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RESOLUTION OF *RS*-ABSCISIC ACID AND THE SEPARATION OF ABSCISIC ACID METABOLITES FROM PLANT TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Attempts to resolve the enantiomers of racemic abscisic acid (ABA) by high-performance liquid chromatography on a chiral stationary-phase column were unsuccessful. However, reduction of *RS*-methyl ABA (*RS*-Me-ABA) with sodium borohydride generates a new chiral centre and one of the two isomeric products, the *RS*-Me-1',4'-*cis*-diol of ABA, was separated into its enantiomers by high-performance liquid chromatography on an optically active Pirkle column.

High-performance liquid chromatography on a μBondapak C_{18} column separated the metabolites and conjugates of [2-^{14}C]ABA fed to tomato shoots. The resolution method was used to measure the relative proportions of *R* and *S* enantiomers in the free acid liberated from conjugates of ABA.

INTRODUCTION

The plant hormone abscisic acid (ABA) occurs naturally as the (+)-*S* enantiomer but most experiments in which ABA has been fed have, perforce, used racemic, synthetic material, although differences in the physiological effects and metabolism of the natural and unnatural enantiomers have been reported [1]. The resolution of racemic ABA was first achieved by fractional crystallization of the (—)-brucine salt of ABA in methanol [2]. The fractions were further enriched by selective solubilization and microsublimation. Acetylcellulose chromatography [3] has also been used to prepare fractions slightly enriched with one or other enantiomer and optically active ABA was isolated by selective solubilization. Recently *R*- and *S*-ABA of high optical purity have been prepared by immunoaffinity chromatography [4]. However, only small amounts of ABA can be resolved and the column has a limited life. The amount of *R*- and *S*-ABA in a sample can be measured by optical rotatory dispersion and circular dichroism (CD) methods [1]. Unfortunately these methods require

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very costly equipment and relatively large quantities of sample with a high degree of ultraviolet (UV) purity. Because of the complexity of the existing methods we have developed a high-performance liquid chromatographic (HPLC) method for *RS*-ABA that completely separates the enantiomers, requires readily available equipment and can produce milligram quantities of the separate enantiomers.

The low endogenous concentrations (ppb) and the wide range of polarities of ABA and its metabolites complicate their isolation from plant tissue. Reversed-phase HPLC has been used for the purification and analysis of ABA [5–9]. We have developed a system that separates the known metabolites of ABA in tomato tissue including the recently characterized metabolites ABA 1′-glucoside (ABAGS) [10], dihydrophaseic acid 4′-glucoside (DPAGS) [11] and the 1′,4′-*trans*-diol of ABA [12].

EXPERIMENTAL

Tomato plants (*Lycopersicon esculentum* cv. Gross Lisse), 150–200 mm high, were used in feeding experiments. The plants were cut just above ground level and the shoots placed in aqueous solutions of *RS*-[2-^{14}C]ABA. A Waters HPLC system consisted of a 6000A pump, an M-45 pump, a U6K injector and a Model 660 solvent programmer. Effluent was monitored with a Model 440 absorbance detector fitted with a 254-nm filter. Solvents for HPLC were purchased from Waters Assoc. (Chippendale, Australia) or were redistilled before use. Distilled water was passed through a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

HPLC resolution of RS-Me-1′,4′-cis-diol of ABA

A 10-mg amount of *RS*-ABA was methylated with ethereal diazomethane and the methyl ABA (Me-ABA) reduced with sodium borohydride in methanol–water (2:1) at 0°C for 30 min. The products were separated by HPLC on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with a mobile phase of ethanol–water–acetic acid (317:517:1) delivered at a flow-rate of 4 ml/min. Me-1′,4′-*trans*-diol of ABA, Me-ABA and Me-1′,4′-*cis*-diol of ABA had retention times of 8.2, 10 and 16 min, respectively.

RS-Me-1′,4′-*cis*-diol of ABA was loaded on a 250 mm × 4.6 mm I.D. Pirkle Type 1-A column (Regis, Morton Grove, IL, U.S.A.) and eluted with hexane–isopropanol (9:1) at a flow-rate of 1 ml/min. Peaks eluted after 21 and 23 min were the 1′*S*,4′*S*- and 1′*R*,4′*R*-*cis*-diol of ABA methyl ester, respectively. A sample of Me-1′,4′-*cis*-diol prepared from natural *S*-ABA gave one peak only (21 min). Improved resolution of *RS*-Me-*cis*-diol with baseline separation between the peaks was achieved by connecting a Pirkle Type 1-A and a Pirkle covalent *R*-phenylglycine column in series, eluting with hexane–isopropanol (97:3) at a flow-rate of 2 ml/min and recycling the eluate up to four times.

R- and *S*-methyl abscisates were regenerated by oxidation of the Me-1′,4′-*cis*-diol of ABA with a ten-fold excess of manganese dioxide. The oxidation mixture in 2 ml dry chloroform was continuously stirred at 20°C. The duration of the oxidation depended on the batch of manganese dioxide with reaction times ranging from 30 min to 72 h. The reaction mixture was loaded on a

200 × 200 mm silica gel 60 F_{254} TLC plate (E. Merck, Darmstadt, F.R.G.) and chromatographed in hexane—ethyl acetate (2:1) to separate Me-ABA from unreacted Me-*cis*-diol and manganese dioxide. ABA was released by alkaline hydrolysis in 2 *M* aqueous potassium hydroxide—ethanol (1:2) at 20°C for 30 min.

Plant feeding and extraction of metabolites

To 30 g tomato (*Lycopersicon esculentum* cv. Grosse Lisse) shoots 2.8 μCi *RS*-[2-^{14}C]ABA (25.6 mCi/mmol) were fed. After two days the plants were homogenised in acetone—acetic acid (99:1) containing 2,6-di-*tert.*-butyl-4-methylphenol (BHT) (100 mg/l). The acetone was evaporated and the aqueous residue extracted three times with diethyl ether. The aqueous phase which contained polar metabolites and conjugates of ABA was put aside for HPLC. An equal volume of water was added to the ether extract and the pH adjusted to 7.0 with saturated sodium hydrogen carbonate. This was repeated three times and the ether phase containing unlabelled neutral material was discarded. The aqueous phase was acidified with 1 *M* sulphuric acid to pH 2.5 and ABA and ether-soluble metabolites were extracted with diethyl ether (three times). The ether extract and the initial aqueous phase were combined, the diethyl ether was evaporated and the sample was concentrated and chromatographed on Sep-Pak C_{18} cartridges (Waters Assoc.).

Four Sep-Pak C_{18} cartridges were connected in series with 1 mm I.D. glass tubing. A peristaltic pump was used to maintain a flow-rate of 5.0 ml/min. The sample, which was in a volume of about 50 ml, was loaded on the Sep-Pak column and washed with 20 ml water—acetic acid (500:1). Only a small amount of radioactivity was present in this fraction and corresponds to uncharacterised polar metabolites of ABA. Then 20 ml ethanol—water—acetic acid (256:475:1) eluted abscisic acid and its metabolites from the column. Washing the column with ethanol removed less polar compounds, which contained no radioactivity, and regenerated the column.

HPLC separation of metabolites

The Sep-Pak fraction containing ABA and its metabolites was evaporated to dryness, dissolved in 50 μl ethanol—water—acetic acid (84:506:1) and loaded on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column. The column was eluted at a flow-rate of 4 ml/min for 22 min with the solvent used to dissolve the sample. This was followed by a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min. After a further 20 min the column was washed with 95% ethanol. Radioactivity in fractions collected was determined by liquid scintillation counting as described elsewhere [13].

Determination of the proportion of R-[^{14}C]ABA to S-[^{14}C]ABA in ABA and its conjugates

Conjugates of ABA were hydrolysed with aqueous ammonium hydroxide (sp.gr. 0.91) at 27°C for 30 min as described by Loveys and Milborrow [10]. Amounts of 400 μg *RS*-ABA were added to the samples of [^{14}C]ABA released by hydrolysis and the samples were methylated with diazomethane. The Me-ABA was purified by HPLC on a μBondapak C_{18} column with ethanol—water—

acetic acid (256:475:1) at a flow-rate of 4 ml/min. The Me-ABA was reduced and resolved as described above. The radioactivity of the *R*- and *S-cis*-diols was determined by liquid scintillation counting.

RESULTS

Attempts to separate the enantiomers of *RS*-ABA and some of its derivatives on a Pirkle column were unsuccessful. Racemic ABA, Me-ABA, 2-*trans*-ABA, Me-2-*trans*-ABA, Me-1′,4′-*trans*-diol of ABA and 4′-O-acetyl-Me-1′4′-*trans*-diol of ABA all chromatographed as single peaks. However, the *RS*-Me-1′,4′-*cis*-diol of ABA and its 4′-O-acetyl derivative were both separated into their enantiomers by HPLC on a Pirkle Type 1-A column with a mobile phase of hexane—isopropanol (9:1) delivered at a flow-rate of 1 ml/min. A sample of Me-1′*S*, 4′*S-cis*-diol of ABA produced from natural *S*-ABA gave a single peak at 21 min and when injected with a sample of the racemic compound caused the peak at 21 min to increase in height while the peak at 23 min remained unchanged. This confirmed that separation of the enantiomers had been achieved and identified the first peak as the *S* enantiomer. The separation of the enantiomers was increased by recycling the column effluent through a Pirkle Type 1-A column and a Pirkle covalent *R*-phenylglycine column in series (Fig. 1). Baseline resolution was achieved after four cycles through the column (248 min). The Pirkle Type 1-A column was found to be more efficient than the Pirkle covalent *R*-phenylglycine column for the resolution of *RS*-Me-1′,4′-*cis*-diol of ABA and a shorter analysis time would be expected if two Type 1-A columns were used in series.

The reduction of Me-ABA to the Me-1′,4′-*cis*- and -*trans*-diols, separation of the enantiomers by HPLC, oxidation of the diols to Me-ABA and saponification to release *R*- and *S*-ABA gave an overall yield of 20% from the original

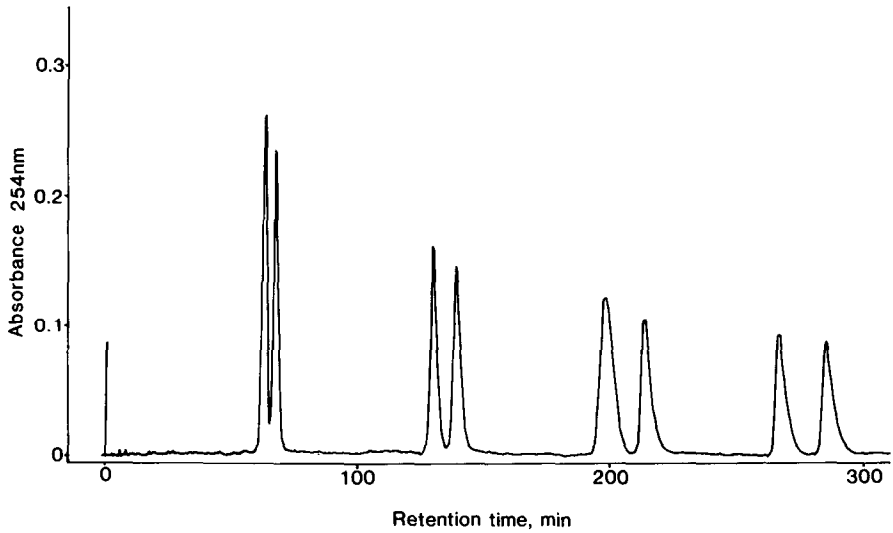


Fig. 1. Resolution of 300 μg *RS*-Me-1′,4′-*cis*-diol of ABA. Two Pirkle HPLC columns were connected in series and eluted with hexane—isopropanol (97:3) at a flow-rate of 2 ml/min and the column effluent was recycled.

ABA (i.e. 10% in each enantiomer). However, half of the ABA is reduced to the 1′,4′-*trans*-diol, which is not resolved. The yield can be increased by re-oxidizing 1′,4′-*trans*-diol to ABA and carrying out a second reduction.

HPLC separation of metabolites

The preliminary chromatography of the tomato extract on C_{18} Sep-Pak proved to be a simple, efficient (98% recovery) method of removing material that interfered with subsequent chromatographic steps. The removal of salts, polar compounds and compounds less polar than ABA made it possible to load samples more concentrated in ABA and its metabolites and facilitated the dissolution of the sample in a small volume for injection. It also removed compounds that bind irreversibly to the stationary phase of the HPLC column.

The metabolites of ABA were separated by one passage through a reversed-phase column with a gradient containing ethanol, water and acetic acid. The separation of the products formed by [2-^{14}C]ABA is shown in Fig. 2. The products were identified by comparison with the retention time of standards (Table I). A mixture of the marker compounds *p*-amino benzoic acid, *p*-hydroxybenzoic acid, benzoic acid 2-*trans*-ABA and 2-*cis*-ABA chromato-

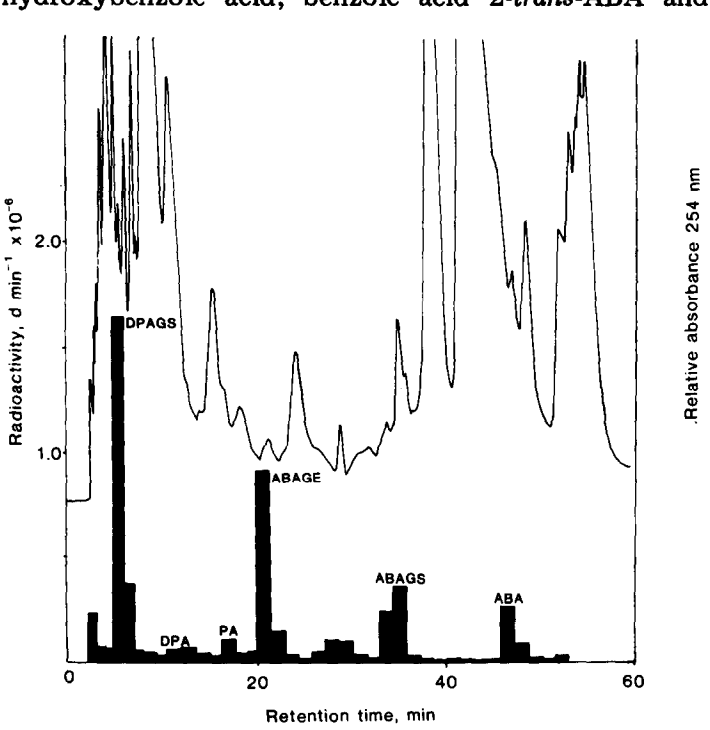


Fig. 2. HPLC separation of the products of *RS*-[2-^{14}C]ABA in tomato shoots. The sample was chromatographed on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with ethanol—water—acetic acid (84:504:1) delivered at a flow-rate of 4 ml/min for 22 min. This was followed by elution with a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min and elution with the final solvent for 20 min. The column was then washed with ethanol—water (19:1). Compounds identified were abscisic acid (ABA), ABA 1′-glucoside (ABAGS), ABA glucose ester (ABAGE), phaseic acid (PA), dihydrophaseic acid (DPA) and DPA 4′-glucoside (DPAGS).

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF STANDARD AND MARKER COMPOUNDS

HPLC on a 300 mm × 7.8 mm I.D. μBondapak C_{18} column with ethanol—water—acetic acid (84:504:1) for 22 min at a flow-rate of 4 ml/min followed by a linear gradient to ethanol—water—acetic acid (158:508:1) over 15 min.

Compound	Retention time (min)
Abscisic acid (ABA)	46.0
2-*trans*-ABA (*t*-ABA)	41.7
ABA 1'-glucoside (ABAGS)	33.0
1',4'-*trans*-Diol of ABA	30.5
epi-Dihydrophaseic acid (*epi*-DPA)	22.7
ABA glucose ester (ABAGE)	21.0
Phaseic acid (PA)	18.0
Dihydrophaseic acid (DPA)	12.0
Dihydrophaseic acid 4'-glucoside (DPAGS)	5.3
p-Aminobenzoic acid	5.7
p-Hydroxybenzoic acid	12.3
Benzoic acid	28.0

graphed under conditions identical to those used to separate the extracts were an effective means to test the performance of the system.

Proportion of R- and S-ABA in conjugates

One of the advantages of the HPLC method of resolution is that it allows the determination of the ratio of *R*-ABA to *S*-ABA in small samples and even allows the specific activity of each enantiomer to be calculated. This is done by hydrolysing the conjugates of ABA and then the ABA released is methylated and reduced to the diols. The *cis*-diol is resolved by HPLC on the Pirkle column and the amount of radioactivity in each anantiomer determined. Conjugates of ABA formed from *S*-[2-^{14}C]ABA contain a preponderance of the *R* enantiomer (Table II). The ratio of *R*-[^{14}C]ABA to *S*-[^{14}C]ABA ranges from 5.8:1 for the glucose ester to 29:1 for ABA glucoside. The free ABA remaining also contains an excess of the *R*-[^{14}C] enantiomer.

TABLE II

PROPORTION OF *R*-[2-^{14}C]ABA AND *S*-[2-^{14}C]ABA in ABA AND ITS CONJUGATES IN EXTRACTS OF TOMATO SHOOTS FED *RS*-[2-^{14}C]ABA

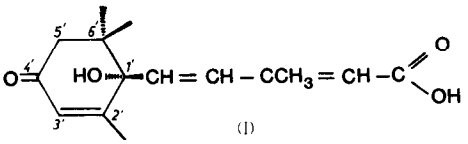
Compound*	*R*-[2-^{14}C]ABA:*S*-[2-^{14}C]ABA
ABA	6.1:1
ABAGE	5.8:1
ABAGS	29.4:1
Uncharacterised conjugate	8.3:1

*For abbreviations see Table I.

DISCUSSION

ABA is intensely optically active so it is surprising, at first sight, that in experiments in which growth is assayed the *R* and *S* enantiomers are equally or

almost equally active. It has been proposed that both *R* and *S* forms can attach to some active site(s). This could occur if the 2′-methyl group of one enantiomer takes the place occupied by a 6′-methyl of the other [14]. The molecule is not intrinsically highly asymmetric and an alternative projection of the structural formula emphasizes this (I).



(I)

Reduction of the 4′-ketone of ABA generates a second chiral centre in close proximity to the 1′ chiral centre and enables *RS*-Me-1′,4′-*cis*-diol to be separated into its enantiomers by HPLC on a column having a chiral stationary phase. It was possible to separate the enantiomers of 4′-O-(−)-camphanyl esters of both *RS*-Me-1′,4′-*cis*- and -*trans*-diols of ABA by normal-phase HPLC although yields were low. The HPLC resolution of *RS*-Me-1′,4′-*cis*-diol of ABA on a chiral-stationary phase column enables large amounts of *R*- and *S*-ABA of extremely high optical purity to be produced at much less cost than existing methods. An added advantage is that the method also allows the proportion of *R*- and *S*-ABA in a sample to be measured. If the ABA is labelled the specific activity of each enantiomer can be determined, though this is limited to samples containing an excess of 1 μg of each enantiomer. However, the proportion of labelled *R*- to *S*-ABA in very small samples of ABA may be determined by adding unlabelled ABA before reducing the sample and is only limited by the sensitivity of the method used to detect the label.

HPLC on μBondapak C_{18} column separated the products formed from [2-^{14}C]ABA by tomato shoots, including three conjugates of ABA. This procedure gives much more information about the metabolites of ABA than methods in which the level of conjugates is measured by assaying the free acids released by basic hydrolysis. Because the free acid metabolites and conjugates are separated in a single step, losses of conjugate by partitioning into solvents used to extract the free acids are avoided and conjugates that are resistant to basic hydrolysis, such as DPA 4′-glucoside [11], can also be measured.

The conjugates were not subjected to basic conditions in the extraction and chromatographic procedures so base-induced rearrangement of ABA glucose ester [15] and basic hydrolysis of conjugates were avoided. The separation of ABA glucose ester, ABA 1′-glucoside and a novel conjugate of ABA, all containing different proportions of *R*-[^{14}C]ABA and *S*-[^{14}C]ABA is evidence against rearrangement of ABA glucose ester to produce the glucoside or the other conjugate. However, stereospecific rearrangement has not been ruled out.

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