High-Performance Liquid Chromatographic Assay of 3′-Phosphoadenosine 5′-Phosphosulfate (PAPS) and UDP-Glucuronic Acid (UDPGA) in Cultured Hepatic Cell Extracts

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3′-Phosphoadenosine 5′-phosphosulfate (PAPS) and UDP-glucuronic acid (UDPGA) in cultured rat hepatocytes were directly assayed by isocratic reverse-phase HPLC. The sample preparation involves the extraction of nucleotide cofactors with cold alkaline solvent and clearing treatment by ultra-filtration. Nucleotides were separated on HPLC equipped with a RPaqueous C-30 column adjusted to 20°C using 100 mM sodium potassium buffer (pH 5.5) as elution solvent and detected by UV absorption at 260 nm. Linear calibration curves were obtained over the ranges from 0.1 to 1.0 μM for PAPS and 1.0 to 10 μM for UDPGA in both distilled water and hepatic cell extracts. Peaks in the cell extracts were identified as PAPS and UDPGA by comparison of the retention times and co-elution with each of their corresponding authentic standards. Assignment of peak identity was additionally supported by the preferential decrease in PAPS and UDPGA in cultured hepatocytes which were incubated with quercetin or D-galactosamine. This HPLC method was found to be sensitive enough to accurately quantify the cellular contents of PAPS and UDPGA far below that normally found in cultured rat hepatocytes.

Key words — flavonoids, 3′-phosphoadenosine 5′-phosphosulfate, UDP-glucuronic acid, HPLC

INTRODUCTION

Phenolic compounds are converted into their more water-soluble metabolites in phase II bioconjugation pathways prior to excretion in vertebrates. The major classes of the conjugation reaction of phenolic compounds are sulfation and glucuronidation using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and UDP-glucuronic acid (UDPGA), respectively, as endogeneous activated reaction cofactors. It has been reported that the sulfate conjugation pathway of phenolic compounds is limited probably due to the poor capacity of PAPS in cells.1–3) Flavonoids, which occur naturally in plant foods and are inevitably ingested by humans through our diet,4,5) are polyphenolic compounds and thereby primarily metabolized by sulfo- and glucurono-conjugation.6–8) Therefore, the intake of large amounts of flavonoids may cause the metabolic consumption of PAPS in the intestine and liver cells, which might affect the biotransformation of phenolic compounds.

Currently, the most widely used methods for PAPS determination rely upon enzymatic assays which measure the formation of sulfo-conjugated substrate from radioisotope-labeled or fluorescent phenolic acceptors and PAPS present in tissue extracts through a sulfotransferase-catalyzed reaction.9–11) Such procedures are time consuming, especially for routine use in conducting kinetic studies of PAPS metabolism. UDPGA concentrations in tissue extracts have also been measured by enzymatic assay,12) although there are only a few reports that employed ion-pair HPLC methods.13,14) In this paper, a simple and sensitive method for the direct determination of low levels of PAPS in hepatic cell extracts by HPLC with simultaneous measurement of UDPGA was developed. This approach could easily be applied to measure the decrease in PAPS and UDPGA contents in cultured rat hepatocytes due to the addition of quercetin or D-galactosamine.

MATERIALS AND METHODS

Materials —— Materials and Chemical reagents were purchased from the following companies: Ultrafree-MC LTK UFC3 filter unit from Millipore Co. (Bedford, MA. U.S.A.); Develosil RPAQUEOUS C-30-UG-3 column (2.0 i.d. × 150 mm) from Nomura Chemical Co. (Aichi, Japan); PAPS and UDPGA from Sigma-Aldrich Co. (St.
and centrifuged at 10,000 rpm. The cell extracts of nucleotides were transferred to ultra-filtration units (Ultrafree-MC LTK Module and 2487 dual λ UV/VIS detector employing an 2.6 µl flow cell (Waters Co. Milford, MA. U.S.A.). A 10 µl aliquot of sample was injected onto a 3 µm Develosil RPAQUEOUS C-30 semimicro column adjusted to 20°C and eluted with 100 mM sodium potassium buffer (pH 5.5). The flow rate of the mobile phase was 0.2 ml/min and elution of nucleotide cofactors was detected by UV absorption at 260 nm. The time between runs was 60 min. The quantitation of PAPS and UDPGA was based upon integration of the peak areas between the start and end of peak.

RESULTS AND DISCUSSION

A long retention of PAPS was achieved on the C-30 column, which led to a satisfactory separation of PAPS from other nucleotide interferences in the hepatic cell extracts. The separation of PAPS was also greatly aided by decreasing the column temperature to 20°C. The pH of the mobile phase phosphate buffer was optimized to 5.5 to obtain a long retention of PAPS in the column. Lower phosphate buffer concentrations than 0.1 M resulted in the peak spreading and loss of PAPS resolution. This procedure also allowed the simultaneous determination of UDPGA. Typical chromatograms for a standard mixture of PAPS and UDPGA and for a cell extract under the above chromatographic conditions are shown in Fig. 1. Authentic UDPGA and PAPS were eluted with retention times of 3.2 and 8.9 min, respectively. PAPS and UDPGA in the cell extracts were separated with the same retention times as their corresponding authentic standards from other cellular components (Fig. 1). ATP, adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) were eluted at 15.4, 19.8 and 51.4 min, respectively, under this HPLC analytical condition. The peaks for PAPS and UDPGA were co-eluted with their corresponding authentic standards which were added to the cell extracts (Fig. 2). When cultured hepatocytes were incubated with 10µM quercetin for 15 min, the peaks for PAPS and UDPGA were preferentially decreased (Fig. 3A). There are a few reports that metabolism of D-galactosamine in rat hepatocytes causes a marked decrease in cellular UDPGA content due to the trapping of uridine phosphates by formation of UDP-galactosamine.16,17) The peak for UDPGA disappeared within 60 min after an addition of 3 mM D-galactosamine to the cultured hepatocytes while the peak for PAPS remained constant during the incubation period (Fig. 3B).

The calibrations of PAPS and UDPGA by peak integration were prepared both in pure water and cell extracts with concentration ranges from 0.1 to

Louis, MO. U.S.A.); Quercetin from Funakoshi Co. (Tokyo, Japan); D-galactosamine hydrochloride and other chemicals used from Wako Pure Chemical Co. (Osaka, Japan).

Cell Culture —— Hepatocytes were isolated by the collagenase perfusion method from male Wistar rats weighing 180–220 g provided with standard rat chow and water ad libitum.15) These cells were re-suspended in Eagle’s MEM containing dexamethasone (1 µM), insulin (0.1 µM), tri-iodothyronine (1 µg/ml), β-aminolevulinic acid hydrochloride (0.2 mM) and 10% fetal calf serum. The cells were seeded in 35 mm plastic culture dishes at a density of 1 × 10⁶ cells/dish, and placed in an incubator in an atmosphere of 5% CO₂-95% air at 37°C. The monolayers of hepatocytes were cultured for 24 hr in Eagle’s MEM containing dexamethasone, insulin, tri-iodothyronine, β-aminolevulinic acid hydrochloride and 10% calf serum. Quercetin was dissolved in dimethyl sulfoxide and added to the cultures at a concentration of 10 µM with the final concentration of dimethyl sulfoxide of 1%. D-galactosamine dissolved in saline was added to the cultures at a concentration of 3 mM.

Sample Preparation —— The monolayers of hepatocytes cultured in dishes were washed with cold phosphate-buffered saline and exposed to 0.45 ml of cold 0.05 M potassium hydroxide. The dishes were placed on ice for 1 min and neutralized with 50 µl of 1.0 M phosphate buffer, pH 6.0. A time of 1 min was considered sufficient for the extraction of endogenous PAPS and UDPGA since longer extraction times produced no increase in the cofactor concentrations. The recoveries of external PAPS and UDPGA added to the pure alkaline extraction solvent were more than 97% within 1 min before neutralization. The cell extract of nucleotides was transferred to ultra-filtration units (Ultrafree-MC LTK UFC3) composed of membrane filters which separate compounds with a molecular weight of 30000 and centrifuged at 10000 x g for 20 min. Samples in the filtrate not analyzed on the day prepared were stored at 4°C and were stable for at least 2 days.

HPLC Analysis —— Separation of nucleotide cofactors was carried out using a Waters Alliance HPLC System equipped with a 2690 Separation Module and 2487 dual λ UV/VIS detector employing an 2.6 µl flow cell (Waters Co. Milford, MA. U.S.A.). A 10 µl aliquot of sample was injected onto a 3 µm Develosil RPAQUEOUS C-30 semimicro column adjusted to 20°C and eluted with 100 mM
1.0 μM for PAPS and from 1.0 to 10 μM for UDPGA, since the concentrations of PAPS and UDPGA in the cell extracts changed within their calibration concentration ranges (Fig. 4). The cell extracts for the calibration of PAPS and UDPGA were obtained from hepatocytes which were treated with 50 μM quercetin for 15 min and from hepatocytes which were treated with 3 mM D-galactosamine for 60 min, respectively. The signal-to-noise ratio was approximately 11 at the concentration of 0.1 μM PAPS in the cell extracts. Recovery was calculated as the ratio of the amount determined in the alkaline cell extracts spiked prior to neutralization to the amount determined in the sample spiked in the final step before HPLC injection. The concentration of PAPS used to spike the sample was 0.5 μM. The within- and day-to-day relative standard deviations were 3.2 and 4.4%, respectively. Calibration curves for PAPS in both pure water and the cell extracts passed through the origin and were linear over the quantified concentration range prepared. Calibration curves of UDPGA in both pure water and the cell extracts were also drawn linearly over the quantified concentration range prepared. The recoveries of UDPGA in the cell extracts were more than 96% with the coefficients of variation less than 4% among all concentrations. The concentrations of PAPS and UDPGA in cultured rat hepatocytes were 0.42 ± 0.05 and 2.2 ± 0.2 nmol/10^6 cells, respectively. These values for PAPS and UDPGA contents in hepato-

Fig. 1. Chromatographic Separation of PAPS and UDPGA in Hepatic Cell Extracts
A) standard mixtures of PAPS (2 μM) and UDPGA (4 μM). B) cell extracts (10^6 cells/0.5 ml extraction solvent). Samples were separated on a 3-μm Develosil RPAQUEOUS C-30 semimicro column. Other operating conditions were as follows: injection volume, 10-μl; mobile phase buffer, 100 mM sodium potassium buffer (pH 5.5); flow rate, 0.2 ml/min; column temperature, 20°C; UV detection, 260 nm.

Fig. 2. Chromatogram of Hepatic Cell Extracts which were Mixed with Authentic PAPS and UDPGA
A) normal cell extracts. B) normal cell extracts (0.5 ml) plus authentic PAPS (400 pmol) and UDPGA (2 nmol).
cytes were comparable to previously reported values assessed by enzymatic assay of liver tissues and isolated hepatocytes, calculated on the basis of 10^6 cells being equal to 10 mg wet weight or 1.6 mg protein.9–14)

Figure 5 shows the time course of the PAPS and UDPGA levels in cultured hepatocytes following an addition of 10 µM quercetin.

Upon the addition of quercetin to the cultured medium, the PAPS content in hepatocytes was almost depleted within 15 min.
The PAPS content began to recover after 30 min but did not recover to the normal level after 180 min. The UDPGA content decreased to as low as about 60% of the initial level at 15 min and returned to the normal level after 60 min. These results suggest that nucleotide cofactors were consumed through the conjugation reaction of quercetin and then biosynthesized in cultured hepatocytes. A few flavonoids including quercetin were examined for their ability to decrease nucleotide cofactors in cultured hepatocytes. The decreasing ability of flavonoids did not depend on the numbers of hydroxyl substitution on flavonoidal nucleus (data not shown). Activities of a number of flavonoids with different hydroxyl substitutions are being tested in our laboratory.

The current method for PAPS quantitation in cell extracts by HPLC offers a simple alternative to enzymatic assays coupled with radioisotope detection.9–11) The direct assay of PAPS by HPLC can be used for the quantitation even in the presence of substances that interfere with enzymatic methods.

The HPLC measurement only requires simple handling of the sample and provides reliable data in the routine assay of a number of samples. The measured PAPS levels in cultured rat hepatocytes by this method corresponded well with the previously reported values obtained by enzymatic assays of liver tissues.9–11)

The second advantage of this method is that UDPGA is simultaneously determined, although the above procedure was optimized for measuring PAPS in the cell extracts. This approach to nucleotide cofactor quantitation could easily be adapted to almost any analytical laboratory.

REFERENCES