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DETERMINATION OF ETRETINATE AND ITS MAIN METABOLITE IN HUMAN PLASMA USING NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An analytical method for the determination of two aromatic retinoids in human plasma (etretinate and its main metabolite) is described, using normal-phase high-performance liquid chromatography. The method is highly sensitive (4 ng/ml) and selective, and allows good separation of isomerization products. Both compounds are well extracted (95%) from plasma in the practically important concentration range (10—1000 ng/ml). After chromatographing the compounds together with an internal standard, they are quantified by spectrophotometry.

INTRODUCTION

Vitamin A analogues have been subjected to considerable investigational effort, which has been intensified in recent years and is still increasing [1—4]. One of the prerequisites for all experimental work are analytical methods for the determination of certain compounds selectively, quantitatively and in very low concentrations in different kinds of biological samples. Many different methods have been developed for the separation of natural vitamin A-related compounds, most of them using liquid chromatography [5—13]. Radioimmunoassays [14] and bioassays [15] for vitamin A have been described.

For one of the best known retinoids, the aromatic compound etretinate, several analytical procedures have been developed, based both on normal-phase as well as on reversed-phase high-performance liquid chromatography (HPLC) [16—18]. However, these methods need to be improved to meet the special requirements encountered in routine analysis during clinical and pharmacokinetic studies. The limit of detection is usually too high, which is a handicap

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when investigating prolonged elimination phases in which the concentrations of both parent compound as well as main metabolites in plasma are low. Good assay reproducibility is a special requirement for all clinical and pharmacokinetic studies.

Closely related compounds, such as *cis—trans* isomers, must be separated. This is a general need in the field of vitamin A analogues, but especially in the case of etretinate, where such isomers are present as biotransformation products. It is the aim of the present report to describe and discuss an analytical method which is, compared to other recently published procedures [16—18], highly sensitive and selective. The use of adsorption chromatography has the advantage of avoiding solvent programming as described in the publications aldready mentioned. In addition, the life time of normal-phase columns, in our hands, was found to be superior to that of reversed-phase columns. The method allows routine determination of both parent drug (etretinate) and its main metabolite (Ro 10-1670), the analogous carboxylic acid. In addition, it provides estimates of various isomerization products of both compounds which, from the point of view of quality control, is important. The method has been used mainly in several clinical pharmacokinetic studies, but also with slightly modified extraction procedures to determine plasma levels in several animal species.

EXPERIMENTAL

Materials

The reagents were of analytical grade unless otherwise stated: *n*-hexane (E. Merck, Darmstadt, G.F.R.), tetrahydrofuran, ethyl acetate, glacial acetic acid, methyl acetate (Merck, synthetic grade); diethyl ether (ad narcosin, Siegfried AG, Zofingen, Switzerland), isopropanol; saturated aqueous sodium sulphate solution at room temperature; nitrogen gas 99.99% (Carba AG, Basle, Switzerland).

Solutions

Mobile phase for HPLC. The mobile phase (*n*-hexane—tetrahydrofuran—glacial acetic acid 200:3:1.2, v/v) was prepared by mixing the three solvents after degassing. The mobile phase was recycled to achieve constant composition.

Standard solutions. Stock standard solutions of all three substances (etretinate, its main metabolite the analogous carboxylic acid Ro 10-1670, and retinoic acid) were prepared separately every two months by dissolivng 10 mg of the solid material in 100 ml of isopropanol to give a concentration of 100 ng/ml.

Working standard solutions containing both etretinate and Ro 10-1670 in two concentrations (10 ng/μl and 1 ng/μl) were freshly prepared every week by diluting 10 ml of each corresponding stock solution to 100 ml with isopropanol, giving a concentration of 10 ng/μl. From this solution 10 ml were then diluted to 100 ml.

Internal standard (retinoic acid) working standard solution was separately prepared by diluting 0.5 ml of the stock standard to 100 ml with isopropanol.

Note: Because of extreme sensitivity of this class of compounds to isomerization under the influence of light, all laboratory work was performed under shortwave light protection and substances were handled in amber or aluminium foil-wrapped glassware.

Plasma standards. Plasma standards were freshly prepared for each assay run, either to establish linearity of the method or for the daily calibration curve. Samples were spiked by pipetting the final volumes of working standard solutions into 0.5 ml of plasma.

Extraction procedure

A 0.5-ml volume of pooled human plasma, delivered by a blood bank, was pipetted into a 30-ml round-bottomed centrifuge tube using an Oxford pipettor. The 100 μl of internal standard working solution were added by an SMI-pettor, and vortexed for 5 sec; 0.5 ml of methyl acetate was added with an Oxford pipettor, and vortexed again for 5 sec. Then 0.5 ml of saturated sodium sulphate solution was added with an Oxford pipettor followed by 10 ml of ethyl acetate and the mixture extracted for 20 min on a Heidolph extractor at 20 rpm. After centrifuging for 10 min at 2000—3000 *g*, the organic phase was transferred into a 30-ml tapered centrifuge tube and evaporated to dryness at 40° C under nitrogen. The glass wall was rinsed with 2—3 ml of ether and again evaporated to dryness. The residue was stable for at least one week in a stoppered tube at 5° C.

High-performance liquid chromatography

Equipment. This comprised a Pye-Unicam LC-UV spectrophotometer (Pye-Unicam, Cambridge, Great Britain) set at 360 nm, a 250 × 3.2 mm column packed with LiChrosorb Si 60, 7 μm particle size (Merck), giving a theoretical efficiency of 10,000—15,000 plates at a flow-rate of 1 ml/min and ambient temperature, a Milton Roy 5000 pump (Milton Roy Company, St. Petersburg, PA, U.S.A.), a Rheodyne 7120 loop injector and a W+W 1100 recorder (Kontron, Zürich, Switzerland).

Procedure. The dry extract was dissolved in 200 μl of mobile phase, vortexed for 15 sec, and 100 μl were injected into the HPLC system.

Retention times were 2 min 50 sec, 4 min, and 6 min 50 sec, for etretinate, retinoic acid, and the main metabolite Ro 10-1670, respectively.

Calibration and calculation

Linearity of the complete assay procedure was established in the range 4—1000 ng/ml of plasma by analyzing spiked human plasma samples covering this range. A calibration curve, consisting of four different concentrations within the expected range of the plasma samples to be analyzed, was generated for each assay run by least-squares regression of the peak height ratios (spiked drug/internal standard) against the concentration of the drug. The unknown concentrations of plasma samples were determined from the calculated peak height ratio by interpolation from this calibration curve.

RESULTS AND DISCUSSION

Selectivity and choice of the internal standard

In developing an analytical method for the separation of closely related retinoids and in the search for an internal standard possessing similar chromatographic properties, several compounds of the vitamin A type were considered as potential candidates. The two main vitamin A components in human plasma, the palmityl ester and the free alcohol (retinol), can easily be separated in any appropriate chromatographic system. Many different human plasma samples from patients and healthy volunteers were screened for possible endogenous compounds which could interfere with the retinoids to be analyzed (Fig. 1, I). Most of these blank samples were completely free of such substances. An additional difficulty with the retinoids is the possibility of rapid isomerization under the influence of UV irradiation. The chromatographic system, therefore, must be able to separate these potential photoisomerization products [19]. Spiked plasma samples were exposed to light before starting the analytical procedure. The chromatograms obtained, compared with those from light-protected samples, showed several additional small peaks grouped around each main substance peak (Fig. 1, II). No effort was made to obtain baseline separation, because quantification of one of the isomers was not a major goal in the development of this method. However, there was one peak with a slightly shorter retention time than that of Ro 10-1670, the main metabolite, which appeared only in the chromatograms from patients undergoing chronic etretinate therapy (Fig. 1, III). As the influence of light could be excluded, formation of metabolite was the most probable explanation. Various synthetic isomers were added to these plasma extracts, and the 13-*cis* isomer of Ro 10-1670 co-chromatographed with this unknown component in two different mobile phases.

The ideal internal standard should have a polarity intermediate between that of etretinate and its main metabolite and should not, of course, be either a metabolite or an isomerization/decomposition product of the parent drug. Retinoic acid fulfils these requirements, as shown in earlier investigations [13].

Although retinoic acid appears regularly as a biotransformation product of vitamin A in plasma, its concentration is negligible compared with the internal standard-spiked concentration. Representative chromatograms are shown in Fig. 2. The average endogenous concentrations of retinoic acid are 1 ng/ml [20]; this is to be compared with an internal standard concentration, used here, of 50 ng/ml.

Recovery

In order to determine the extraction efficiency of both parent drug and its main metabolite, human plasma samples were spiked with ^{14}C-labelled material according to the spiking procedure described (see Experimental). Plasma samples at concentrations of 10, 100 and 500 ng/ml were prepared, extracted, and an aliquot of the final organic solution was counted in a liquid scintillation counter (W+W, Kontron) (Table I). The percentage of recovered radioactivity was about 97% for both compounds at all concentrations.

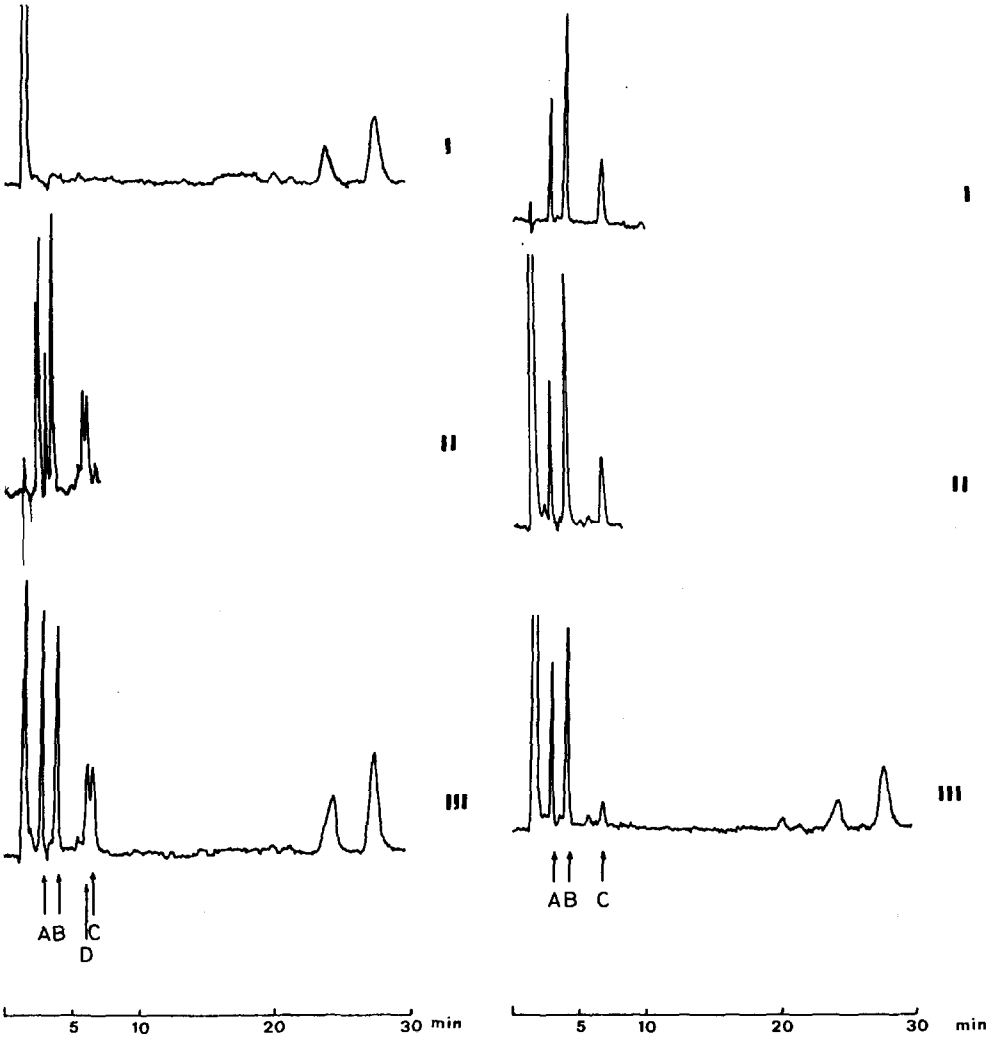

Fig. 1. (I) Chromatogram of a human blank plasma. (II) Chromatogram of a spiked human plasma sample, which was exposed to short-wavelength fluorescent light for 10 min. (III) Chromatogram of plasma from a patient undergoing chronic dosing. The 13-*cis* isomer (D) of Ro 10-1670 is shown.

Fig. 2. (I) Chromatogram of a mixture of etretinate (A), retinoic acid (B) and Ro 10-1670 (C) in mobile phase. (II) Chromatogram of human blank plasma, spiked with all three compounds (see Fig. 1), at a concentration of 25 ng/ml for A and C and 50 ng/ml for B. (III) Chromatogram of a volunteer's plasma 2.5 h after a single dose of 25 mg of etretinate.

Limit of detection

A signal-to-noise ratio of 3:1 corresponded to a concentration of 4 ng/ml etretinate and main metabolite.

TABLE I

EXTRACTION YIELD OF [^{14}C]ETRETINATE AND ^{14}C-LABELLED Ro 10-1670 FROM PLASMA

Spiked conc.* (ng/ml)	Etretinate			Ro 10-1670 (main metabolite)		
	Recovery (%)	C.V.** (%)	No. of replicates	Recovery (%)	C.V. (%)	No. of replicates
10	93.1	2.4	3	96.4	2.0	3
100	96.6	1.0	3	95.5	0.5	3
500	96.5	0.3	3	96.2	0.6	3

*Solvent concentration in spiked plasma samples was <1%.
**C.V. = coefficient of variation.

Linearity

Calibration samples were prepared from plasma samples taken from volunteers taking part in pharmacokinetic studies or from patients undergoing etretinate therapy. A linear correlation between peak height ratios and concentration over the range 4—2000 ng/ml was found. Coefficients of correlation calculated from individual time-independent calibration curves showed acceptable variation. Since 90% of the analyzed samples showed concentrations below 500 ng/ml, standard curves were routinely set up from 10 to 500 ng/ml.

Stability

Spiked plasma samples were stored under varying conditions over different periods of time and analyzed for etretinate. No degradation was found after eight weeks of storage at −20°C, neither did repeated thawing and freezing give rise to degradation. For the same storage period, about 20% loss was observed at +4°C; part of the degradation was due to hydrolysis to Ro 10-1670.

In order to obtain information about possible enzymatic degradation in both whole blood and plasma, biological samples were spiked with etretinate or Ro 10-1670 and incubated at 37°C for 1 h and 16 h, respectively. No Ro 10-1670 could be detected, either in blood after 1 h or in plasma after 16 h. The concentrations of etretinate remained constant for at least 8 h.

Accuracy and precision

In Table II reproducibility data are summarized. Spiked plasma samples covering a concentration range of 10—1000 ng/ml were analyzed in duplicate on five different days over a period of four weeks. The intra-assay reproducibility was calculated from the variation of duplicates, whereas the inter-assay reproducibility was established from the variation of the means of duplicate values. Inter-assay precision for etretinate and its main metabolite Ro 10-1670, calculated for the whole concentration range, was less than 4% and 3%, respectively. Inter-assay accuracy was 95%.

TABLE II

REPRODUCIBILITY DATA

Etretinate						Ro 10-1670 (main metabolite)					
Conc. added (ng/ml)	Found (%)	Found — expected (%)	No. of replicates	C.V. (%)		Conc. added (ng/ml)	Found (%)	Found — expected (%)	No. of replicates	C.V. (%)	
				Inter-assay	Intra-assay					Inter-assay	Intra-assay
10	94	—6	5	5.8	3.1	10	110	+10	5	6.4	14.4
100	95	—5	5	3.8	1.0	100	100	±0	5	1.5	1.3
500	94	—6	5	4.2	2.5	500	94	—6	5	3.5	1.6
1000	95	—5	5	3.2	1.7	1000	96	—4	5	2.5	1.9

Application of the method to biological samples

The method was developed primarily to analyze human plasma samples, either from patients on chronic etretinate therapy in whom plasma concentrations have to be controlled for safety reasons from time to time, or from volunteers who were administered etretinate in order to establish the pharmacokinetics of the drug. The method has also been used, with certain minor modifications, to determine etretinate concentrations in animal plasma and different tissues. The main problems hampering the method under these circumstances were endogenous compounds extracted from the tissues which interfere, chromatographically, with the compounds to be determined.

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