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IMPROVED METHOD FOR THE DETERMINATION OF N-ACETYLCYSTEINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved method for the determination of N-acetylcysteine by paired-ion reversed-phase high-performance liquid chromatography has been developed. Following incubation with dithiothreitol to release bound N-acetylcysteine, free N-acetylcysteine and the internal standard N-acetylpenicillamine were derivatised with 2,4-dinitro-1-fluorobenzene. The samples were then deproteinised by ultrafiltration. The dinitrophenyl derivatives were extracted from acidified ultrafiltrate into diethyl ether and purified by using a back-extraction step. They were then separated from naturally occurring plasma components and reagent impurities by high-performance liquid chromatography, utilising an Ultrasphere ODS (5 μm) reversed-phase column and detection at 360 nm.

INTRODUCTION

N-acetylcysteine (NAC) has been shown to be effective as an antidote for the treatment of acetaminophen poisoning, giving protection against liver damage[1]. When administered orally to patients suffering from chronic obstructive bronchitis[2], N-acetylcysteine influences the consistency and volume of sputum.

Recently, a sensitive high-performance liquid chromatographic (HPLC) method for the determination of N-acetylcysteine in human plasma and urine[3] has been published. This method includes a reduction step with dithiothreitol to release N-acetylcysteine bound to protein and existing in the dimer form, *i.e.*, bis-N-acetylcysteine and allows the determination of total compound in samples generated from both intravenous and oral pharmacokinetic studies.

Since the procedure did not include an internal standard considerable expertise was required to maintain good reproducibility.

Several modifications to this method have been introduced to overcome this problem. N-Acetylpenicillamine (NAP) has been included as an internal standard, the chromatographic conditions were further optimised, and a back-extraction step was included in the sample preparation. The modified method is presented together with full validation data.

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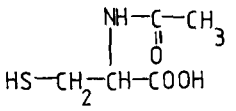
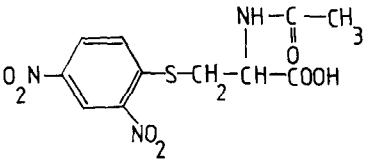
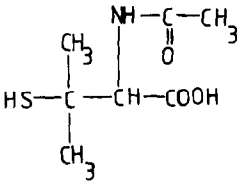
N-Acetylcysteine



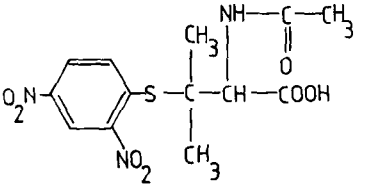
2,4-dinitrophenyl derivative



N-Acetylpenicillamine



2,4-dinitrophenyl derivative



EXPERIMENTAL

Reagents and materials

All reagents used were of analytical grade (BDH, Poole, U.K.) and aqueous reagents were prepared with doubly glass-distilled water (DGDW). Diethyl ether and methanol were HPLC Grade (Rathburn Chemicals, Walkerburn, U.K.).

The 0.067 *M* phosphate buffer pH 8.0 containing 0.005 *M* tetrabutyl ammonium hydrogen sulphate (TBAHS, Sigma, Poole, U.K.), used in the mobile phase, was prepared by placing 11.43 g disodium hydrogen phosphate ($2H_2O$), 0.336 g of potassium dihydrogen phosphate and 1.698 g TBAHS in a 1-l volumetric flask and dissolving in 500 ml DGDW. The pH was adjusted to 8.0 with 5 *M* sodium hydroxide solution and the flask made up to volume with DGDW.

2,4-Dinitro-1-fluorobenzene (DNFB) was of Biochemical grade (BDH, Poole, U.K.) and was recrystallised from diethyl ether before use.

Pure N-acetylcysteine and N-acetylpenicillamine were purchased from Sigma. Standard solutions of 7.5 mg/100 ml N-acetylcysteine and 60.0 mg/100 ml N-acetylpenicillamine in 1% sodium hydrogen carbonate solution were made up fresh daily.

The ultrafiltration cones used were of a membrane type CF50A Centriflo cones (Amicon, Stonehouse, U.K.) and were conditioned immediately prior to use by immersion in DGDW for 1 h and centrifuged for 10 min at 800 *g* to remove excess water.

Extraction procedure

Aliquots (1.5 ml) of test, control and calibration samples were pipetted into 15.0 ml glass extraction tubes provided with PTFE liners and 100 μl of N-acetylpenicillamine internal standard solution was added to all tubes to give a concentration of 40 μg/ml in plasma. A volume of 125 μl of 10 mg/ml dithiothreitol solution was added to each tube which was then vortex mixed, capped and placed in a water bath at 37°C for 30 min. To each tube was then added 1.0 ml of 1% sodium hydrogen

carbonate solution and 180 μl of 10% DNFB in methanol, the tubes were vortex mixed, capped loosely and placed in a water bath at 60°C for 30 min. The tube contents were then decanted into ultrafiltration cones which were placed in 30-ml glass vials and centrifuged at 1000 *g* for 20 min at 20°C. A volume of 400 μl of the ultrafiltrates obtained was pipetted into 15-ml screw-capped extraction tubes containing 3.0 ml of 0.2 *M* hydrochloric acid solution. Diethyl ether (8.0 ml) was added to each tube and the tubes were shaken mechanically at 250 cycles/min for 5 min followed by centrifugation at 750 *g* for 5 min at 10°C. The upper ethereal layers were then transferred with Pasteur pipettes into further 15-ml screw-capped extraction tubes containing 2.0 ml of 1% sodium hydrogen carbonate solution. The tubes were shaken and centrifuged as before and the upper ethereal layers aspirated to waste. A volume of 100 μl of 6 *M* hydrochloric acid solution and 8.0 ml diethyl ether was then added to each tube which was shaken and centrifuged as described previously. The ether layers were transferred with Pasteur pipettes to glass vials and evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried residues were stored at −20°C until they were reconstituted in 100 μl of HPLC mobile phase just prior to chromatography when 20 μl of the reconstituted residue was injected onto the column.

Calibration standards

Calibration samples were prepared from pooled control (drug-free) plasma. 1.5-ml aliquots were spiked by the addition of N-acetylcysteine standard solution to produce a concentration range of 0.125–5.0 μg/ml plasma. Calibration samples were then Vortex mixed, allowed to equilibrate for 5 min and extracted by the procedure detailed previously.

Chromatography and instrumentation

Chromatography was performed on a 25 cm × 4.6 mm I.D. stainless-steel column prepacked with Ultrasphere ODS 5 μm material (Beckman Instruments, High Wycombe, U.K.). The mobile phase consisted of 50% (v/v) methanol and 50% (v/v) 0.067 *M* phosphate buffer containing 0.005 *M* TBAHS. The mobile phase was filtered through a 0.5-μm PTFE membrane filter (Whatman Labsales, Maidstone, U.K.), degassed by sonication, purged with pure helium and delivered to the column at a flow-rate of 1.0 ml/min by a LDC Constametric III HPLC pump. The eluate stream was coupled to an LDC Spectromonitor III detector Model 1204D with a Max N, Series LS 23 high-efficiency cell set at a wavelength of 360 nm and an attenuation of 0.05 a.u.f.s. The signal from the detector was recorded on a Rikadenki chart recorder (Rikadenki Mitsui, Machinery Sales, Chessington, U.K.) set at a range of 10 mV and a chart speed of 12 cm/h.

The sample extracts were introduced into the chromatograph with a Rheodyne valve injector (Type 7125, Rheodyne, Cotati, CA, U.S.A.) with a 20-μl sample loop.

Typical chromatograms of a blank plasma sample and of plasma samples spiked with N-acetylcysteine and N-acetylpenicillamine are shown in Fig. 1. Under the conditions described in the analytical procedure the retention times for N-acetylcysteine and N-acetylpenicillamine were 7.8 min and 13.3 min respectively.

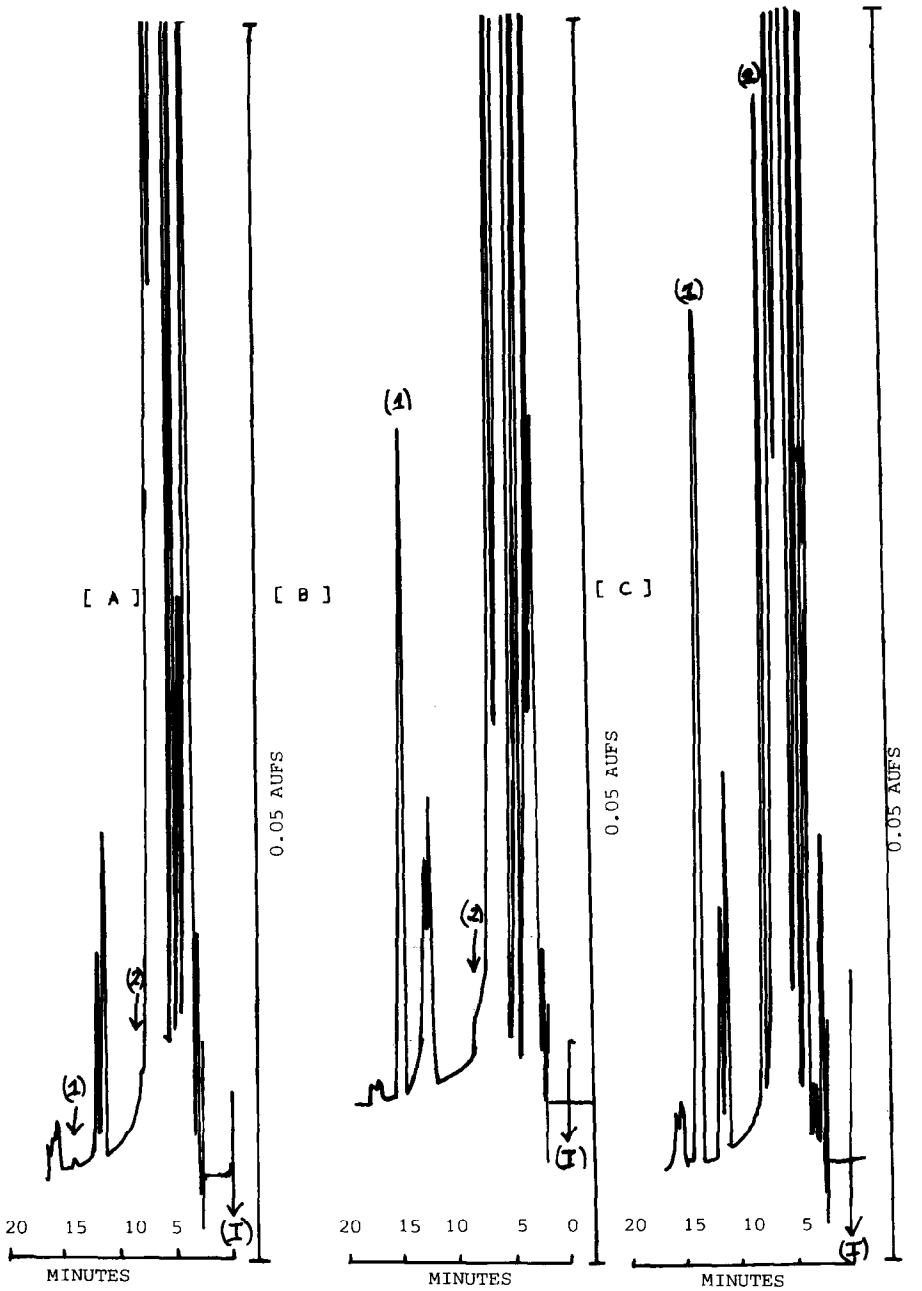


Fig. 1. (A) Chromatogram of control drug-free human plasma. (B) Chromatogram of spiked plasma, containing 40 μg/ml N-acetylpenicillamine. The 2,4-dinitrophenyl derivative of N-acetylpenicillamine (1) is indicated. (C) Chromatogram of spiked plasma containing 40 μg/ml N-acetylpenicillamine and 5 μg/ml N-acetylcysteine. The 2,4-dinitrophenyl derivatives of N-acetylpenicillamine (1) and N-acetylcysteine (2) are indicated. I indicates the point of injection.

RESULTS

Calibration

Standard calibration lines were constructed by plotting the peak height ratio of the 2,4-dinitrophenyl derivative of N-acetylcysteine to the 2,4-dinitrophenyl derivative of N-acetylpenicillamine against the concentration of N-acetylcysteine over the range 0.125–5.0 μg/ml. Table I shows the mean values of peak height ratios obtained with the calculations of between batch coefficient of variations (C.V.).

TABLE I

REPLICATE PLASMA CALIBRATION LINES

	Concentration of N-acetylcysteine (μg/ml)	*Number of determinations*	*Mean peak height ratios*	*Standard deviation (S.D.)*	*Coefficient of variation (C.V.)*
S0	0	5	–	–	–
S1	0.125	5	0.029	0.004	12.4
S2	0.25	5	0.061	0.009	14.6
S3	0.50	5	0.132	0.017	12.7
S4	1.25	5	0.289	0.029	10.2
S5	2.50	5	0.489	0.038	7.9
S6	3.75	5	0.790	0.078	9.8
S7	5.00	5	0.982	0.061	6.2

From this data linear regression analysis of plasma concentrations of N-acetylcysteine (X) against peak height ratios (Y) gives: $Y = 0.1989X + 0.016$. The mean correlation coefficient was found to be 0.9984.

Recovery

The absolute recovery of N-acetylcysteine as the dinitrophenyl derivative was calculated over the calibration range by comparing peak heights for DGDW-spiked standards to plasma-spiked standards and taken through the complete analytical procedure. The recovery data are calculated over the calibration range by comparing peak heights for DGDW-spiked standards to plasma-spiked standards and taken through the complete analytical procedure. The recovery data are tabulated in Table II which gives the following: The mean absolute recovery of N-acetylcysteine = 82.1% (5 determinations), S.D. = 4.7, C.V. = 5.1%.

Reproducibility

Randomly numbered drug-free plasma samples spiked with N-acetylcysteine were assayed blind by the described analytical procedure.

Within-batch quality assurance data is shown in Table III. Between-batch data is given in Table IV for sets of spiked samples assayed on consecutive days.

Detection limits

The minimum detectable level (with 80% confidence limits) was computed for N-acetylcysteine for each calibration. The mean minimum detectable level is 0.26

TABLE II

RECOVERY STUDY DATA

N-Acetylcysteine concentration (μg/ml)	*Plasma standards peak height (mm) 2,4-dinitrophenyl derivative of N-acetylcysteine*	*Double glass-distilled water standards (DGDW) peak height (mm) 2,4-dinitrophenyl derivative of N-acetylcysteine*	*Recovery of N-acetylcysteine from plasma (%)*
0.51	18	21	85.7
1.28	43	57	75.4
2.55	83	95	87.4
3.83	126	156	80.8
5.10	137	169	81.1

TABLE III

WITHIN-BATCH QUALITY ASSURANCE DATA

Number of determinations	*N-Acetylcysteine concentration*			
	Spiked value (μg/ml)	*Mean value found (μg/ml)*	*S.D.*	*C.V.*
10	0.93	0.86	0.09	10.5
10	2.08	2.19	0.15	7.0
10	4.17	4-32	0.18	4.3

TABLE IV

BETWEEN-BATCH QUALITY ASSURANCE DATA

Number of determinations	*N-Acetylcysteine concentration*			
	Spiked value (μg/ml)	*Mean value found (μg/ml)*	*S.D.*	*C.V.*
6	1.25	1.37	0.12	8.5
6	2.50	2.49	0.13	5.1
6	3.75	3.76	0.14	3.7

μg/ml (S.D. = 0.10 μg/ml). The observed detection limits defined as the minimum peak discernible over twice the level of baseline noise varied between 0.02 and 0.15 μg/ml.

DISCUSSION

The chromatographic conditions were optimised to give the best compromise between chromatographic resolution and peak symmetry and relatively short retention times so good accuracy and precision were achieved. For this purpose an ion-

pair modifier TBAHS was added to the mobile phase and an Ultrasphere ODS 5-μm column was used.

Both within- and between-batch precision and accuracy were improved by the addition of NAP as the internal standard. This also allowed a back-extraction clean-up step to be included in the procedure which minimised background interference.

It is thought that the variation found in the observable detection limits is caused by endogenous NAC, but a further investigation needs to be carried out to confirm this.

CONCLUSION

A sensitive specific and reproducible HPLC procedure has been described for the determination of NAC in human plasma. The method with a detection limit of approximately 0.1 μg/ml is sufficiently sensitive to determine the drug in human plasma following single oral dosing of NAC.

Good reproducibility, between- and within-batch precision and accuracy has been established.

N-Acetylpenicillamine has been shown to be suitable as an internal standard.

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