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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ASSAY FOR LOW LEVELS OF RETINOIC ACID IN HUMAN BLOOD

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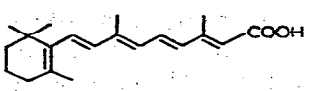
SUMMARY

A rapid and sensitive method of analysis for retinoic acid in human blood has been developed using gas chromatography—mass spectrometry for separation and detection. The retinoic acid is isolated by solvent extraction into petroleum ether, and converted to methyl retinoate by reacting with dimethylformamide dimethylacetal. The method has been applied to the study of retinoic acid in human blood after subtotal inunction, total inunction and intravenous injection of retinoic acid. The sensitivity limit of 1 ng/ml blood is realized with a 10-ml blood sample.

INTRODUCTION

Retinoic acid is an active compound of Tretinoin (Johnson & Johnson, New Brunswick, NJ, U.S.A.), a topical application acne drug. The ability to monitor low levels of retinoic acid in human blood in a relatively short time would be of considerable value in studying the metabolism of retinoic acid.



retinoic acid

Retinoic acid concentrations in plasma following oral administration have been measured in humans by Jurkowitz [1] and in pigs by Nelson et al. [2] using a colorimetric method. The limit of detection reported by these authors was in the order of submicrogram quantities of retinoic acid per ml of plasma. Liquid—gel partition chromatography has also been used by Ita et al. [3] in separating retinoic acid from its analogs in plasma samples from rats. Recently, high-performance liquid chromatography has become a popular method for

separation and identification of natural retinoids [4–7]. The limits of detection claimed by these authors varied from 1 ng [4] to 6.7 ng [5] of retinoic acid in standard solutions; however, no limit of detection was reported from actual experimental samples using nonradioactive retinoic acid.

A gas chromatographic—mass spectrometric (GC—MS) method was developed for detecting as little as 2 ng of nonradioactive retinoic acid per ml of human plasma. The purpose of this study was to develop a method for detection and quantitation of retinoic acid in human plasma. This method requires a simple clean-up and derivatization prior to GC—MS analysis; thus it allows the analysis of a large number of clinical samples, with the limit of detection down to 1 ng retinoic acid per ml of blood.

EXPERIMENTAL

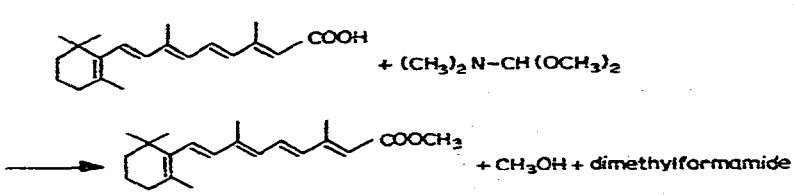
Extraction

Blood samples were drawn from patients into J-Vac (Jelco Lab.) tubes containing anticoagulant ethylenediaminetetraacetic acid and antimycotic agent potassium sorbate. Blood of ^{14}C-labeled (at the carboxylic group) retinoic acid (Amersham Corp., Amersham, Great Britain) (3.8 ng/ml) was added to the blood samples as an internal standard when internal standard was used. The Amersham material was a mixture of 38% of ^{14}C-labeled retinoic acid and 62% of nonlabeled retinoic acid as determined by our mass spectrometer. This ratio was used to calculate the amount of nonradioactive retinoic acid originally in the samples by correcting for contribution from the 62% nonlabeled retinoic acid in the added internal standard.

Plasma samples were obtained by centrifuging approximately 30 ml of blood at 2600 *g* for 25 min. To each 10 ml of plasma in a glass-stoppered centrifuge tube, 1 ml of 2 *N* HCl, 10 ml of ethanol and 25 ml of petroleum ether (b.p. 39—56°C) were added. After shaking on a mechanical shaker for 10 min, the tube was cooled and centrifuged at 2600 *g* for 5 min. The petroleum ether layer was evaporated to dryness in a 2-ml amber vial with a stream of nitrogen gas, and the vial was immediately capped with a PTFE-lined rubber seal. All steps were performed in the absence of direct light due to the lability of retinoic acid.

GC—MS method

Fifty microliters of dimethylformamide dimethylacetal (Aldrich, Milwaukee, WI, U.S.A.) were added to the vial to convert retinoic acid to methyl retinoate [8]. The conversion of retinoic acid to the volatile methyl retinoate derivative enables separation on a glass GC column (6 ft. × 2 mm I.D.) packed with 3% SE-30 on 80—100 mesh Chromosorb W HP (Supelco, Bellefonte, PA,



U.S.A.) at a column temperature of 230°C and injector temperature of 250°C. Three microliters of the dimethylformamide dimethylacetal solution were used for each GC—MS injection. The molecular ions of methyl retinoate at *m/z* 314 [9] and methyl ^{14}C-labeled retinoate at *m/z* 316 were monitored by a Varian MAT 311A mass spectrometer equipped with a two-stage Watson-Biemann separator, and operating at 70 eV in the electron-impact mode. The separator temperature was 250°C and the helium flow-rate was 50 ml/min. Signal output from the electron multiplier was recorded on a Varian A-25 potentiometer strip chart recorder.

A typical selected ion monitoring trace of *m/z* 314 from a sample containing all-*trans*-retinoic acid is shown in Fig. 1A. For samples which also contained 13-*cis*-retinoic acid the methyl 13-*cis*-retinoate was clearly separated from the

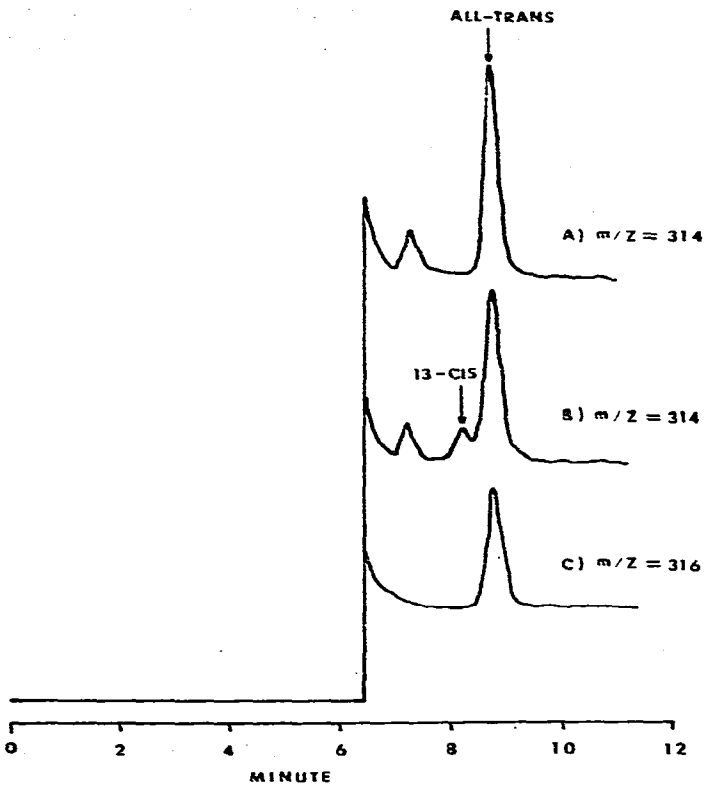


Fig. 1. Selected ion monitoring traces of methyl retinoate from samples: (A) *m/z* = 314 trace of sample with all-*trans*-retinoic acid; (B) *m/z* = 314 trace of sample with mixture of all-*trans*- and 13-*cis*-retinoic acids; (C) *m/z* = 316 trace of sample with ^{14}C-labeled (at the carboxylic group) all-*trans*-retinoic acid as an internal standard. Column: glass column (6 ft. × 2 mm I.D.) packed with 3% SE-30 on 80—100 mesh Chromosorb W HP at 230°C.

methyl all-*trans*-retinoate (Fig. 1B). A typical *m/z* 316 trace of ^{14}C-labeled (at the carboxylic group) retinoic acid used as an internal standard is shown in Fig. 1C.

In order to determine the degree of esterification of retinoic acid a known amount of retinoic acid was dissolved in dimethylformamide dimethylacetal.

Peak intensity of methyl retinoate from this solution was compared with a standard solution made by dissolving a known amount of pure methyl retinoate* in dimethylformamide dimethylacetal. It was found that the esterification of retinoic acid with dimethylformamide dimethylacetal was complete without heating. This made it possible to work with small amounts of retinoic acid in biological systems because the procedure does not require heating, extensive extraction or separation. These operations usually cause the loss of small amounts of labile retinoic acid in the samples.

RESULTS AND DISCUSSION

Percent recovery from spiked blood samples

In order to determine the efficiency of extracting retinoic acid from blood, blood samples were spiked with known amounts of nonradioactive retinoic acid (Table I), and extracted according to the procedure described under

TABLE I

RETINOIC ACID CONCENTRATION MEASUREMENTS FROM BLOOD SAMPLES SPIKED WITH VARIOUS LEVELS OF RETINOIC ACID

Level of spiking (ng retinoic acid per ml plasma)	Concentration measured (ng retinoic acid per ml plasma)*	Percent recovery**
49	28.9	59
20	11.2	56
10	6.0	60
6.9	3.0	43
3.4	1.6	47
1.7	0.8	47
1.6	0.7	44

*Average result from two measurements.
**S.D. = 6.6%.

Experimental without using internal standard. Percent recovery of the spiked retinoic acid measured by the GC—MS method described above was found to be between 40 and 60% with standard deviation of 6.6%. The percent recovery of retinoic acid from spiked plasma samples was also found to be between 40 and 60%, thus indicating no significant difference between the blood and plasma samples in recovery.

Clinical samples

Subtotal inunction. Ten female human subjects were applied with 0.025% Tretinoin Cream (a Johnson & Johnson product containing 0.025% retinoic acid) to the entire arms, legs and back two times a day, at least 8 h apart, for 28 consecutive days. Blood specimens were collected for analysis before application started (day 0), 14 days and 28 days after application started (day

*Obtained from R.J. Gander, Organic and Polymer Chemistry Dept., Johnson & Johnson Research Center.

14 and day 28, respectively). All subjects fasted for 12 h before blood specimens were collected. The specimens were analyzed according to the procedure described under Experimental, first with internal standard and then without internal standard. The results show that no retinoic acid at a limit of detection of 2 ng retinoic acid per ml of plasma was found from any of these specimens.

Total inunction. A male human volunteer was whole body treated with 1% retinoic acid cream, and blood specimens were collected for analysis during the treatment. No retinoic acid was detected in the blood samples of this rather extensive and drastic topical application of retinoic acid (see Table II).

TABLE II

RETINOIC ACID IN BLOOD FOLLOWING TOTAL INUNCTION AND INTRAVENOUS INJECTION

Sample No.*	ng retinoic acid per ml plasma**
1	not detected***
2	14.6
3	5.4

*No. 1: 5.9 ml of plasma from blood taken from patient being treated topically (whole body) with 1% retinoic acid cream. No. 2: 11.8 ml of plasma from blood taken 5 min after intravenous injection of 0.5 mg of retinoic acid into human volunteer. No. 3: 3.5 ml of plasma from blood taken 1 h after injection described in No. 2.
**Average result from two measurements.
***The limit of detection was 2 ng retinoic acid per ml plasma.

Intravenous injection of retinoic acid solution. A male human volunteer was intravenously injected with a total of 0.5 mg of retinoic acid dissolved in solution. Blood specimens were collected for analysis 5 min and 1 h after the injection. The results show a level of 14.6 ng and 5.4 ng retinoic acid per ml of plasma, respectively (see Table II).

CONCLUSIONS

A relatively rapid, sensitive and specific method for analysis of retinoic acid in human blood at a limit of detection of 2 ng per ml of plasma was developed. Results indicated that no detectable amount of retinoic acid was found in normal human blood or after topical application of retinoic acid. Low levels of retinoic acid were detected in blood following 0.5 mg intravenous injection. The non-detectable level of retinoic acid in blood following topical applications suggests low level of absorption and/or rapid metabolism of retinoic acid.

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