calculated by comparing the peak areas of the standard solutions injected directly onto the analytical column (Fig. 3e). The results indicated a satisfactory recovery of both 8-hydroxyquinoline 91.2–102.3% and its glucuronide 101.2–107.4% and the CV values were 0.1–0.8 and 0.3–4.9%, respectively (Table 2). These findings confirm that the column-switching LC system provides excellent repeatability and reliability. Thus, trace amounts of drugs in bile samples can be successfully identified using the LC system with UV detection.

3.2. LC–LC–MS analysis of propranolol and its metabolites in bile

The sample pre-treatment method and automated on-line system were validated by using 8-hydroxyquinoline and its glucuronide. Then, the automated on-line system was also applied to the measurement of propranolol and its metabolites in bile. Propranolol was selected as a test compound of in vivo

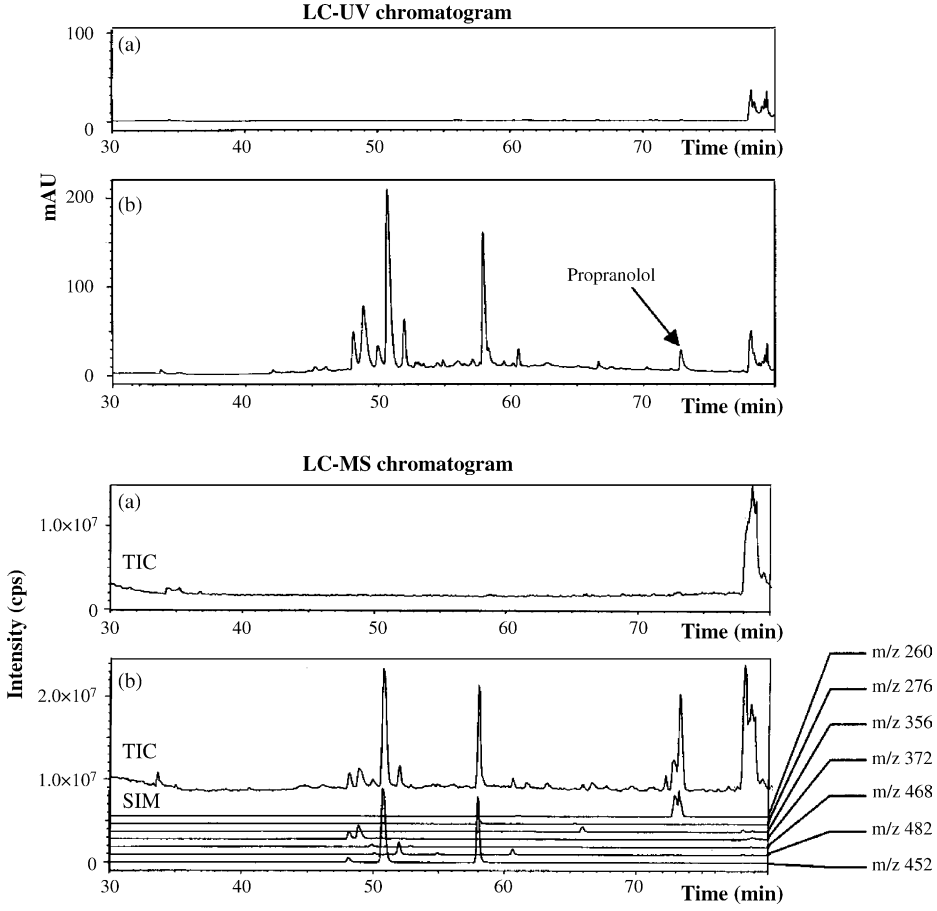


Fig. 8. LC–LC–UV and LC–LC–MS chromatograms of a blank bile sample (a) and a bile sample collected 0–0.5 h after the intravenous administration of propranolol (2.5 mg/kg) to the rat (b). The injection volume of each sample was 100 μL.

sample because its hepatic elimination and metabolism are well established [14].

Fig. 8 shows the LC–LC–UV and LC–LC–MS chromatograms obtained for the blank bile samples and the bile samples. A linear relationship was observed for both the standard solution and the spiked bile samples at concentrations ranging from 0.05 to 10 μg/mL. The peak area of propranolol was linearly related to the six different concentrations. Although no internal standard was used, there was a strong correlation between the peak area of propranolol (y) and its concentrations (x) ($y = 319{,}990x - 13{,}215$, $r = 1.000$ for the standard solution; $y = 329{,}258x - 20{,}462$, $r = 1.000$ for the spiked bile samples). These results indicated a satisfactory recovery of propranolol 93.1–109.4% and the CV values were 0.1–5.9% (Table 3). Recoveries from the spiked bile samples were calculated by comparing the peak areas of the standard solutions injected directly into the analytical column (Fig. 3e). After eluting propranolol and its metabolites to the MAYI-SCX column from the C4 column (Fig. 3b), the latter was analysed by connecting it directly to the analytical ODS column (Fig. 3d); no peaks for propranolol or its metabolites were observed on the C4 column. The UV and MS chromatograms revealed that propranolol and its metabolites were clearly identified, and no matrix interference was observed for the bile samples. The scan mode detected both propranolol (260 m/z) and its metabolites (corresponding to 276, 356, 372, 452, 468 and 482 m/z). These results are consistent with the four major propranolol metabolites: propranolol glucuronide, naphthoxylactic acid, and the glucuronic acid and sulphate conjugates of 4′-hydroxypropranolol [14].

3.3. Pharmacokinetics of propranolol

The technique developed in this study was also used to measure the concentration of propranolol in bile following the intravenous administration of 2.5 mg/kg to rats. Fig. 9 shows

Table 3
The recovery of propranolol from a spiked bile sample

Concentration (μg/mL)	Recovery (%, mean)	CV (%)
0.05	93.1	5.9
0.1	109.4	3.4
0.5	99.4	0.7
1.0	102.2	0.4
5.0	98.5	0.6
10.0	97.2	0.1

Recovery (%) for an average of three determinations. CV (%) for peak area ($n = 3$). The injection volume of each sample was 100 μL.

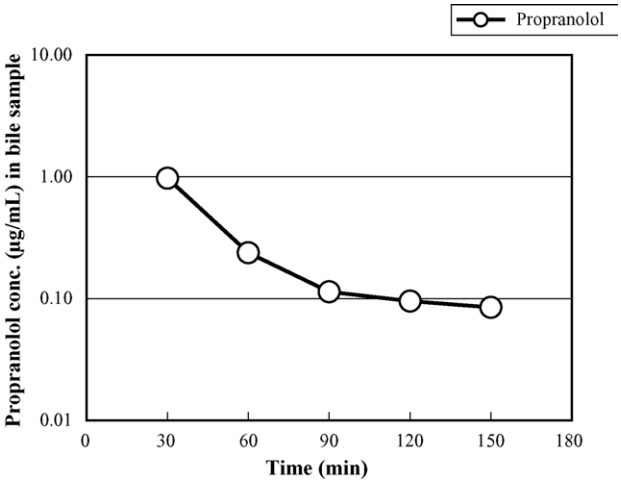


Fig. 9. Time-course of changes in the bile propranolol concentrations after the intravenous administration of 2.5 mg/kg to two rats. Bile samples were collected over a 0.5 h period. Each value represents the mean of two experiments.

representative bile-concentration profiles obtained during toxicokinetic analyses of rat bile. The concentration of propranolol was calculated by comparing the peak areas of the standard solutions injected directly into the analytical column. The results confirm that this method is suitable for characterizing pharmacokinetic parameters in pre-clinical studies. These findings also suggest that column-switching LC–LC–MS analysis might be successfully applied to therapeutic drug monitoring.

By the way, the endurance of this MAYI-SCX pre-treatment column, a reduction in chromatographic performance in the LC system were not apparent in this study, which involved 100 injections of 100 μL bile samples over a 1-week period.

4. Conclusions

This report describes an effective on-line purification method for cationic compounds in rat bile using column-switching LC. C4 and MAYI-SCX columns were utilised for pre-treatment, in order to eliminate endogenous components from the rat bile. The use of a tandem pre-treatment column, on which the sample was cleaned before elution onto the analytical column, provided a simple and useful means of sample introduction, resulting in effective quantitation without the loss of target compounds. Structural estimation of the target metabolite was successfully achieved using mass spectrometry with this system; structural estimation using NMR is ongoing. In addition, methylcellulose-immobilised materials with anion-exchangeable functions are currently being investigated for use in the analysis of acidic compounds in rat bile.

Acknowledgements

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