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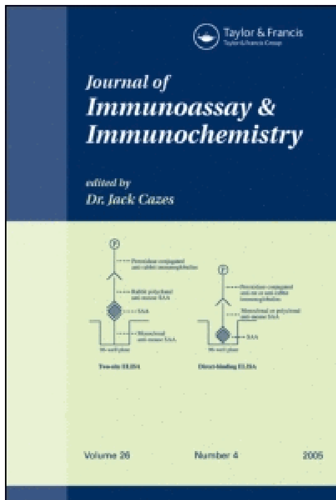

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ON-LINE IMMUNOAFFINITY EXTRACTION FOLLOWED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY FOR A NOVEL RETINOBENZOIC ACID, AM-80, IN HUMAN PLASMA

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ON-LINE IMMUNOAFFINITY EXTRACTION FOLLOWED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY FOR A NOVEL RETINOBENZOIC ACID, AM-80, IN HUMAN PLASMA

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ABSTRACT

On-line coupled immunoaffinity chromatography and reversed-phase high performance liquid chromatography (on-line IAC-HPLC) with detection by radioimmunoassay (RIA) was developed for 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-carbamoyl] benzoic acid (Am-80) in human plasma. A 0.05-mL sample was directly loaded onto an immunoaffinity pre-column packed with immobilized polyclonal antibodies against Am-80. The immunoaffinity extract was then automatically introduced to reversed-phase

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HPLC column for separation by column switching. The immunoreactivity in the HPLC-eluted fraction was measured by competitive RIA.

The within-assay precision and accuracy of the method were 4.0–10.6% and −6.0−+8.3%, respectively, and between-assay precision and accuracy were 6.8–11.9% and −16.0−+9.0%, respectively. The limit of quantitation was 0.5 ng/mL in human plasma. The proposed method yields highly purified analyte in complex matrices, detects it with high sensitivity, not only by radioimmunoassay, but also by other methods, such as mass-spectrometry, and can be used for routine assay of many samples without manual sample extraction and purification.

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INTRODUCTION

Sensitive and labor-saving analytical methods are needed in many fields, but sufficient sensitivity is often difficult to attain when determining trace amounts of analytes in biological matrices or some other complex matrices because of interferences. In order to reduce interfering components, one popular method is the use of small pre-columns packed with, for example, hydrophobic or hydrophilic stationary phases or ion-exchange materials. Immunoaffinity techniques, which are based on antigen-antibody interaction of a highly selective retention mechanism, have recently been used for sample cleanup where samples or extracts from urine, bile, serum, and tissue are applied onto these immunoaffinity systems, with little or no pre-treatment, followed by elution of the analytes from the immunoaffinity column. The eluate can be further separated directly by on-line HPLC or off-line HPLC after fractionation.(1–4)

Immunoaffinity columns have also been utilized for sample preparation prior to gas chromatography-mass spectrometry (GC-MS) (5), capillary electrophoresis (CE),(6) or high-performance liquid chromatography-mass spectrometry (LC-MS).(7–9) Such sensitive detection methods can be used for clinical-related work.

Retinoids, such as vitamin A, play an important role in growth, differentiation, and development of normal tissues.(12) 4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-carbamoyl] benzoic acid (Am-80) is a novel retinobenzoic acid derivative, synthesized by K. Shudo *et al.* in 1988.(13) Am-80 shows a high affinity for retinoid receptors in the nucleus and good absorption through the skin by comparison with the conventional retinoic acids.(14)

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In clinical use, about 10 g of 0.008% ointment of Am-80 is applied. This is predicted to lead to a very low plasma concentration of Am-80, of less than 1 ng/mL. Previously, we reported the determination of Am-80 in human plasma by a combination of RIA and reversed-phase (RP) HPLC.(15,16) But, the method was complicated and involved conventional solid-phase extraction, so-called, off-line SPE-HPLC-RIA. One way to solve these problems would be to use on-line immunoaffinity extraction with a column switching technique. An on-line technique should offer the advantages of direct sample injection without sample pretreatment, avoidance of extraction losses during the evaporation and reconstitution steps, better reproducibility, loss contamination, and avoidance of direct handling of toxic solvents. This paper presents an on-line immunoaffinity sample pre-treatment step, in a method using RIA and an automated HPLC column switching system.

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EXPERIMENTAL

Reagents

Am-80 was obtained from Shionogi Research Laboratories (Osaka, Japan). Bovine serum albumin (BSA), trifluoroacetic acid (TFA), and normal human plasma were purchased from Sigma (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), and COSMO BIO CO., Ltd. (Tokyo, Japan), respectively. Immobilized anti-rabbit second antibody (Immunobead®) and affi-gel protein A MAPS-II kit were purchased from Bio-Rad Laboratories (Richmond, CA).

An activated carrier for high-performance immunoaffinity chromatography, TSKgel Tresyl-5PW, and ChemcoPak Nucleosil 5C18 (4.6 × 150 mm) column were purchased from Tosoh (Tokyo, Japan) and Chemco Scientific (Tokyo, Japan), respectively.

All solvents used were of HPLC grade and other reagents were of analytical grade.

Preparation of Immunoaffinity Pre-column

A 1 mL of anti-Am-80 antiserum (F187) was purified with affi-gel protein A MAPS-II-kit and then the isolated IgG (immunoglobulin G) fraction was concentrated with Centricon 30 (Amicon, Beverly, MA) in 1 M phosphate buffer (pH 8.1). The IgG fraction was coupled to TSKgel Tresyl-5PW (0.5 g) as recommended by the manufacturer. After the purified IgG-coupled TSKgel Tresyl-5PW was packed into a stainless steel HPLC column

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(6.0 × 40 mm I.D.), the column was blocked with 0.1 M Tris-HCl buffer (pH 8.5) and equilibrated with 0.2% (w/v) BSA and 0.02% (w/v) sodium azide in 0.01 M phosphate buffer (pH 7.0). The column was stored at 4°C.

Automated On-Line Extraction and HPLC Preparation Procedure

The on-line IAC-HPLC system is shown schematically in Fig. 1. Human plasma sample was mixed with the same volume of equilibration buffer [0.2% (w/v) BSA and 0.02% (w/v) sodium azide in 0.01 M phosphate buffer (pH 7.0)]. After centrifugation at 10,000 × g for 5 minutes, 0.1 mL of the mixture was loaded into the on-line IAC-HPLC system at a flow rate of 1.0 mL per minute, following prior equilibration with the buffer described above.

After the immunoaffinity column was washed with distilled water for 15 minutes, the Am-80 fraction was eluted with 50% (v/v) acetonitrile and, using a column switching technique, this fraction (12 mL) was introduced into the line of the RP-HPLC column. When the elution was completed and the valve was changed to the immunoaffinity column line, the column was washed successively with 80% (v/v) acetonitrile and distilled water for 15 minutes each, and equilibrated for the next injection.

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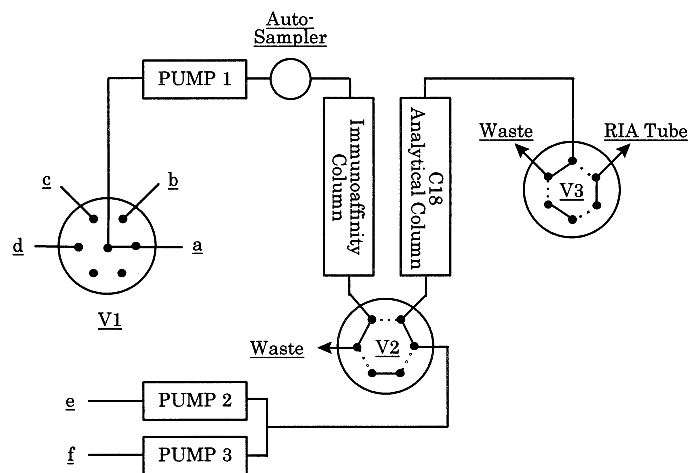

Figure 1. Schematic diagram of the on-line IAC-HPLC system. Valves V1–V3 are shown in their initial positions. Solvents were as follows: a, equilibration buffer; b, water; c, 50% (v/v) acetonitrile; d, 80% (v/v) acetonitrile; e, 0.1% (v/v) TFA; f, 0.1% (v/v) TFA in acetonitrile.

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The immunoaffinity-extracted Am-80 fraction was further purified by RP-HPLC, with gradient elution, using 0.1% (v/v) TFA (mobile phase A) and 0.1% (v/v) TFA in acetonitrile (mobile phase B). The linear gradient profile adopted was from %B = 50 (0 minute) to %B = 100 (20 minutes) at a flow rate of 1.0 mL per minute. Two-milliliter fractions of the unchanged form of Am-80 were collected at the retention time of 9 to 15 minutes under these conditions. After evaporation of the solvent at 70°C under a stream of nitrogen gas, the residue was dissolved in 0.5 mL of an assay buffer [0.2% (v/v) BSA and 0.02% (w/v) sodium azide in 0.1 M phosphate buffer (pH 7.0)].

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Radioimmunoassay

The RIA procedure was reported in detail previously.(17) A 0.1-mL portion of the labeled antigen (300,000 cpm/mL), 0.1 mL of the standard Am-80 solution (9.37 to 10000 pg/mL) and diluted antiserum (antiserum No. F187, 1:50,000) were added to an assay tube containing 0.4 mL of the assay buffer in order to determine the standard curve. In the case of unknown samples, a 0.2-mL portion of the samples described above, labeled antigen, and diluted antiserum were added to an assay tube containing 0.3 mL of the assay buffer, successively. The mixture was incubated at room temperature for more than 16 h; then, 0.1 mL suspension of immobilized anti-rabbit second antibody (Immunobead, 1 mg/mL) was added to the solution. After further incubation at room temperature for 2 h, the mixture was centrifuged at 2,000 × g for 10 minutes. The supernatant was aspirated off and the radioactivity of the residue was measured with an Aloka ARC-600 gamma system (Tokyo, Japan).

RESULTS

Preparation of Immunoaffinity Column

A 1-mL portion of anti-Am-80 antiserum (F187) was purified to IgG (yield: 19.3 mg); the IgG was used to prepare the immunoaffinity column. We then determined the antigen-binding capacity of the immunoaffinity column by loading an excess dose of antigen to the column. When 2 mg of Am-80 was applied and eluted with 50% (v/v) acetonitrile after washing, about 0.5 mg of Am-80 was recovered and the remainder was found in non-retained fractions (determined by RIA, data not shown). The binding capacity was thought to be sufficient but, in the case of a high

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dose, the sample could be diluted with the equilibration buffer before injection.

Automated On-Line Extraction and HPLC Procedure

As shown in Fig. 2, Am-80 was specifically trapped on the immunoaffinity column and eluted with high recovery (88%) compared to that of non-specific rabbit IgG-immobilized column under the conditions described in Experimental. We preferred unique organic solvent to aqueous solutions in the elution step, which made the HPLC purification method, previously reported,(15) applicable with few modifications. Next, we designed an on-line purification system using RP-HPLC with a column switching technique. The upper panel in Fig. 3 of the immunoaffinity chromatogram determined by RIA shows no Am-80 immunoreactivity and indicates that the entire Am-80 fraction was introduced to the RP-HPLC column line by column switching. On the other hand, the lower panel of the HPLC chromatogram shows a reproducible single immunoreactive peak of Am-80 at a retention time of 11 minutes from the start of the gradient program. Am-80 was satisfactorily extracted by the immunoaffinity column and its unchanged form was obtained using the automated HPLC purification system of the on-line column switching technique without any manual handling.

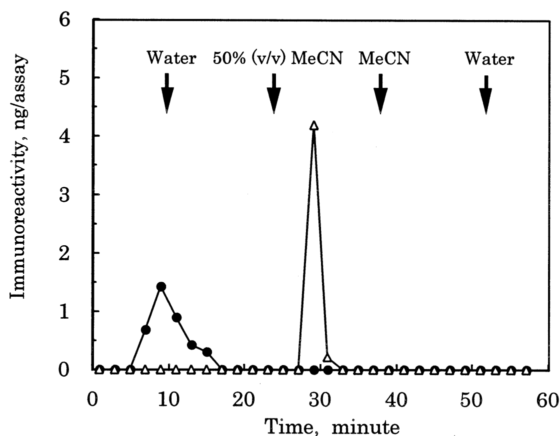

Figure 2. Chromatographic profile of Am-80 in an immunoaffinity column. Am-80 was specifically trapped on the immunoaffinity column (open triangles) compared to non-specific rabbit IgG-immobilized column (filled circles). The immunoreactivity of the fraction was measured by RIA.

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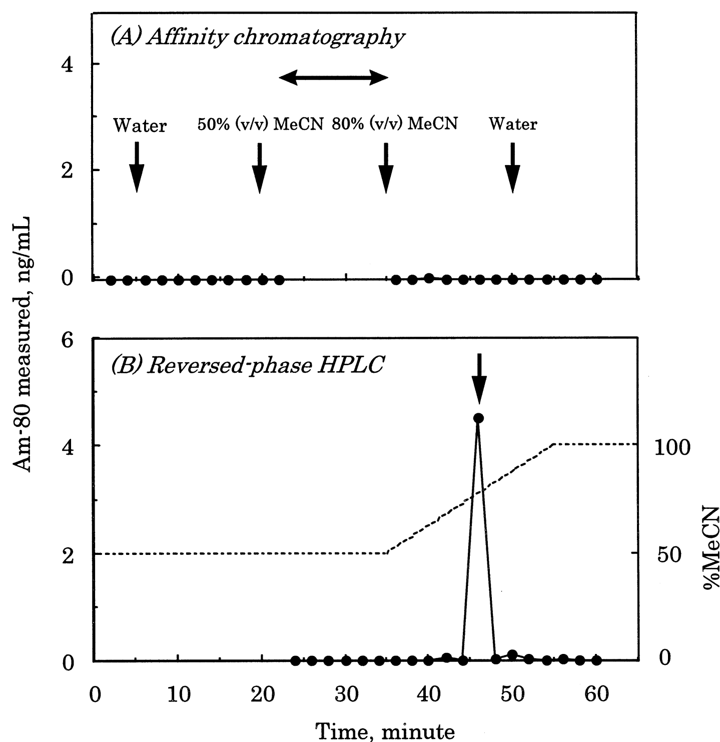

Figure 3. Chromatographic profile of Am-80 in the on-line IAC-HPLC system. (A) After loading with Am-80, the immunoaffinity column was washed and eluted. The eluate was introduced to reversed-phase HPLC with column switching as shown in the area indicated by arrows. (B) The immunoaffinity-purified fraction was further separated with gradient elution. The immunoreactivity in the fractions was measured by RIA. The arrow indicates the reproducible single peak of Am-80.

Accuracy and Precision

The correlation between the Am-80 added to human plasma (x, ng/mL) and the measured value (y, ng/mL) was linear, $y = 0.889x + 0.055$ ($r = 0.9992$, ranging from 0.50 to 10.0 ng/mL). The measured values were corrected for insufficient recovery, 88.9%. For assay precision, the within- and between-assay precision and accuracy are shown in Table 1. From the results, the limit of quantitation and the working range, without dilution, of the automated on-line IAC-HPLC-RIA were 0.5 ng/mL and 0.5 to 10 ng/mL for human plasma, respectively.

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Table 1. Between- and Within-Assay Precision and Accuracy of Am-80 in Human Plasma by On-Line IAC-HPLC-RIA

Am-80 Added ng/mL	Am-80 Measured Mean ± SD, ng/mL	RSD %	% Bias[a]
Between-assay (n = 5)			
0.50	0.42 ± 0.05	11.9	− 16.0
1.00	1.09 ± 0.13	11.9	+ 9.0
3.00	3.09 ± 0.21	6.8	+ 3.0
10.0	9.24 ± 1.09	11.8	− 7.6
Within-assay (n = 5)			
0.50	0.47 ± 0.05	10.6	− 6.0
1.00	1.04 ± 0.11	10.6	+ 4.0
3.00	3.25 ± 0.27	8.3	+ 8.3
10.0	10.1 ± 0.4	4.0	+ 1.0

[a](measured-added) × 100/added.

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Correlation Between Off-Line SPE-HPLC-RIA and On-Line IAC-HPLC-RIA

As shown in Fig. 4, the correlation between the values measured by off-line SPE-HPLC-RIA previously reported (ng/mL) and that of the on-line IAC-HPLC-RIA described here (ng/mL) was linear, $y = 0.981x - 0.765$ ($r = 0.997$, $n = 12$), and no significant difference between the two sides was found ($p > 0.01$) on regression analysis.

DISCUSSION

On-line immunoaffinity extraction coupled with RP-HPLC and RIA was developed to determine Am-80 in human plasma. Although the limit of quantitation, 0.5 ng/mL, was not as good as that of the solid-phase extraction-HPLC-RIA previously reported,(15) the limit of quantitation was thought to be reasonable, considering the lesser amount of the sampling volume of 0.05 mL per injection. The sensitivity could be improved by increasing the plasma sampling volume. The immunoaffinity column prepared in this study with the sampling volume of 0.05 mL, maintained its performance even after more than 200 injections. In the system, about 30 samples could be successfully treated per day, but better performance could be attained by refining the chromatographic conditions, e.g., taking

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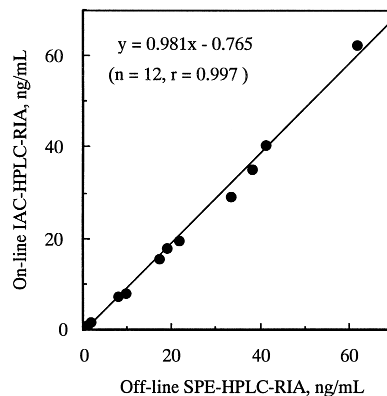

Figure 4. Correlation between off-line SPE-HPLC-RIA and on-line IAC-HPLC-RIA. After preparation of plasma samples containing different several doses of Am-80, these samples were measured by both off-line SPE-HPLC-RIA and on-line IAC-HPLC-RIA. The measured values were calculated by linear regression analysis.

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advantage of semi-micro columns in the isocratic mode in the reversed-phase HPLC step.

This on-line clean-up system made a valuable contribution to labor-saving analytical operations, compared to off-line SPE-HPLC-RIA with batch operations or other conventional pretreatment methods. Here, we used Am-80 as a model compound in the on-line IAC-HPLC system. This on-line clean-up system should be much more suitable for routine assay of other compounds in complex matrices with high condensation procedures, especially for trace amounts of environmental hormones or other hormones in food analysis.

The on-line IAC-HPLC was capable of pre-treating many samples with high accuracy, avoiding extraction losses during the evaporation and reconstitution steps, and is thought to be more useful, not only for immunological analysis, but also for other sensitive assays, e.g., mass-spectral analysis.

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