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Note

Determination of aspirin and its major metabolites in plasma by high-performance liquid chromatography without solvent extraction

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Aspirin (acetylsalicylic acid, ASA) is widely used for its analgesic, anti-inflammatory and antipyretic effects. It has been considered to be the drug of first choice in the treatment of rheumatoid arthritis [1, 2]. Aspirin has also been found to interfere with platelet aggregation and is now approved by the U.S. Food and Drug Administration as a therapeutic substance for reducing the risk of transient ischaemic attacks of stroke. In man, aspirin is rapidly hydrolyzed to salicylic acid (SA) which is further metabolized to salicyluric acid (SU), gentisic acid (GA), gentisuric acid (GU), salicyl acyl glucuronide (SAG), and salicyl phenolic glucuronide (SPG). Since aspirin, salicylic acid, salicyluric acid and gentisic acid have all been reported to show differing pharmacological effects as analgesics, antiplatelet agents and as inhibitors of prostaglandin synthetase in vitro [3, 4], it is desirable for analytical procedures to be able to quantify each substance in a blood sample from a subject after ingestion of aspirin.

The most frequently used analytical procedures for aspirin and its metabolites in biological fluids are colorimetry [5], fluorometry [6, 7] and gas—liquid chromatography [8]. Colorimetric procedures are limited by their poor specificity for salicylate and their dependence on a differential hydrolysis of aspirin to salicylic acid for quantification of aspirin. Fluorometric procedures have also depended on the hydrolysis of aspirin to salicylic acid for quantification. Gas—liquid chromatographic procedures have been

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found to be time consuming and are complicated by partial hydrolysis of aspirin during derivatization [9].

High-performance liquid chromatography (HPLC) is the most recent analytical technique used for the determination of salicylates in biological fluids [10—14]. In two of these methods aspirin has been determined [10, 14] but these involved time consuming extraction and evaporation processes. A specific, rapid and sensitive method for the determination of aspirin, salicylic acid, salicyluric acid and gentisic acid in plasma is now presented which requires only a simple protein precipitation step. In addition, the same chromatographic system can be used to quantify the total amount of salicylate excreted in the urine.

EXPERIMENTAL

Standards and reagents

Aspirin (acetylsalicylic acid), salicyluric acid, gentisic acid, phthalic acid and *o*-methoxybenzoic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Salicylic acid (AnalaR grade) and *p*-toluic acid were obtained from BDH Chemicals (Poole, Great Britain) and Hopkins and Williams (Essex, Great Britain), respectively.

Acetonitrile and methanol were specially purified for HPLC and supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were analytical grade.

Instrumentation

Reversed-phase HPLC was performed using a Waters Model M6000A solvent delivery system and a U6K universal injector. A C_{18} μBondapak column (300 × 3.9 mm I.D., 10 μm average particle size) and a guard column (23 × 3.9 mm I.D.) packed with μBondapak C_{18}/Porasil B (also from Waters Assoc.) were used in all studies. The absorbance of the eluent was determined using a Waters Model 450 variable wavelength UV absorption detector. The absorbance was recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). Injections were made with a 50-μl Hamilton syringe.

Assay procedure for the measurement of aspirin and its metabolites in human plasma

Plasma samples were processed by transferring a 200-μl quantity into a glass tube (disposable borosilicate glass culture tubes, 6 × 50 mm, Kimble, IL, U.S.A.), and adding 20 μl perchloric acid (30%) solution containing the internal standard, *p*-toluic acid (0.02%). To this solution, 200 μl of methanol were then added. The sample was vortexed for 2 min and centrifuged at 1500 *g* for 5 min. An aliquot (20 μl) of clear supernatant was injected onto the column. The mobile phase consisted of acetonitrile—0.03% phosphoric acid, pH 2.5 ± 0.1 (30:70). The flow-rate was 1 ml/min. Absorbance was monitored at a wavelength of 237 nm.

Assay procedure for the measurement of total salicylate in urine

Total salicylate was estimated from the salicylic acid resulting from the hydrolysis of salicylate conjugates. For this hydrolysis process, 2 ml of urine were mixed with 2 ml of concentrated hydrochloric acid (10 *N*) in a 10-ml glass ampoule. The air inside the ampoule was removed by flushing with oxygen-free nitrogen immediately prior to sealing the ampoule. The ampoule and its contents were autoclaved at 120°C for 3 h. On cooling, the contents of the ampoule were vortexed for 10 sec and 20—100 μl of hydrolysate were made up to 300 μl with distilled water. To this solution, 200 μl acetonitrile were added. This solution was vortexed for 30 sec and centrifuged at 1500 *g* for 5 min. An aliquot (20 μl) of supernatant was injected onto the column. Chromatographic conditions used for the analysis of total salicylate in urine were identical to those used for the determination of aspirin and metabolites in plasma, except that the absorbance of the eluent was monitored at a wavelength of 313 nm.

Preparation of standard curves

Plasma standards were prepared by spiking drug-free plasma with known amounts of each analyte to produce concentrations of 0.5—200 μg/ml. The standards were then assayed in the described manner. Standard curves were then prepared by plotting the ratio of the peak height of analyte to peak height of internal standard versus concentration of analyte. Standards for salicylic acid, salicyluric acid and gentisic acid were prepared monthly and stored at −20°C between use. Standards for aspirin were prepared immediately prior to assay by spiking drug-free plasma with freshly prepared solutions of aspirin in methanol then vortexing vigorously for 30 sec. The strengths of the aspirin solutions were such that the amount of methanol added to the plasma never exceeded 1%.

To obtain standard curves for the hydrolysed urine, aqueous standard solutions of salicylic acid (2—500 μg/ml) were subjected to the same hydrolytic procedure as urine samples to be analyzed for total salicylate. Standard curves were prepared by plotting the peak height of salicylate versus concentration.

RESULTS AND DISCUSSION

Plasma samples

Typical chromatograms are shown in Fig. 1. The retention times for gentisic acid, salicyluric acid, aspirin, salicylic acid and *p*-toluic acid (internal standard) are 5.2, 6.2, 8.0, 11.2 and 13.6 min, respectively. With the present solvent system, a given analysis is completed in about 15 min.

The wavelength of 237 nm used in the present assay gave the best detector response for each salicylate component. This wavelength approximates to the optimal absorbance wavelength for gentisic acid, salicyluric acid and salicylic acid. Although the detector sensitivity is maximal for aspirin at 229 nm, the noise-to-signal ratio in the detector response is excessive at this wavelength. At the other absorbance maximum of 280 nm for aspirin, the detector response for aspirin is approximately one fifth the response obtained at 237 nm. At 237 nm, the limits of detection (with 20 μl supernatant) for gentisic

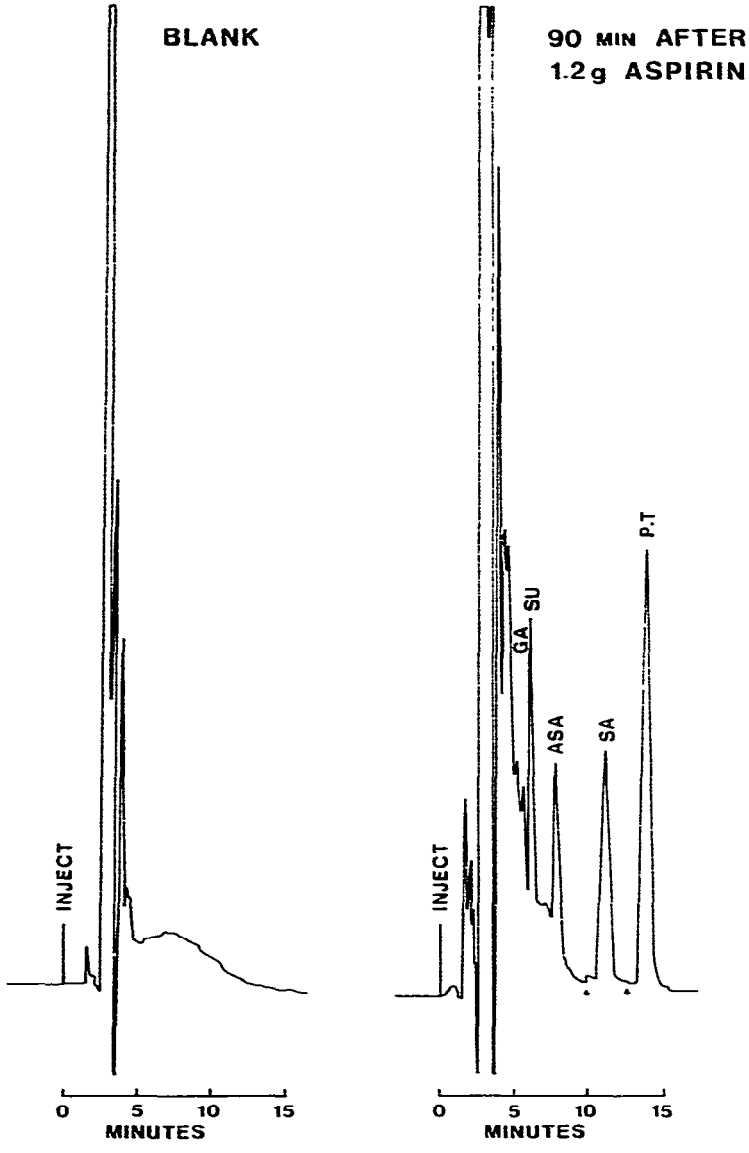

Fig. 1. Chromatograms of blank plasma and of plasma from the same subject 90 min after ingestion of 1.2 g soluble aspirin. The plasma concentrations of gentisic acid (GA), salicyluric acid (SU), aspirin (ASA) and salicylic acid (SA) are estimated to be 0.36, 2.4, 3.2 and 88.9 μg/ml, respectively; *p*-toluic acid (P-T) is the internal standard. Arrows indicate change in absorbance scale.

acid, salicyluric acid, aspirin and salicylic acid are 0.2, 0.5, 0.1 and 0.1 μg/ml, respectively.

As aspirin has been shown previously to exhibit weak fluorescence [15], an attempt was made to improve the sensitivity of the assay using a fluorescence detector (Schoeffel, FS970, Schoeffel, Westwood, NJ, U.S.A.). With an emission filter (KV320), the fluorescence of aspirin was found to

be optimal at an excitation wavelength of 229 nm but was insufficient to provide better detection sensitivity than found with the monitoring of eluent absorbance at 237 nm in the present assay system.

Linearity of response was found to be good (> 0.99) and consistently reproducible for standard curves based on both peak height ratio of salicylate compound to peak height of internal standard and for absolute peak height of a given salicylate compound following the injection of a known volume of aliquot. The reproducibility of the assay is given in Table I.

TABLE I

PRECISION OF ASSAY FOR ASPIRIN (ASA), SALICYLIC ACID (SA), SALICYLURIC ACID (SU) AND GENTISIC ACID (GA) IN PLASMA (n = 10)

	ASA	SA	SU	GA
Mean (μg/ml)	20.09	23.52	23.20	5.91
Standard deviation	0.914	0.595	0.736	0.132
Coefficient of variation (%)	4.55	2.53	3.17	2.23

Perchloric acid, used routinely for protein precipitation in other HPLC assays [16], was found to give complete protein precipitation. Only partial extraction of salicylate compounds from precipitated protein was achieved when methanol was not added. Addition of methanol and vortexing for 2 min was necessary for complete extraction of salicylate compounds from plasma protein. Complete extraction was indicated by standard curves (absolute peak height) of the salicylate compounds and *p*-toluic acid prepared in plasma being identical to standard curves for these compounds prepared in water. The supernatant of the present assay protein precipitation procedure gave a good, almost noise-free baseline after the initial plasma peaks, only one endogenous plasma peak was sometimes found at 8.8 min. This peak did not interfere with any of the compounds under investigation. Acetonitrile used as a protein precipitant in other HPLC assays for salicylate [13] was found to be unsuitable in the present assay. The supernatant of acetonitrile-extracted plasma resulted in a noisy baseline with interfering peaks being observed in the chromatogram.

The internal standard (*p*-toluic acid) chosen in the present plasma assay eluted after the other salicylate compounds and was not affected by endogenous plasma peaks. It was found, however, that recycling of the mobile phase resulted in an increase in the height of the *p*-toluic acid peak possibly due to some interaction with active binding sites on the column. No change in the peak height of *p*-toluic acid was found when the mobile phase was not recycled. Internal standards used in some other HPLC plasma salicylate assays were found to be unsuitable for the present plasma assay. Phthalic acid used by Peng et al. [10] overlapped with the gentisic acid peak. *o*-Methoxybenzoic acid has also been used as an internal standard in HPLC methods for the determination of salicylate [11—13], but was found to co-chromatograph with aspirin using the present system.

Interference by other drugs was studied by analyzing plasma samples from patients taking various drugs. Table II lists the drugs being taken by these patients. None of these drugs nor altered blood constituents, such as elevated bilirubin, interfered with the assay.

Since aspirin is rapidly hydrolyzed in whole blood to salicylic acid, it is essential to collect blood in a manner so as to minimize hydrolysis. Potassium fluoride (5 mg/ml) was found to slow aspirin hydrolysis in whole blood at 37°C more effectively than the cholinesterase inhibitor, ecothiopate (0.25 and 0.025 mg/ml). The rate of aspirin hydrolysis was halved by ecothiopate and reduced to a quarter with potassium fluoride. This extent of inhibition was also greater than that reported for physostigmine [17]. The greater effectiveness of the weak cholinesterase inhibitor potassium fluoride may result from its better penetration into red blood cells. The technique of collecting blood samples into chilled sample tubes containing potassium fluoride [8] was therefore adopted in the present assay system. As the half-life of aspirin in frozen plasma containing potassium fluoride is about 23.5 days [18], its is desirable to analyze plasma as quickly as possible after collection.

TABLE II

DRUGS TESTED FOR INTERFERENCE IN PLASMA

Allopurinol	Ibuprofen	Prazosin
Amiloride	Imipramine	Prochlorperazine
Ampicillin	Indomethacin	Quinidine
Chloral hydrate	Metamucil	Salbutamol
Chlormethiazole	Multivite	Spironolactone
Dextropropoxyphene	Nitrazepam	Sulindac
Diazepam	Paracetamol	Theophylline
Digoxin	Phytomenadione	Thiamine
Folic acid	Potassium chloride	Trimipramine
Frusemide		

The present assay system was used to monitor plasma aspirin levels with time from a healthy male adult given 1.2 g soluble aspirin in 100 ml of water. Blood samples from this subject were collected into chilled containers containing potassium fluoride [8]. The resultant plasma concentration—time profiles for aspirin, salicylic acid, salicyluric acid and gentisic acid are shown in Fig. 2. This plasma concentration—time profile is similar to those reported by other workers for comparable doses of aspirin [8, 13, 19].

Urine samples

As virtually all aspirin absorbed into the body can be accounted for by the recovery of salicylic acid in urine after hydrolysis of its conjugates [20, 21], the quantification of salicylic acid in urine using the HPLC conditions of the plasma aspirin assay permits a rapid evaluation of the extent of aspirin absorption. Fig. 3 shows chromatograms of hydrolyzed urine samples collected from a subject before and after a dose of aspirin. Salicylic acid is well separated from all urine peaks and can be quantified to as low as 1 μg/ml with an intra-assay coefficient of variation of 4.1%. Gentisic acid, which

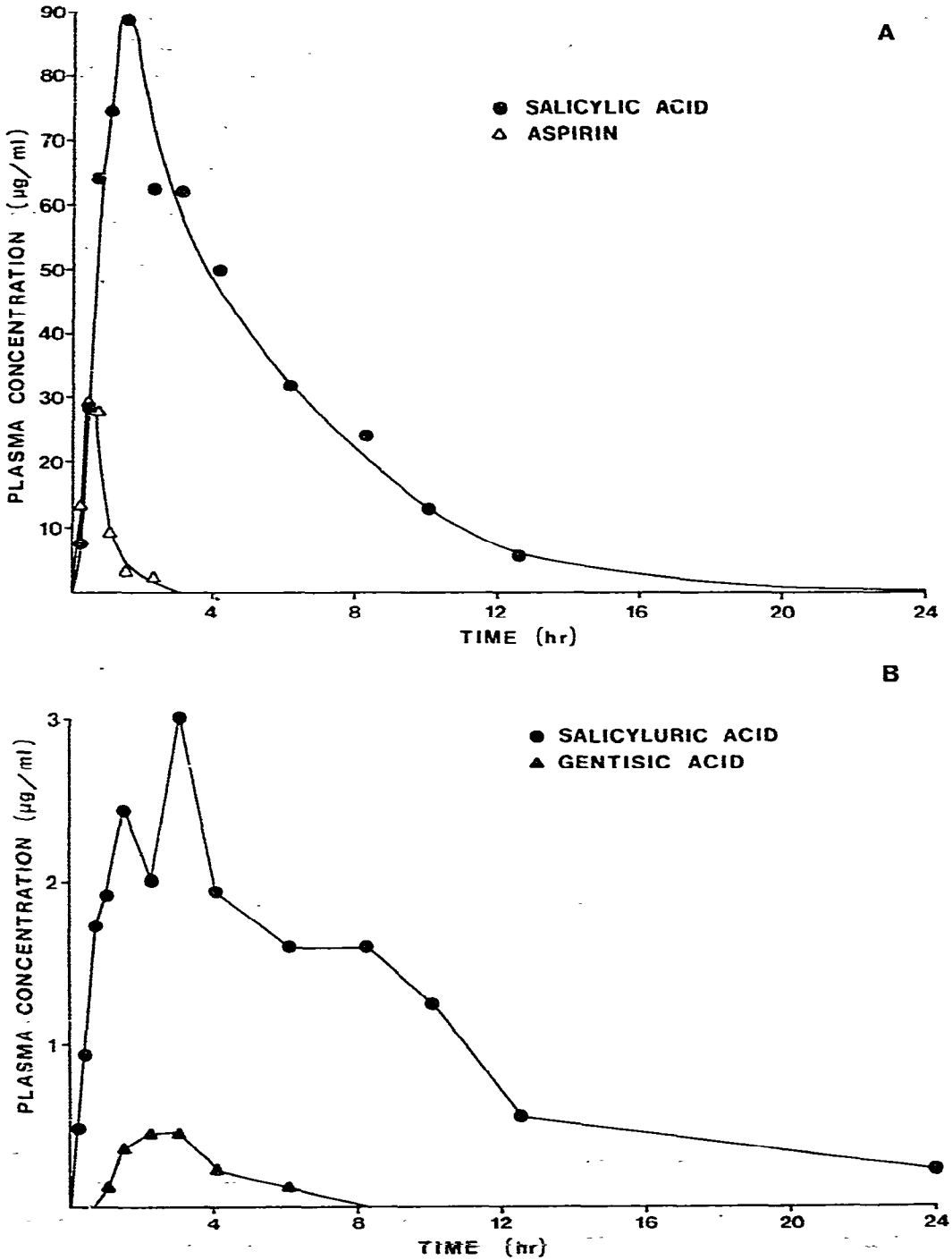

Fig. 2. Time course of (A) plasma aspirin and salicylic acid concentrations and (B) plasma salicyluric acid and gentisic acid concentrations found in a subject after ingestion of 1.2 g soluble aspirin.

Fig. 3. Chromatograms of blank hydrolyzed urine and of hydrolyzed urine collected from the same subject 4 h after ingestion of 600 mg soluble aspirin. The concentration of salicylic acid (SA) in the hydrolyzed urine is estimated to be 186 μg/ml. Arrow indicates change in absorbance scale.

accounts for 1—3% of the dose of aspirin recovered in the urine after hydrolysis [20, 21], is poorly resolved from endogenous urine peaks and therefore not readily quantified with the present HPLC assay. Salicylic acid was completely recovered from urine samples and gave a linear standard curve of peak height versus salicylate concentration at the less intense but more

selective detector wavelength of 313 nm used to minimize interference in the urine assay. The total amounts of salicylate recovered in the urine of subjects after doses of aspirin were comparable to values obtained by other analytical procedures [6, 20—23].

CONCLUSION

The present procedure for quantifying aspirin in plasma differs from previous analytical procedures in that the time consuming extraction processes are replaced by a protein precipitation step. In addition, the use of the same chromatographic conditions for both plasma and hydrolyzed urine allows the pharmacokinetics of aspirin after oral administration to be readily followed.

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REFERENCES

1 M.D. Muirden, Curr. Ther., 21 (1980) 215.
2 R.H. Rumble, P.M. Brooks and M.S. Roberts, Brit. J. Clin. Pharmacol., 9 (1980) 41.
3 R.J. Flower and J.R. Vane, in H.J. Robinson and J.R. Vane (Editors), Prostaglandin Synthetase Inhibitors, Raven Press, New York, 1974, p. 9.
4 C. Patrono, G. Ciabattoni, F. Pugliese, E. Pinca, G. Castrucci, A. De Salvo, M.A. Satta and M. Parachini, Agents Actions Suppl., 4 (1979) 138.
5 L.A. Muni, J.L. Leeling, R.J. Helms, N. Johnson, J.J. Bare and B.M. Phillips, J. Pharm. Sci., 67 (1978) 289.
6 D. Schatcher and J. Manis, J. Clin. Invest., 37 (1958) 800.
7 J. Putter, Arzneim.-Forsch., 25 (1975) 941.
8 M. Rowland and S. Riegelman, J. Pharm. Sci., 56 (1967) 715.
9 S.L. Ali, Chromatographia, 8 (1975) 33.
10 G.W. Peng, M.A.F. Gadalla, V. Smith, A. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 710.
11 D. Blair, B.H. Rumack and R.G. Peterson, Clin. Chem., 24 (1978) 1543.
12 C.P. Terweij-Groen, T. Vahlkamp and J.C. Kraak, J. Chromatogr., 145 (1978) 115.
13 B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff and M. Rowland, Clin. Chem., 25 (1979) 1420.
14 L.Y. Lo and A. Bye, J. Chromatogr., 181 (1980) 473.
15 C.I. Miles and G.H. Schenk, Anal. Chem., 42 (1970) 656.
16 P.I. Andriaenssens and L.F. Prescott, Brit. J. Pharmacol., 6 (1978) 87.
17 D.M. Imhoff, B.E. Cham, L.M. Cotter and F. Bochner, Proc. Aust. Physiol. Pharmacol. Soc., 10 (1979) 327P.
18 L.J. Walter, D.F. Biggs and R.T. Coutts, J. Pharm. Sci., 63 (1974) 1754.
19 G.G. Graham, G.D. Champion, R.O. Day and P.D. Paull, Clin. Pharmacol. Ther., 22 (1977) 410.
20 B.E. Cham, F. Bochner, D.M. Imhoff, D. Johns and M. Rowland, Clin. Chem., 26 (1980) 111.
21 G. Levy, T. Tsuchiya and L.P. Amsel, Clin. Pharmacol. Ther., 13 (1972) 258.
22 G. Levy, J. Pharm. Sci., 54 (1965) 959.
23 M.A. Page, R.A. Anderson, K.F. Brown and M.S. Roberts, Aust. J. Pharm. Sci., NS3 (1974) 95.