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Analysis of ibuprofen metabolites by semi-microcolumn liquid chromatography with ultraviolet absorption and pulsed amperometric detectors[1]

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Abstract

Semi-microcolumn (1.5 mm i.d.) liquid chromatography (LC) system with ultraviolet (UV) and pulsed amperometric detector (PAD) was constructed for analysis of ibuprofen metabolites in human urine. PAD was connected in series with the UV detector, and an alkaline solution was post-column added to a mobile phase after the UV detector. By a gradient elution, five ibuprofen metabolites were detected with UV detection from 1 μl of human urine at 3.5 h after the administration, and information concerning their glucuronation was simultaneously obtained by PAD response. © 1997 Elsevier Science B.V.

Keywords: Semi-microcolumn liquid chromatography; Pulsed amperometric detector; Ibuprofen

1. Introduction

In the early stage of the drug development process, it is important to determine the structure and amount of metabolites of drug candidates in order to find out their physiological effect and toxicity. Among the common metabolic conversions is glucuronic acid conjugation. Glucuronic acid conjugation may occur not only on intact drug molecules but also on their metabolites, which, with the coexistence of unglucuronated molecules, usually contributes to the generation of a complicated metabolic pathway. A method to rapidly distinguish glucuronated metabolites in biological liquids—such as urine and bile—must be useful in clinical pharmacokinetic studies.

Carbohydrates, including glucuronic acid, have been considered difficult to handle in analytical chemistry, lacking a useful chromophore for common optical detectors. Pulsed amperometric detector (PAD) was introduced as a sensitive detector for carbohydrates by Johnson and coworkers [1–3], which does not require a chemical derivatization. Although, a sensitivity obtained with PAD may not be as impressive as those based on fluorescent derivatization [4,5], PAD shows a sensitivity of nearly two orders of

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magnitude higher than that obtained with a conventional differential refractometer, the only alternative for un-derivatized sugars [4,5]. One complexity in PAD, however, is that carbohydrates should be placed in a strong alkaline condition to let their hydroxyl groups dissociate and become electrochemically active, which has been a limiting factor in the choice of mobile phase and a mode of chromatographic separation.

Semi-microcolumn liquid chromatography (LC) has been accepted as a sensitive analytical tool for biologically important substances because of its concentrating effect of narrow bore columns (typically, 1.0–1.5 mm i.d.) [6–8]. Low flow rates employed in semi-microcolumn LC (typically, 0.05–0.1 ml min^{-1}) seem convenient for its application to carbohydrates and related substances with PAD, because a strong alkaline condition can be easily attained by post-column adding a moderate flow rate of an alkaline solution to a mobile phase used in separation.

In this paper, a construction of a semi-microcolumn LC system with PAD for monitoring substances having a carbohydrate moiety in their structure, and its application to ibuprofen metabolites will be discussed.

2. Experimental

2.1. Reagent

Ibuprofen was purchased from Kaken Pharmaceutical (Tokyo, Japan).

2.2. Dosing and sample collection

A normal, healthy, male subject (age 27) was dosed orally with a single (therapeutic) dose of ibuprofen (2-(4-isopropylphenyl)-propionic acid, 400 mg). Urine samples were collected into plastic containers and stored frozen until analysis.

2.3. HPLC conditions

HPLC was performed on Capcell Pak UG C18 (1.5 mm i.d. × 250 mm, Shiseido, Tokyo, Japan). An HPLC system was constructed with components of NANOSPACE series (Shiseido, Tokyo, Japan). Two Inert Pumps 2001, a column Oven 2004, an Auto Sampler 2003, a UV-VIS Detector 2005, Pulsed Amperometoric Detector 2016 and a Degassing Unit (Shiseido, Tokyo, Japan) were arranged as shown in Fig. 1. All of these units use polyetheretherketone (PEEK)-made parts to eliminate a contact between a mobile phase and metal parts normally used in other HPLC instruments. The system was operated by SYSCON (Shiseido, Tokyo, Japan), a system controller based on a personal computer with Windows 95™ (Microsoft, USA). Signal from the UV detector and PAD was recorded with S-MicroChrom 4.1 (Shiseido), an integrator that operates in parallel with SYSCON in the same computer. PEEK tubings (0.13 mm i.d., Shiseido, Tokyo, Japan) were used for all the connections among the HPLC components. (Windows 95: Trademark of Microsoft, USA).

2.4. Gradient elution

Gradient elution was performed using (A) 0.05 M potassium dihydrogen phosophate (pH = 2.45) in water (purified with a Milli-Q system, Nihon Millipore Kogyo, Tokyo, Japan) and (B) acetonitrile (HPLC grade from Nakalai Tesque, Tokyo, Japan). A liner gradient elution from 2–60% acetonitrile was employed over 70 min at 40°C.

2.5. Mass spectrometry (MS)

LC-MS was performed by connecting the outlet of the UV detector to an inlet of Model M-1000 LC API (atmospheric pressure ionization) (Hitachi, Japan). The entire eluent was introduced to the mass spectrometer.

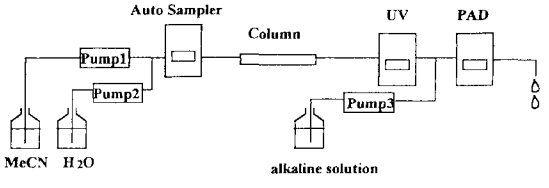

Fig. 1. UV-PAD 2-channel analytical system.

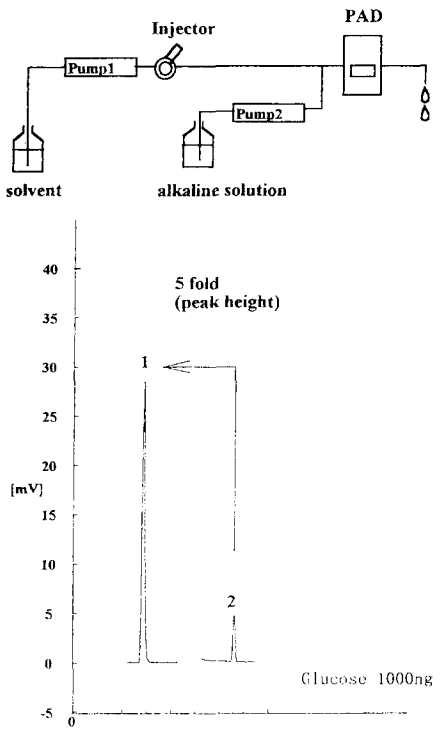

Fig. 2. Flow rate dependence of PAD response measured with flow-injection arrangement (1) semi-microcolumn, 1.5 mm i.d. (flow rate: 0.1 ml min^{-1}), (2) conventional column, 4.6 mm i.d. (flow rate: 1.0 ml min^{-1}).

3. Results

Before the analysis of the glucuronic acid conjugates, the sensitivity of PAD was measured with a simplified flow-injection arrangement (Fig. 2) with a glucose solution as a standard sample. Two different flow rates (0.1 and 1.0 ml min^{-1}) were compared using 1.5 and 4.6 mm i.d. columns respectively. The response for glucose increased as a flow rate of carrier solvent decreased. Approximately, a 5-fold increase was observed at 0.1 ml min^{-1}, in comparison with 1 ml min^{-1}.

The influence of carrier solvent type on PAD response was also examined with the same flow injection system. A carrier flow rate was set to 0.1 ml min^{-1}. Glucose peaks were compared using three different carrier solvents, water, phosphate buffer, and organic-containing solution. The phosphate buffer (pH = 2) at the low flow rate could easily be converted to an alkaline condition after being mixed with the alkaline solvent, to provide the same glucose response as that obtained with water as a carrier solvent. When a carrier solvent was altered to 70% acetonitrile, the response decreased to approximately 30% that obtained with water.

The metabolic pathway of ibuprofen has already been reported and is shown in Fig. 3 [9,10]. Urine at 3.5 h after the administration (ibuprofen 400 mg) was analyzed with the system already described (Fig. 1). Results are shown in Fig. 4. The UV detector adjusted at 210 nm is expected to respond to the conjugated double bonds of ibuprofen and related compounds, and PAD is expected to respond to a glucuronic acid moiety of metabolites.

The upper chromatogram in Fig. 4 was obtained at the UV detector (Channel 1), where five metabolites sharing a para-substituted benzene structure are detected. The middle chromatogram was obtained at PAD (Channel 2). Glucuronated metabolites are successfully detected at both chromatograms, while non-glucuronated ones appear only in the upper chromatogram. The lower chromatogram was obtained in a separate experiment, where an ibuprofen solution was injected. These results show the absence of intact ibuprofen after the metabolic pathway, which agrees with the previous reports.

Peak assignments of the five metabolites shown in Fig. 4 were carried out with the aid of previous reports [10], and directly by an LC/MS measurement with an API interface. Mass spectra of the five metabolites are shown in Fig. 5. Psudo-molecular ions (negative ions) corresponding to the five structures were successfully observed.

Reproducibility of results with the system was examined through repetitive injections of the urine sample. Relative standard deviations of retention time, peak height at UV, and peak height at PAD (mean of the five metabolites) were 0.8, 3.1 and 3.2% ($n = 10$), respectively.

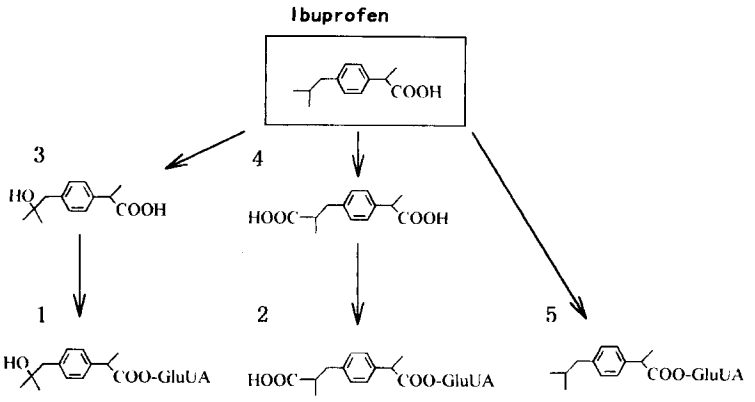

Fig. 3. Metabolic pathway of Ibuprofen. (1) HMPPA glucuronide, (2) CMPPP glucuronide, (3) HMPPA, (4) CMPPP, (5) Ibuprofen glucuronide, HMPPA: 2-(4-(2-hydroxy-2-methylpropyl)phenyl)propionic acid; CMPPP: 2-(4-(2-carboxy-2-methylpropyl)phenyl)propionic acid; GluUA: glucuronic acid.

4. Discussion

It is known that only a limited portion of analytes can diffuse into a working electrode surface in an electrochemical cell during a chromatographic elution. The enhanced response at a low flow rate (Fig. 2) can be explained by an increased reaction yield of glucose because a lower flow rate allows glucose molecules to stay in a flow cell for a longer time.

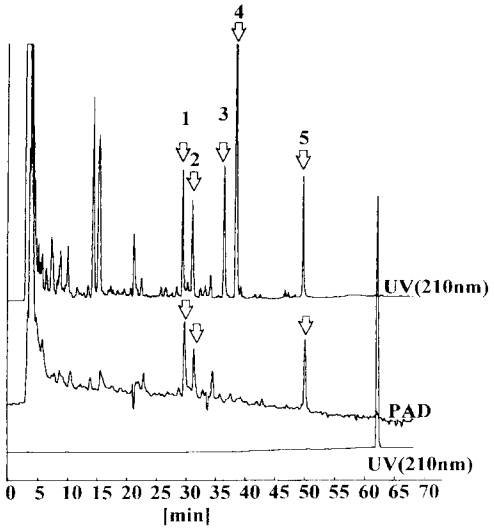

Fig. 4. Chromatograms of Ibuprofen metabolites and Ibuprofen standard HPLC conditions, Column: Capcell Pak C18 UG120 (1.5 mm i.d. × 250 mm), Mobile phase: 50 mM Potassium dihydrogen phosphate pH 2.45 $MeCN^{-1}$ Gradient elution, Reagent: 0.6 N NaOH, Column temp: 40°C, Detectors: UV detection at 210 nm, PAD potentials (measuring pulse at 0.150 mV, cleaning pulses at 0.650 and −0.950 mV).

Another advantage of low flow rates in semi-microcolumn LC seems to be an extended mobile phase choice. Although a certain decrease from the best response might be expected for different mobile phases, it seems that various solvents can be used with PAD in the same format (post-column addition of alkaline solution), as long as the mobile-phase flow rate is kept at a low level. The same strategy in conventional LC is expected to end up with a high total flow rate after mixing with an alkaline solution, which will be disadvantageous in sensitivity as shown in Fig. 2.

The semi-microcolumn LC system presented here seems unique in that a carbohydrate moiety, which has been considered difficult to detect, is utilized for the selective detection. While mass spectrometry provides exact information concerning mass number, the information about presence of carbohydrate moiety, which is derived by 'chemistry', might be useful when a decisive fragmentation is not available.

While ibuprofen metabolites were chosen as model compounds to discuss the analytical methodology, applications to real drug candidates will need to involve validation processes, such as

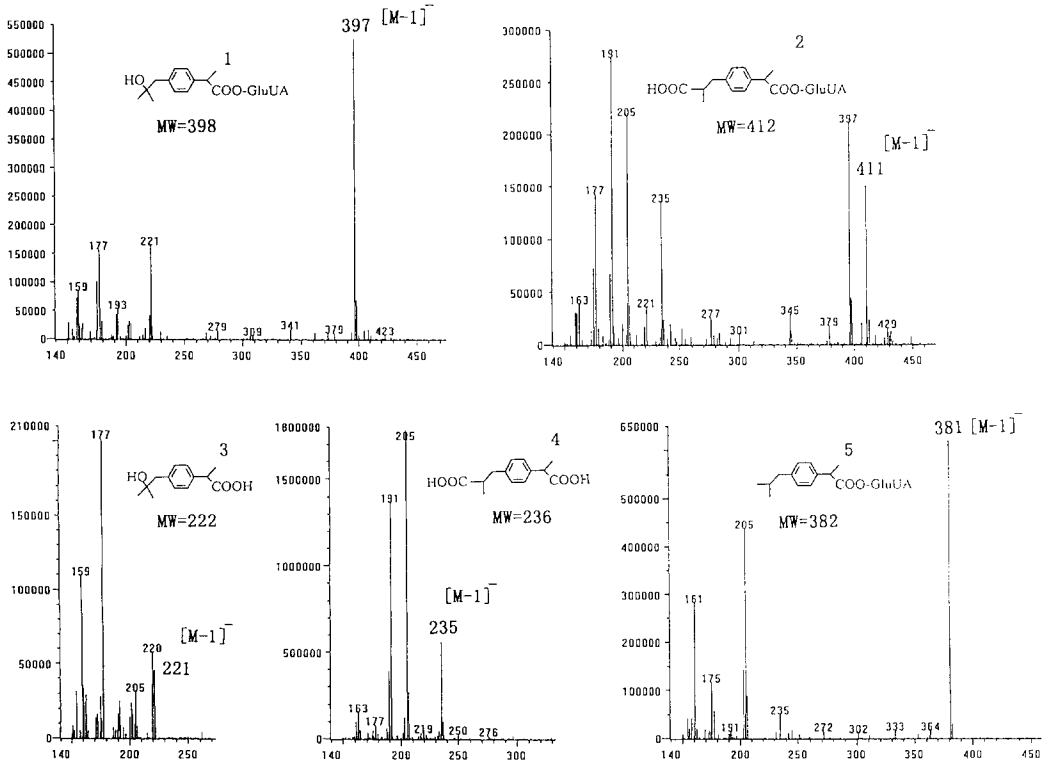

Fig. 5. Mass spectra of the five Ibuprofen metabolites structure and peak number correspond to those shown Fig. 3.

tests of linearity, detection limits, accuracy, and ruggedness with the use of synthesized standards for corresponding metabolites. The detection limits of singly-glucoronated metabolites on PAD are roughly estimated as 60 μg ml^{-1} (injection volume: 1 μl, S/N = 3) which was calculated by using peak area at UV detection peak height and noise level at PAD, under the assumption that all the metabolites share the same extinction coefficient at 210 nm as that of intact ibprophen, the only available standard.

Besides the analysis of glucoronic acid conjugates, the system seems able to be applied to various biological mixtures related to carbohydrates, such as peptides derived from glycoproteins and plant extracts containing various glycosylated components. Optimization of the system for different purposes will be attempted in the future.

5. Conclusion

The semi-microcolumn LC system with UV detector and PAD could provide information concerning glucuronic acid conjugation in the analysis of ibuprofen metabolites in human urine. Low flow rates of semi-microcolumn LC were suitable in its combination with PAD, a selective detection technique for carbohydrates.

Acknowledgements

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