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EXTRACTIVE ALKYLATION OF SULPHONAMIDE DIURETICS AND THEIR DETERMINATION BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

The extractive alkylation of 11 sulphonamide diuretics has been evaluated using tetrabutylammonium, tetrapentylammonium or tetrahexylammonium as counter ion at different pH values and methyl iodide in methylene chloride as the organic phase. The sulphonamides are methylated within 20 min with tetrahexylammonium as counter ion in 0.2 *M* sodium hydroxide at 50°. The derivatives have been identified by mass spectral and nuclear magnetic resonance analysis. Relative retentions of the derivatives are given using 1% SE-30 as the stationary phase. A contaminant, dimethylsulphuric acid, occurs in methyl iodide and seriously disturbs the gas chromatographic analysis. The application of the extractive alkylation to biological samples is demonstrated by the direct analysis of acetazolamide in serum. 0.1 *M* tetrapentylammonium in 0.5 *M* sodium hydroxide is suitable as the aqueous phase with 1.6 *M* methyl iodide as alkylating reagent in methylene chloride. The trimethyl derivative of acetazolamide formed has been determined by electron-capture gas chromatography down to 0.5 μg/ml in a 0.1-ml serum sample. The relative standard deviation at the 10 μg/ml level is 6.6% ($n = 10$).

INTRODUCTION

Extractive alkylation can be considered as a two-stage process comprising the extraction of an organic acid as an ion pair to an organic solvent in which the alkylation takes place. The technique has been particularly useful in the conversion of low concentrations of acidic drugs into derivatives amenable to gas chromatographic analysis. Extractive alkylation of carboxylic acid[1–3], phenols[4,5], barbituric

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acids[6–8] and sulphonamides[9–11] has been demonstrated. The application of this technique to the determination by electron-capture gas chromatography (EC–GC) of these compounds in biological specimens has also been demonstrated.

One of the first analytical procedures based on extractive alkylation was developed for the sulphonamide diuretic, chlorthalidone[12]. Under the same conditions, other sulphonamide diuretics have been derivatized and determined by EC–GC, *e.g.*, furosemide[13], hydrochlorothiazide[14] and bendroflumethiazide[15]. The present paper evaluates extractive alkylation conditions for 11 sulphonamide diuretics. The application of this method to the determination of diuretics in biological specimens is demonstrated with acetazolamide.

EXPERIMENTAL

Apparatus

A Pye GCV and a Packard-Becker 409 gas chromatograph were used with flame ionization or ^{63}Ni electron-capture detectors. The glass columns (150 × 0.18 cm) were filled with 3% QF-1 on Gas-Chrom Q (100–120 mesh), 3% OV-17 on Gas-Chrom Q (100–120 mesh), or 1% SE-30 on Gas-Chrom Q (80–100 mesh) and operated at 250°, 280° and 230°, respectively. The injector and detector temperatures were 280°. The flow-rate of nitrogen carrier gas was 40 ml/min.

The methylated sulphonamides were identified on an LKB 2091 gas chromatograph–mass spectrometer. The glass column contained 1% SE-30 on Gas-Chrom Q (80–100 mesh). The ionisation energy was 70 eV.

Nuclear magnetic resonance (NMR) spectra were recorded on Varian XL 100 and Jeol Fx 100 spectrometers in deuteriochloroform as solvent and with tetramethylsilane as reference.

Reagents and chemicals

0.1 *M* tetrabutylammonium and 0.05 *M* tetrahexylammonium ion solutions were prepared by neutralization of the corresponding hydrogensulphate (Labkemi, Stockholm, Sweden) and purified, if necessary, by washing four times with methylene chloride and twice with heptane. The solutions were diluted to volume with buffer or water. 0.2 *M* tetrapentylammonium solution was made from the iodide salt (Eastman-Kodak, Rochester, N.Y., U.S.A.) by shaking overnight with an equivalent amount of silver oxide and, after filtration, diluting to volume with water. Methyl iodide was supplied by E. Merck (Darmstadt, G.F.R.) or Hopkin & Williams (Chadwick Heath, Great Britain). Methylene chloride and toluene (E. Merck) were distilled before use. Silver sulphate (E. Merck) was prepared as a saturated solution in water. A standard solution of acetazolamide (2-acetamido-5-sulphamoyl-1,3,4-thiadiazole) was obtained by dissolving the compound in water and diluting to 100 μg/ml. Aliquots of 0.05, 0.1, 0.2 and 0.4 ml of this solution were diluted to 1.0 ml with serum. 2-[2-Chlorophenyl]-5-sulphamoyl-1,3,4-thiadiazole dissolved in 0.1 *M* sodium hydroxide and diluted to 20 μg/ml with water was used as the internal standard.

Identity of methylated sulphonamides

The following spectroscopic data were obtained from the sulphonamides after methylation.

Trimethyl acetazolamide. Mass spectrum (MS): *m/e* (percentage relative abundance) = 43 (70), 83 (39), 108 (45), 250 (100), 264.

Tetramethyl bendroflumethiazide. MS: *m/e* = 42 (21), 91 (12), 278 (23), 386 (100), 477 (M^+, *m/e ca.* 0). NMR: δ = 2.82 (s, 3H), 2.89 (s, 6H), 2.96 (s, 3H), 3.1–3.72 (m, 2H), 4.84 (t, 1H, *J* 8 Hz), 7.1 (s, 1H), 8.36 (s, 1H).

Trimethylbumetanide. MS: *m/e* = 44 (44), 254 (56), 318 (44), 363 (100), 406 (M^+, *m/e* 99). NMR: δ = 0.8 (t, 3H, *J* 6, 3 Hz), 0.96–1.6 (m, 4H), 2.76 (s, 6H), 3.08 (t, 2H, *J* 6, 3 Hz), 3.91 (s, 3H), 6.82–7.36 (m, 5H), 7.5 (d, 1H, *J* 2 Hz), 7.89 (s, 1H, *J* 2 Hz).

Tetramethylchlorthalidone. MS: *m/e* = 176 (43), 287 (100), 363 (86), 394 (M^+, *m/e ca.* 0). NMR: δ = 2.76 (s, 3H), 2.9 (s, 6H), 2.96 (s, 3H), 7.08–7.58 (m, 5H), 7.87 (dd, 1H, J_1 5.6 Hz, J_2 3 Hz), 8.21 (d, 1H, *J* 2 Hz).

Trimethylclopamide. MS: *m/e* = 55 (26), 91 (42), 11 (84), 112 (100), 141 (97), 387 (M^+, *m/e ca.* 0). NMR: δ = 1.15 (d, 6H, *J* 6 Hz), 1.3–1.9 (m, 6H), 2.2–2.55 (m, 2H), 2.92 (s, 9H), 7.11 (d, 1H, *J* 8 Hz), 7.98 (dd, 1H, J_1 8 Hz, J_2, 2 Hz).

Tetramethylcyclopenthiazide. MS: *m/e* = 352 (100), 436 (M^+, *m/e* 3.3). NMR: δ = 1.0–1.35 (m, 2H), 1.5–2.1 (m, 9H), 2.75 (s, 3H), 2.87 (s, 6H), 3.05 (s, 3H), 4.86 (dd, 1H, J_1 10.4 Hz, J_2 4.6 Hz), 6.8 (s, 1H), 8.3 (s, 1H).

Trimethylfurosemide. MS: *m/e* = 81 (100), 96 (16), 372 (M^+, *m/e* 48). NMR: δ = 2.8 (s, 6H), 3.84 (s, 3H), 4.4 (d, 2H, *J* 5.6 Hz), 6.2–6.35 (m, 2H), 6.81 (s, 1H), 7.35 (s, 1H), 8.53 (s, 1H).

Tetramethylhydrochlorothiazide. MS: *m/e* = 42 (89), 44 (89), 138 (55), 202 (47), 218 (40), 288 (37), 310 (100), 353 (M^+, *m/e* 87). NMR: δ = 2.79 (s, 3H), 2.87 (s, 6H), 3.08 (s, 3H), 4.9 (s, 2H), 6.81 (s, 1H), 8.28 (s, 1H).

Dimethylmefruside. MS: *m/e* = 43 (75), 85 (100), 410 (M^+, *m/e ca.* 0). NMR: δ = 1.23 (s, 3H), 1.6–2.1 (m, 4H), 2.93 (s, 9H), 3.06 (s, 2H), 3.7–3.95 (m, 2H), 7.65 (d, 1H, *J* 8.2 Hz), 7.84 (dd, 1H, J_1 8.2 Hz, J_2 2 Hz), 8.38 (d, 1H, *J* 2 Hz).

Trimethylpolythiazide. MS: *m/e* = 44 (100), 73 (23), 182 (15), 198 (15), 245 (22), 290 (19), 372 (10), 398 (6), 481 (M^+, *m/e* 54). NMR: δ = 2.88 (s, 6H), 2.91 (s, 3H), 3.13 (s, 3H), 2.78–3.80 (m, 4H), 4.58–4.78 (dd, 1H, J_1 9.4 Hz, J_2 5.6 Hz), 6.81 (s, 1H), 8.29 (s, 1H).

Tetramethylpolythiazide. MS: *m/e* = 44 (86), 73 (92), 196 (51), 304 (100), 495 (77). NMR: δ = 2.74–3.35 (m, 19H), 6.77 (s, 1H), 8.21 (s, 1H).

Tetramethylquinethazone. MS: *m/e* = 44 (51), 208 (33), 316 (100), 345 (M^+, *m/e* 2). NMR: δ = 0.88–1.00 (t, 3H, *J* 7 Hz), 1.66–1.92 (m, 2H), 2.86 (s, 6H), 3.03 (s, 3H), 3.12 (s, 3H), 4.58 (t, 1H, *J* 4.8 Hz), 6.61 (s, 1H), 8.44 (s, 1H).

Extractive alkylation of sulphonamide diuretics

(A) Tetrabutylammonium as counter ion. The sulphonamide was dissolved in water to a concentration of *ca.* 5 mg/ml. A 0.1-ml aliquot of this solution was added to 1 ml of 0.1 *M* tetrabutylammonium in 1 *M* phosphate buffer (pH = 10.5) and 1 ml of methylene chloride containing tridecane (0.5 mg/ml) as internal standard. Methyl iodide (0.1 ml) was added and the mixture shaken at room temperature at 60 strokes per min. An aliquot of the organic phase was washed with 0.1 *M* phosphoric acid and a few microlitres were taken for analysis by gas chromatography with flame ionization detection. The ratio of the height of the peak of the methylated product to that of the internal standard was calculated.

(B) Tetrapentylammonium as counter ion. The sulphonamide solution (0.1 ml), as above, was mixed with 1 ml of 0.2 *M* tetrapentylammonium, 0.2 ml of 5 *M* sodium hydroxide and diluted with water to 2 ml. To this solution, 1 ml of methylene chloride with the internal standard and 50 μl of methyl iodide was added. The mixture was shaken at room temperature and then treated as under (A) above.

(C) Tetrahexylammonium as counter ion. The sulphonamide dissolved in 2 ml of 0.2 *M* sodium hydroxide, 0.05 ml of 0.1 *M* tetrahexylammonium and 5.0 ml of of 3% (0.5 *M*) methyl iodide in methylene chloride was shaken for 20 min at 50°. After centrifugation, an aliquot of the organic phase was transferred to another tube and evaporated to dryness. Toluene–hexane (1:3) was added and a few microlitres were taken for analysis by EC–GC.

Determination of acetazolamide in serum

To a serum sample of acetazolamide (0.1 ml), 0.1 ml of internal standard solution, 1 ml of 0.2 *M* tetrapentylammonium, 0.2 ml of 5 *M* sodium hydroxide and water to give a volume of 2 ml were added. This solution was shaken for 1 h with 1 ml of methylene chloride containing 5% of methyl iodide. After centrifugation at 500 *g* for 15 min, *ca.* 100 μl of the organic phase were transferred to another tube and evaporated in a stream of nitrogen. A 1-ml volume of toluene was added and this solution was washed with hot saturated silver sulphate in water. A few microlitres of the organic phase were taken for analysis by EC–GC. A standard curve was constructed by treating 0.1 ml of the standard samples according to the procedure above.

RESULTS AND DISCUSSION

Extractive alkylation of acetazolamide

In extractive alkylation procedures it is essential to use conditions which will minimize hydrolysis of the reagent. Acetazolamide was chosen as a model sulphonamide diuretic since forced conditions were required. A distribution ratio of 1.9 was obtained for acetazolamide as an ion pair with 1 *M* tetrabutylammonium between methylene chloride and the aqueous phase at pH 10. Extractive methylation using this ammonium ion yielded two products. the dimethyl and the trimethyl derivatives. The formation of the trimethyl derivative was promoted by a high hydroxide concentration in the organic phase.

Trimethylacetazolamide was formed as the only product when 0.1 *M* tetrapentylammonium in 0.5 *M* sodium hydroxide was used as the aqueous phase. A lower concentration of hydroxide or tetrapentylammonium left some dimethyl derivative. The formation of trimethylacetazolamide was complete in 1 h and it was stable in the reaction medium for more than 4 h.

The derivatives of acetazolamide were formed in approximately equal amounts when tetrahexylammonium (2.5×10^{-4} *M*) in 0.2 *M* sodium hydroxide was used as the aqueous phase with treatment for 1 h at room temperature. An increase in the concentration of the quaternary ammonium ion increased the proportion of the trimethyl derivative. Some dimethylacetazolamide was, however, always obtained in the reaction mixture. If the reaction temperature was raised to 50° with tetrahexylammonium in 0.2 *M* sodium hydroxide as the aqueous phase, only one product

from acetazolamide was found. The promotion of the extractive methylation by a temperature increase has also been noticed with furosemide using tetrabutylammonium at pH 10 as the aqueous phase. At room temperature only *ca.* 10% was found after 1 h, whereas at 50° the reaction was complete in less than 20 min. This indicates that a small temperature increase is very favourable to the reaction rate.

Methyl iodide with dimethylsulphuric acid

In several batches of the methyl iodide reagent a contaminant was found. It was identified as dimethylsulphuric acid by means of IR, NMR and MS analysis. The presence of dimethylsulphuric acid was easily detected in the proton NMR spectrum where its protons appeared as a singlet at $\delta = 3.96$ in methyl iodide–deuteriochloroform (1:1). Long tailing solvent fronts in the gas chromatograms were seen with this contaminant in mixtures of biological samples of hydrochlorothiazide and chlorthalidone and quantitative analysis was impossible. Distillation of methyl iodide before use was therefore essential in the analysis in the low concentration range.

Extractive alkylation of sulphonamide diuretics

Extractive alkylation with tetrapentylammonium or tetrahexylammonium as counter ion in sodium hydroxide increases the hydroxide concentration in the organic phase which causes hydrolysis of the alkylating agent. An hydrolysis of alkylated derivatives has also been demonstrated under these conditions[3]. Conditions for the extractive alkylation of sulphonamide diuretics were evaluated for three methods.

An aqueous phase containing 0.1 *M* tetrabutylammonium at pH 10 has been found to give negligible solvolysis of the alkylating reagent[16]. Methylation of the acidic hydrogens with this aqueous phase within 1 h was observed with bumetanide (III), clopamide and mefruside (IX) (Table I). Hydrochlorothiazide (VIII) required 2 h, while bendroflumethiazide (II), chlorthalidone (IV) and furosemide (VII) required more than 4 h reaction time. 0.1 *M* tetrapentylammonium in 0.5 *M* sodium hydroxide as the aqueous phase gave complete methylation within 1 h of the above compounds with the exception of III and VII. These two compounds have a carboxylic acid group in common and it is likely that a hydrolysis of the formed methyl esters will occur.

Tetrahexylammonium in 0.2 *M* sodium hydroxide as the aqueous phase with an increased reaction temperature was found to be the most suitable method and gave complete methylation of all the sulphonamides within 20 min. Three other sulphonamide diuretics, methylchlothiazide, trichloromethiazide and chlorothiazide, were also tested, but they gave several peaks in all cases and were not investigated further.

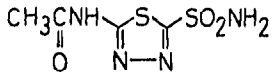
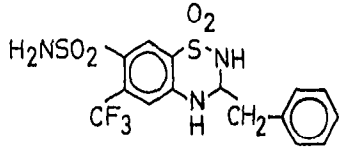
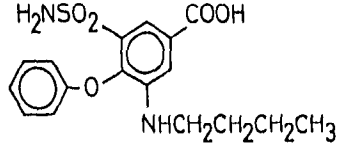
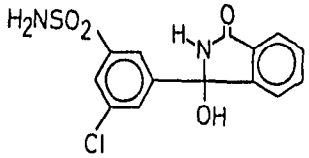
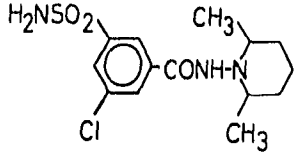
Identity of sulphonamide diuretics after methylation

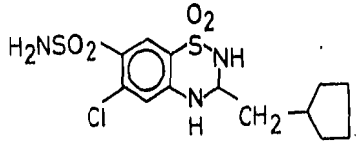
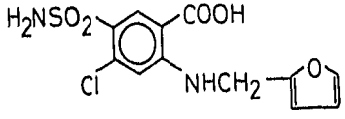
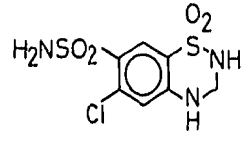
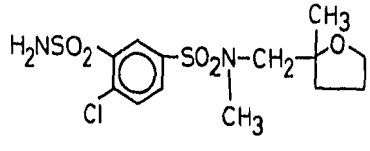
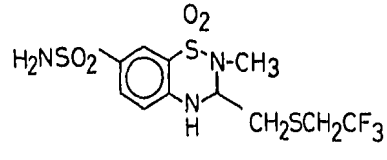
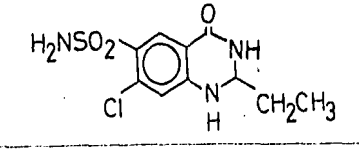
Methylation of the acidic hydrogens of the sulphonamide diuretics occurred during the extractive alkylation. The hydrogen atoms of the sulphonamide and the amide groupings were replaced by methyl groups. The carboxylic acid groups in compounds III and VII and the hydroxyl group in IV were also methylated.

The secondary amino group in the thiazides and in quinethazone (XI) was methylated. These groups are weakly acidic, and the formation of a quaternary

TABLE I

EXTRACTIVE METHYLATION OF SULPHONAMIDE DIURETICS

No.	*Generic name*	*Structure*	*Derivative after extractive methylation*	*Retention relative to furosemide derivative on SE-30*	*Time (min) for extractive methylation (see Experimental)*		
					Method A	*B*	*C*
I	Acetazolamide		trimethyl	0.1	two derivatives	60	20
II	Bendroflumethiazide		tetramethyl	3.9	240	60	20
III	Bumetanide		trimethyl	1.5	60	two derivatives	20
IV	Chlorthalidone		tetramethyl	1.1	240	60	20
V	Clopamide		trimethyl	0.8	15	60	20

VI	Cyclopenthiazide		tetramethyl	7.8	—	—	20
VII	Furosemide		trimethyl	1.0	300	two derivatives	20
VIII	Hydrochlorthiazide		tetramethyl	2.3	120	60	20
IX	Mefruside		dimethyl	1.1	15	60	20
X	Polythiazide		tetramethyl	3.7	—	—	20
XI	Quinethazone		tetramethyl	2.7	—	—	20

ammonium compound was not observed. On the other hand, the secondary amino group in compounds VII and III was not methylated with methyl iodide. Furosemide was treated with both dilute ^2HCl and dilute NaO^2H prior to and during methylation without exchange of the hydrogen atom.

Methylation of analytical amounts ($<500\ \mu g$) of polythiazide (X) using tetrahexylammonium in 0.2 *M* sodium hydroxide gave a tetramethyl derivative. A proposed structure is given in Fig. 1. When the amount of polythiazide was increased, the trimethyl derivative was obtained. The position of the extra methyl group in the tetramethyl derivative was elucidated from the NMR spectrum. The proton appearing at $\delta = 4.58$–4.78 in the spectrum of the trimethyl derivative was absent in the spectrum of the tetramethyl derivative, indicating substitution of this proton.

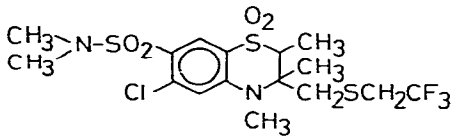


Fig. 1. Proposed structure for tetramethylpolythiazide.

Gas chromatography and electron capture response of methylated sulphonamide drugs

The dimethyl and trimethyl derivatives of acetazolamide were not separated with OV-17 as stationary phase. The more selective QF-1 was therefore used. The derivatives of the other sulphonamide diuretics could not be chromatographed on this stationary phase owing to long retention times, even when the column was operated at the upper temperature limit. A column with a low content of SE-30 as stationary phase was used. The relative retentions of the derivatives to trimethylfurosemide are given in Table I. The derivatives of compounds VIII and XI gave tailing peaks on this stationary phase.

The sulphonamide grouping has a high electron capture response, particularly when it is directly attached to an aromatic ring[11]. The minimum detectable concentration of trimethylacetazolamide was 2.3×10^{-16} mol/sec at a detector temperature of 270°. This minimum was found to be dependent on the detector temperature and increased by a factor of four over the range 180–350°. The minimum detectable concentration of the other methylated sulphonamide diuretics were of the same order of magnitude.

Purification of the reaction mixture

Before analysis by EC–GC the excess of reagent must be removed. Methylene chloride and methyl iodide are most easily removed by evaporation. The iodide of the quaternary ammonium ion is formed as a side product in the reaction and gives rise to long tailing fronts in the chromatograms. Two methods have been proposed to avoid this disturbance. Washing of the final organic solvent with hot saturated silver sulphate has greatly improved the chromatograms[5,8]. This method was found to be detrimental to the yield of some of the derivatives, *e.g.*, the methyl derivatives of compounds VII and III. An alternative method is to reconstitute the residue after

evaporation in hexane, since the quaternary ammonium iodide has a low solubility in this solvent.

If the residue after methylation of furosemide was dissolved in methylene chloride and injected into the gas chromatograph, the trimethylfurosemide peak decreased. A new peak with longer retention appeared in the gas chromatogram. Mass spectral analysis of this peak revealed that the methyl ester was converted into the hexyl ester if tetrahexylammonium was used in the extractive alkylation. The butyl ester was formed when using tetrabutylammonium (Fig. 2). A possible explanation of this reaction is hydrolysis of the methyl ester followed by flash alkylation caused by the presence of tetraalkylammonium hydroxide and the corresponding iodide in the gas chromatographic injector. The reaction did not occur on injection into the gas chromatograph of a mixture of trimethylfurosemide and tetraalkylammonium hydrogensulphate dissolved in methylene chloride.

Determination of sulphonamide diuretics in biological samples

The application of the extractive alkylation to biological samples of sulphonamide diuretics has been demonstrated[12–15]. A direct extractive alkylation of

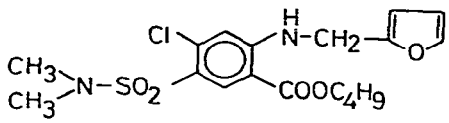


Fig. 2. Butyl ester of methylated furosemide generated in the injection into the gas chromatograph.

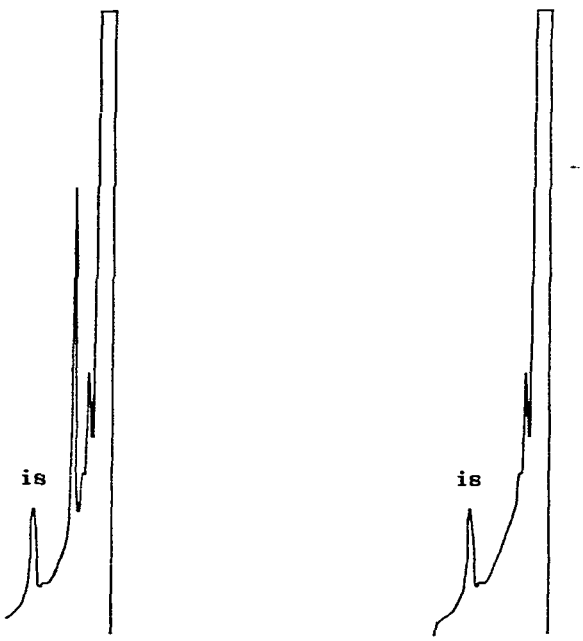


Fig. 3. Chromatograms of a plasma sample containing acetazolamide (25 μg) and internal standard (is, 20 μg) analyzed according to the recommended method (a), and of a blank plasma sample containing internal standard (b).

the sulphonamides[5,11] from a biological sample was not possible below 0.1 μg/ml owing to disturbances from the biological sample itself. A purification step was therefore included comprising extraction of the drug as an acid from the biological sample and re-extraction into an alkaline aqueous phase before extractive alkylation.

Direct extractive alkylation of acetazolamide from biological samples could be achieved by the method developed. Fig. 3 shows chromatograms of plasma samples. The internal standard in the determination was 2-[2-chlorophenyl]-5-sulphamoyl-1,3,4-thiadiazole, which was extracted and derivatized simultaneously. Concentration of acetazolamide down to 0.5 μg/ml in a 0.1 ml serum sample were determined by this method. The relative standard deviation at the 10 μg/ml level was 6.6% ($n = 10$).

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