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CHROMATOGRAPHIC DETERMINATION OF N-ACETYL-DL-TRYPTOPHAN AND OCTANOIC ACID IN HUMAN ALBUMIN SOLUTIONS

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SUMMARY

Chromatographic procedures have been developed for determination of the stabilizers N-acetyl-DL-tryptophan and octanoic acid in human albumin solutions. N-Acetyl-DL-tryptophan and the internal standard, N-formyl-DL-tryptophan, were separated by liquid chromatography on a reversed-phase column with UV detection at 280 nm. Deproteinization and extraction were carried out with methanol. The extraction recovery at the level of 4.9 m*M* was 92.5 ± 2.5% (S.D.) (n = 10), and the average coefficient of variation (C.V.) for replicate analyses of albumin solutions ($\bar{x}$ = 2.57, 10.44 and 17.10 m*M*) was 1.10% (n = 27). Octanoic acid was determined gas chromatographically as its methyl ester, with nonanoic acid as the internal standard. The sample pretreatment included acidification, extraction with hexane and derivatization with methanol–sulphuric acid. The relative recovery from albumin solutions was 89.7 ± 5.8% (S.D.) (n = 6), and replicate determinations of the compound yielded a C.V. of 5.5% ($\bar{x}$ = 14.82 m*M*, n = 9).

INTRODUCTION

Human albumin solutions for therapeutic use commonly contain the additives N-acetyl-DL-tryptophan and octanoic acid to enhance the thermal stability of the protein during heat treatment[1]. Quantitation of these stabilizers has recently been recognized as an essential part of the quality control of concentrated protein solutions[1]. To this end, spectrophotometric and gas chromatographic procedures have been reported for N-acetyl-DL-tryptophan and octanoic acid, respectively[1].

This paper describes new analytical methods for the determination of both these components in albumin solutions. A liquid chromatographic (LC) method was developed for N-acetyl-DL-tryptophan as an alternative to the less specific spectrophotometric assay. Octanoic acid was determined gas chromatographically as its methyl ester rather than as the free acid[1]. This approach permits a wider choice of common stationary phases, as opposed to the limited number of specially treated phases that are useful for chromatography of free C_2–C_6 fatty acids.

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MATERIALS AND METHODS

Chemicals

N-Acetyl-DL-tryptophan, octanoic acid and nonanoic acid were obtained from Fluka (Buchs, Switzerland). N-Formyl-DL-tryptophan and N-acetyl-DL-tyrosine were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol (Hoechst, Frankfurt, F.R.G.) was chemically pure and redistilled before use. All other reagents were analytical grade and purchased from Merck (Darmstadt, F.R.G.).

Liquid chromatography

The liquid chromatograph used consisted of a Varian Model 8500 syringe pump (Varian, Palo Alto, CA, U.S.A.), a Valco N60 injector (Valco, Houston, TX, U.S.A.), fitted with a 50-μl loop and a Varian Varichrom variable wavelength detector. A 25 × 0.46 cm stainless-steel column, packed with 5-μm Zorbax ODS (DuPont, Wilmington, DE, U.S.A.), was eluted with water–methanol–phosphoric acid (72:28:0.1, v/v) at a flow-rate of 1 ml/min (pressure drop 12.06 MPa or 1750 p.s.i.) and at ambient temperature. The detector was set at 280 nm (0.16 a.u.f.s.).

Gas chromatography

A HP 5830A gas chromatograph (Hewlett Packard, Avondale, PA, U.S.A.), equipped with a flame ionization detector and a HP 18850A integrator (Hewlett Packard), was used. The conditions were as follows: column, 1.8 m × 2 mm I.D., packed with 3% OV-25 on Gas-Chrom Q (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.); injection temperature, 220°C; column temperature, 100°C; detector temperature, 300°C; carrier gas (nitrogen) flow-rate, 11 ml/min.

Analytical procedure for N-acetyl-DL-tryptophan

A 100-μl aliquot of the albumin solution was mixed with 50 μl of a methanolic solution of the internal standard, N-formyl-DL-tryptophan (8.6 m*M*). Proteins were precipitated by the addition of 500 μl of methanol, followed by thorough mixing. After centrifugation (10 min at 750 *g*), a 300-μl aliquot of the supernatant was diluted in 800 μl of water and 50 μl of this final solution were injected in the liquid chromatograph.

For calibration, 100-μl aliquots of stabilizer-free, 20% (w/v) bovine serum albumin were supplemented with increasing amounts (0.243–0.486 μmol) of a methanolic standard solution of N-acetyl-DL-tryptophan (9.74 m*M*), mixed with the internal standard and analyzed as described above. Calibration curves were constructed by plotting peak height ratios (N-acetyl-DL-tryptophan/internal standard) *versus* the corresponding molar concentrations of N-acetyl-DL-tryptophan. The extraction recovery of the latter was determined by analyzing 100-μl aliquots of blank bovine albumin, supplemented with $4.9 \cdot 10^{-4}$ mmol of N-acetyl-DL-tryptophan but with addition of the internal standard after the extraction step *i.e.*, following dilution of 300 μl of the supernatant in 800 μl of water. As a reference, a sample containing 100 μl of water, 500 μl of methanol and the same amount of N-acetyl-DL-tryptophan was prepared; 300 μl were isolated, diluted in water, mixed with the internal standard and treated likewise. The loss of N-acetyl-DL-tryptophan upon deproteinization and extraction with methanol was estimated by comparing the respective peak height ratios ($n = 10$) with those of the reference samples ($n = 3$).

Analytical procedure for octanoic acid

To 500 μl of albumin solution was added 100 μl of a methanolic solution of nonanoic acid (internal standard, $6.45 \cdot 10^{-2}$ m*M*), 400 μl of 1 *M* hydrochloric acid and 10 ml of *n*-hexane. After thorough mixing, 400 μl of 1 *M* hydrochloric acid were added to ensure complete protein precipitation. The solution was re-mixed and centrifuged for 10 min at 3000 rpm. The organic layer was isolated, evaporated to dryness at room temperature under a stream of dry air and the residue reconstituted with 200 μl of 5% (v/v) methanolic sulphuric acid. This mixture was incubated overnight at 65°C in a heating block. After addition of 100 μl of ice–water, the fatty acid methyl esters were extracted with 400 μl of *n*-hexane. A 0.5–1 μl aliquot of this hexane layer was injected into the gas chromatograph.

Calibration was carried out by analyzing mixtures containing increasing amounts (3.4–20.5 μmol) of octanoic acid stock solution ($6.8 \cdot 10^{-2}$ m*M*) and 100 μl of the internal standard stock solution. The analytical (relative) recovery of octanoic acid was determined by supplementing 500 μl of 20% (w/v) albumin solution with octanoic acid (3.4, 6.8 and 10.2 μmol, respectively). After addition of the internal standard, the mixtures were analyzed as outlined above. As a reference, the same amounts of octanoic acid and nonanoic acid were mixed and directly derivatized without prior extraction. The relative recovery of octanoic acid was calculated from a comparison of the two sets of peak height ratios.

RESULTS AND DISCUSSION

N-Acetyl-DL-tryptophan

N-Acetyl-DL-tryptophan in albumin solutions has previously been determined spectrophotometrically, by taking advantage of its absorption band at 280 nm[1]. The aim of this study was to develop a more specific method to deal with the potential interference of other UV-absorbing compounds. Initial attempts to develop a LC system for N-acetyl-DL-tryptophan and its structural analogues N-formyl-DL-tryptophan and N-acetyl-DL-tyrosine using reversed-phase columns eluted with water–methanol or water–acetonitrile invariably resulted in severely tailing peaks. The addition of small amounts of phosphoric acid, however, led to a considerable improvement in peak shape. Other N-acetyl amino acids have previously been chromatographed on a reversed-phase column using mixtures of water–acetonitrile–phosphoric acid[2]. In the present study, substitution of acetonitrile by methanol was found to yield a further improvement. The capacity factors, k', of N-acetyl-DL-tyrosine, N-formyl-DL-tryptophan and N-acetyl-DL-tryptophan under the present conditions were 0.9, 6.5 and 7.2, respectively. In view of the structural similarity between N-acetyl-DL-tryptophan and its formyl analogue, as well as their similar elution positions and near-baseline separation, the latter compound was selected as an internal standard.

For the analysis of albumin solutions, sample pretreatment was initially based on the method of Yu and Finlayson[1] and included deproteinization with perchloric acid, followed by neutralization with saturated potassium carbonate. The latter modification was necessary to protect the LC column. However, because the close structural analogue N-acetyl-DL-tyrosine, unlike N-acetyl-DL-tryptophan, was completely lost during the extraction, perchloric acid was abandoned in favour of methanol as

TABLE I

LINEARITY DATA FOR N-ACETYL-DL-TRYPTOPHAN ANALYZED ON FIVE DIFFERENT DAYS

Concentration (mM)	*Peak height ratio*					$\bar{X}$	*S.D.*	*C.V. (%)*
	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*			
2.43	0.547	0.562	0.586	0.536	0.530	0.552	0.022	4.0
2.92	0.644	0.664	0.678	0.631	0.629	0.649	0.021	3.2
3.41	0.743	0.767	0.775	0.737	0.730	0.750	0.020	2.7
3.89	0.842	0.865	0.883	0.833	0.830	0.851	0.023	2.7
4.38	0.941	0.969	0.972	0.934	–	0.954	0.019	2.0
4.86	1.031	1.073	1.085	1.032	1.031	1.050	0.026	2.5

a protein-denaturing agent. In addition, chromatography showed that alkaline N-acetyl-DLtryptophan stock solutions, as recommended by Yu and Finlayson[1], should be avoided. When these solutions were used to supplement albumin solutions, the resulting N-acetyl-DL-tryptophan peak was found to display a significant shoulder on its descending slope, which is presumably indicative of degradation. This phenomenon was not observed with methanolic stock solutions.

With the modified sample preparation, involving extraction with methanol, the quantitative performance was satisfactory. Linearity data are summarized in Tables I and II. The extraction recovery (± S.D.) of N-acetyl-DL-tryptophan following protein precipitation was found to be 92.1 ± 2.4% ($\bar{x}$ = 4.9 m*M*, n = 10). Three commercial albumin solutions (Schwab, Vienna, Austria), containing 3.5, 20 and 25% (w/v) albumin, respectively, were analyzed. The latter two solutions were diluted four- and five-fold, respectively, in 0.9% sodium chloride, before analysis. Table III lists the results. The relative standard deviation for replicate determinations averaged 1.10% (n = 27).

TABLE II

CALIBRATION CURVES FOR N-ACETYL-DL-TRYPTOPHAN

Equation: $y = ax + b$.

Day	*Slope, a*	*Intercept, b*	*Correlation coefficient, r*
1	0.2005	0.060	0.9999
2	0.2098	0.051	0.9999
3	0.2049	0.082	0.9996
4	0.2049	0.036	0.9999
5	0.2065	0.027	0.9999
Mean	0.2053	0.051	
S.D.	0.0034	0.021	
C.V. (%)	1.66	41.1	

TABLE III

PRECISION OF N-ACETYL-DL-TRYPTOPHAN DETERMINATION IN COMMERCIAL ALBUMIN SOLUTIONS

Protein concentration (%, w/v)	*Concentration of N-acetyl-DL-tryptophan (mM)*		*S.D.*	*C.V.** (%)	*n*
	Expected	*Exptl.*			
3.5	2.8	2.57	0.036	1.38	10
20**	16	10.44	0.051	0.49	7
25	20	17.10	0.245	1.43	10

* Mean C.V. 1.10%.
** Deteriorated solution.

The superiority of LC over spectrophotometry is illustrated in Fig. 1A, which depicts a chromatogram of an old, 20% (w/v) albumin solution. This particular sample had been stored at room temperature for several weeks and showed a marked cloudiness. As evidenced from Table III, the experimentally determined N-acetyl-DL-tryptophan concentration was about 35% lower than that expected. The significant extra peak ($k' = 5.5$) in front of the internal standard peak is probably attributable to a degradation product. Because this by-product obviously absorbs at 280 nm, the spectrophotometric assay would have yielded a falsely high result. Fig. 1B shows a chromatogram of a 25% (w/v) albumin solution stored at 4°C. The unidentified extra peak is virtually absent.

Octanoic acid

Low-molecular-weight fatty acids (C_2–C_6) can be gas chromatographed without prior derivatization on such special phases as FFAP (free fatty acid phase) or

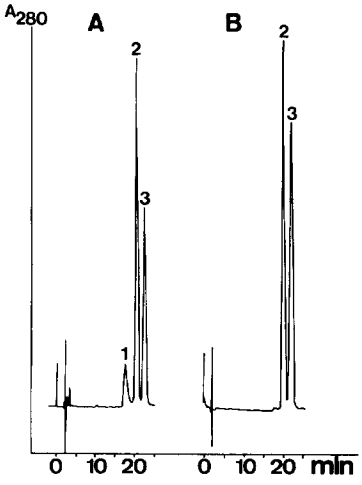

Fig. 1. Liquid chromatographic profile of (A) a deteriorated, 20% (w/v) albumin solution and (B) a 25% (w/v) albumin solution, stored under optimal conditions. Peaks: 1 = unidentified by-product; 2 = N-formyl-DL-tryptophan (internal standard); 3 = N-acetyl-DL-tryptophan.

polyethylene glycol (Carbowax), coated on specially deactivated supports, *e.g.*, graphitized carbon black treated with phosphoric acid[1,3]. According to some authors, these phases would allow the direct chromatography of heptanoic and octanoic acid[1], or even of medium and long chain fatty acids[4], but the performance strongly depends on the quality of the column packing and overall inertness of the chromatographic system. In the present study, the use of FFAP-like phases for the chromatography of octanoic acid yielded broad, considerately tailing peaks. Alternatively, methylation of the acid, after extraction from albumin, was attempted. Of the three different derivatization procedures tested, *i.e.*, flash alkylation with tetramethylammonium hydroxide, dimethylformamide dimethyl acetal treatment or reaction with methanolic sulphuric acid, the latter was superior in terms of reproducibility, simplicity and chromatographic convenience (absence of interfering reagent peaks). Derivatization with diazomethane was not considered in view of the risks involved in its preparation. Fatty acid methyl esters have the important advantage of yielding symmetrical peaks on most common stationary phases.

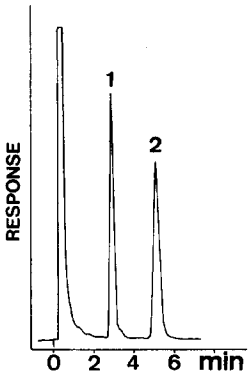

Fig. 2. GC profile of an octanoic acid-containing albumin solution. Peaks: 1 = octanoic acid methyl ester; 2 = nonanoic acid methyl ester (internal standard).

Under the present experimental conditions, the capacity factors, k', of octanoic and nonanoic acid were 4.6 and 8.1, respectively. A representative chromatogram of an extract of a stabilizer-containing albumin solution is shown in Fig. 2. A plot of peak height ratios *versus* concentration was linear (correlation coefficient, r = 0.9996). The equation of the standard curve was $y = ax + b$, where a = 0.031 and b = 0.012. The analytical (relative) recovery of octanoic acid from albumin solutions at the 6.83, 13.6 and 20.48 mM levels averaged 89.7 ± 5.8% (n = 6), respectively. The coefficient of variation for replicate determinations of the compound in albumin solution was 5.5% ($\bar{x}$ = 14.82 mM, n = 9). The results of the analysis of three lots of commercial albumin solutions are presented in Table IV. There is good agreement between the experimentally determined and the expected octanoic acid concentrations.

TABLE IV

DETERMINATION OF OCTANOIC ACID IN COMMERCIAL ALBUMIN SOLUTIONS

Protein concentration (%, w/v)	*Concentration of octanoic acid (mM)*		*S.D.*	*C.V. (%)*	*n*
	Expected	*Exptl.*			
3.5	2.8	2.82	0.06	2.14	3
20	16	15.01	0.17	1.12	3
25	20	19.07	0.03	0.16	3

CONCLUSION

The described analytical methods for the determination of N-acetyl-DL-tryptophan and octanoic acid are sufficient for the quality control, on a routine basis, of commercial albumin solutions for human use. They differ in two respects from the previously reported methods[1]. The N-acetyl-DL-tryptophan assay is considerably more specific than the spectrophotometric procedure. This is of particular interest in connection with deteriorated solutions. Though somewhat more tedious than its earlier counterpart, the described octanoic acid assay is more convenient in practice because it does not require special precautions with regard to the choice of the GC column.

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