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QUANTITATIVE DETERMINATION OF DRUGS IN BIOLOGICAL MATERIALS BY MEANS OF EXTRACTIVE ALKYLATION AND GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Analytical methods utilizing extractive alkylation followed by gas–liquid chromatography are described for the quantitative determination of (neo)sulfalepsine and its metabolites, chlorthalidone and chlorquinaldol.

The applications described indicate that, for the use of this technique, careful optimization and the introduction of a suitable internal standard at the beginning of the analytical manipulations are essential.

INTRODUCTION

Extractive alkylation has become a well known technique for the quantitative measurement of drugs in biological materials. A number of methods have been published in which the technique is used in combination with gas–liquid chromatography (GLC) (carboxylic acids and phenols[1], pentazocine[2], chlorthalidone[3,4], furosemide[5], hydrochlorothiazide[6], theophylline[7], sulfonamides[8], clioquinol[9]). An extensive review of the theory and application of the technique was compiled by Jonkmann[10].

The experience gained in our laboratories, where analytical methods are used in the processing of large numbers of samples, showed that the extractive alkylation is a great asset for the analytical laboratory.

Internal standardization is essential in quantitative drug assays using extractive alkylation procedures. Internal standards must have physicochemical properties that are almost identical with those of the compound being measured, except that there is a difference in retention volume great enough to allow good peak resolution. The addition of a known amount of internal standard to the biological sample prior to any manipulation permits a quantitative determination regardless of losses of material during extraction and derivatization procedures. Concentrations are calculated from the amount of standard added and the peak-area (or peak-height) ratios.

In this paper the application of the technique to the determination of (neo)-sulfalepsine (CGP 8426, Geistlich PB 385), a new anticonvulsant, is reported. Using differential extraction prior to the alkylation, the unchanged compound as well as the

assumed major metabolite Ba 589J can be quantified using the same biological sample.

The method for assaying the diuretic chlorthalidone (Hygroton, Ciba-Geigy) reported by Ervik and Gustavii[4] has been modified by optimization of the extraction conditions, which is especially important when analyzing whole blood, and the introduction of a suitable internal standard. The procedure for the antibacterial agent clioquinol (Vioform, Ciba-Geigy) has already been published[9], but has been extended to chlorquinaldol (Sterosan, Ciba-Geigy) simply by using clioquinol as the internal standard.

PRINCIPLE AND CONDITIONS

The compounds and their internal standards can be extracted in the form of their tetrahexylammonium salts into dichloromethane where, in the presence of iodomethane (or other alkylating agents), the methyl derivatives are formed almost spontaneously. However in most instances a purification step is necessary. Either the compounds are extracted first, using a conventional organic solvent extraction, or the compounds are extracted as ion pairs, alkylated and then purified.

Direct extractive alkylation from biological material, without purification, produces high background signals that make GC quantification difficult or impossible. The conventional organic solvent extraction procedures are optimized first by screening various suitable solvents followed by the evaluation of the optimal pH with respect to the GC background and recovery.

Both (neo)sulfalepsine and chlorthalidone are extracted prior to alkylation, using methyl isobutyl ketone, and then back-extracted into dilute sodium hydroxide solution. After addition of the tetrahexylammonium hydrogen sulfate, ion-pair extraction and alkylation are carried out.

Clioquinol can easily be alkylated directly after ion-pair extraction from the biological material. A purification step was added in the form of a base-specific extraction procedure. However, when the extractive alkylation procedure is applied to biological materials directly, the sample volume is very critical. For instance, the yield of the procedure decreases considerably if plasma samples larger than 0.5 ml are used. Owing to the properties of a suitable internal standard, the method is still workable but new calibration graphs must be prepared. Attempts to overcome this problem by increasing reagent concentrations or reaction time did not succeed.

EXPERIMENTAL

(Neo)sulfalepsine (CGP 8426, Geistlich PB 385)

The succinimide ring (Fig. 1, I) is easily hydrolyzed to form two different succinic acid monoamides (Fig. 1, II and III). The hydrolysis is extremely pH-dependent. Owing to instability of the compound at pH > 7, alkylation was not possible without simultaneous opening of the succinimide ring.

According to mass spectrometric data, the alkylated product is a trimethylated derivative (Fig. 1, V). The same derivative is obtained when subjecting compound Ba-589J (metabolite and hydrolysis product) to the alkylation reaction. This means

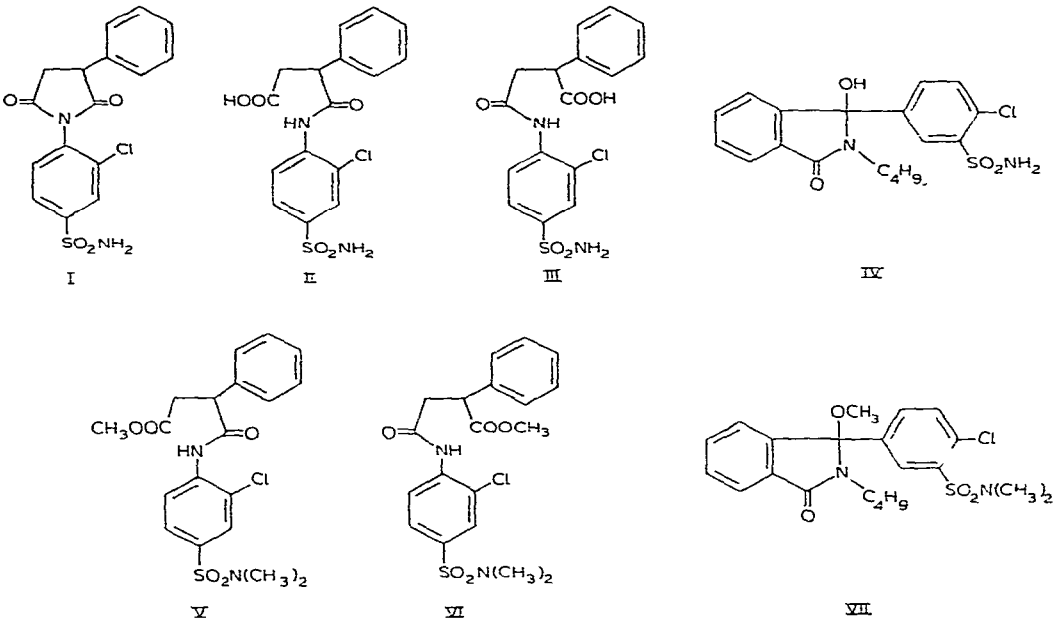

Fig. 1. Formation of the methyl derivative of (neo)sulfalepsine, the metabolites and the internal standard. I = (Neo)sulfalepsine; II = Ba 589J; III = Li/Ba 589; IV = N-butylchlorthalidone; V = trimethyl derivative of (neo)sulfalepsine and Ba 589J; VI = trimethyl derivative of Li/Ba 589; VII = trimethyl derivative of N-butylchlorthalidone.

that differentiation between unchanged (neo)sulfalepsine and the metabolite Ba 589J is not possible unless the two compounds are separated prior to alkylation.

It was found that toluene extracts unchanged (neo)sulfalepsine, as well as the internal standard, selectively. Strongly acidic conditions must be used when whole-blood samples are to be analyzed. Owing to binding of (neo)sulfalepsine to erythrocytes, extraction is not possible at neutral pH. The hydrolysis product (or metabolite) Ba 589J can be extracted with methyl isobutyl ketone, and it is also possible to extract both the unchanged (neo)sulfalepsine and Ba 589J together with methyl isobutyl ketone. The isomer of Ba 589J, the compound Li/Ba 589 (Fig. 1, III), displays the same extraction properties as Ba 589J but the retention time of its trimethyl derivative (Fig. 1, VI) in GC is different from that of the trimethyl derivative of Ba 589J and therefore the two compounds can easily be differentiated. However, only trace amounts of Li/Ba 589 have been found in both plasma and urine after oral administration of (neo)sulfalepsine to humans, and therefore the emphasis was put on the measurement of unchanged (neo)sulfalepsine and the metabolite Ba 589J.

The internal standard used (Fig. 1, IV) was chosen because of its almost quantitative extractability with both toluene and methyl isobutyl ketone.

Extraction and derivatization. The flow diagram for the simultaneous determination of unchanged (neo)sulfalepsine and metabolite Ba 589J is as follows:

0.2 ml of whole blood (or 1 ml urine or plasma)
+ 0.1 ml of aqueous solution of internal standard (25 ng per sample)
+ 2.0 ml of 1 *N* HCl (or 2 ml of pH 7 buffer* for urine)
(or 2 ml of pH 3 buffer** for plasma).
Extracted with 2 × 3.5 ml of toluene by shaking at 200 rpm for 10 min. Centrifuged briefly and toluene phase transfered via a filter plug of cotton.

For (neo)sulfalepsine:
Combined toluene phase extracted with 3 ml of 0.1 *N* NaOH by shaking at 320 rpm for 10 min; centrifuged briefly, toluene phase removed by aspiration and discarded.

For Ba 589J:
Aqueous phase + 0.1 ml of internal standard solution (as above) extracted with 5 ml of methyl isobutyl ketone by shaking at 200 rpm for 10 min, followed by brief centrifugation.

↓

Organic phase extracted with 3 ml of 0.1 *N* NaOH by shaking at 320 rpm for 10 min; centrifuged briefly, organic phase removed by aspiration and discarded.

↓

Aqueous phase + 0.1 ml of 0.05 *M* solution of tetrahexylammonium hydrogen sulfate + 2.0 ml of 1 *M* solution of iodomethane in dichloromethane shaken for 40 min at 100 rpm. Following brief centrifugation, aqueous phase removed by aspiration and discarded. Organic phase passed through a cotton filter and evaporated to dryness under a stream of nitrogen at 40°.

↓

Dry residue re-dissolved in 2 ml of *n*-heptane; after standing for 20 min at room temperature, insoluble particles removed by filtration through a cotton plug.

↓

2–5 µl of the *n*-heptane solution injected into the chromatograph.

Gas–liquid chromatography. A Philips Pye-GCV gas chromatograph equipped with a linear ^{63}Ni electron capture detector was used. The column was a Pyrex glass column, 1.5 m × 4 mm I.D., packed with 3% OV-1 on Supelcoport (80–100 mesh). The carrier gas was nitrogen at a flow-rate of 90 ml/min. The temperatures were: column, 250°; injector, 255°; detector, 350°. The retention times under these condi

* Buffer solution of pH 7: 0.041 *M* disodium hydrogen phosphate + 0.028 *M* potassium hydrogen phosphate.

** Buffer solution of pH 3: 0.050 *M* potassium hydrogen phthalate + 0.021 *M* hydrochloric acid.

tions were 6.5 min for (neo)sulfalepsine (or Ba 589J), 7.9 min for the internal standard and 8.5 min for Li/Ba 589.

Calibration graphs. If both the unchanged (neo)sulfalepsine and the metabolite Ba 589J were to be determined, calibration graphs were prepared by mixing equal amounts of both compounds with blank blood or plasma samples followed by analysis as described. However, as the extraction procedure as well as the extractability itself for blood differ from those for plasma and urine, separate calibration graphs must be prepared for each type of biological fluid.

A calibration graph prepared with whole blood is shown in Fig. 2. The peak-height ratios (H_x) were plotted against the actual concentrations of either (neo)sulfalepsine or Ba 589J. The graph shown was prepared by using 0.2 ml of human blood. The corresponding graphs for plasma or urine are similar, but the slopes are slightly different.

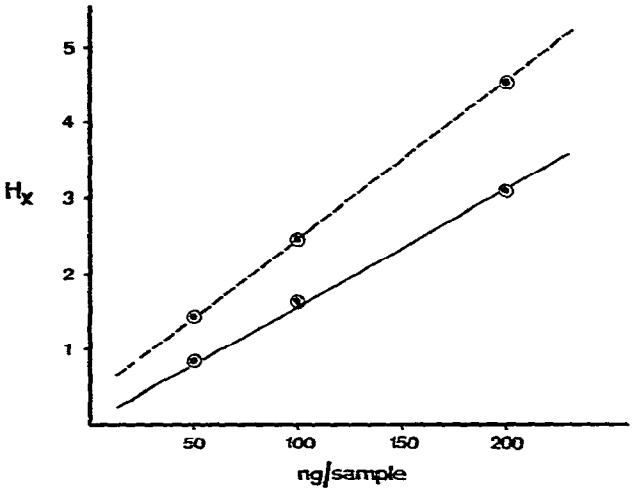

Fig. 2. Calibration graphs for the entire analytical procedure to determine unchanged (neo)sulfalepsine (◉—◉) and metabolite Ba 589J (◉—◉) in fresh human blood (0.2 ml). Each sample contained 25.0 ng of N-butylchlorthalidone as internal standard. H_x is the ratio of the peak height of either the (neo)sulfalepsine or the Ba 589J derivative to that of the internal standard derivative.

Accuracy and precision. The method was tested by analyzing prepared samples of whole blood, the (neo)sulfalepsine and the Ba 589J concentrations in which were unknown to the analyst. Samples containing various concentrations of both compounds were analyzed for each component independently.

The results (Table I) show that this method is capable of determining both the unchanged (neo)sulfalepsine and the metabolite Ba 589J in the same sample. However, a slight carry-over effect occurs if there is a large difference in the concentrations of the two components.

When analyzing unknown samples for both components, the analysis of the total of unchanged compound plus metabolite provides additional information on the accuracy of the assay.

TABLE I

RECOVERIES OF UNCHANGED (NEO)SULFALEPSINE AND Ba 589J FROM SPIKED BLOOD SAMPLES

Sample volume: 0.2 ml.

Given		*Found [$n = 3$; $\bar{x} \pm s(x)$]*	
(Neo)sulfalepsine (ng per sample)	*Ba 589J (ng per sample)*	*(Neo)sulfalepsine (ng per sample)*	*Ba 589J (ng per sample)*
0	200	9.0 ± 2.52	179 ± 20
30	100	30.0 ± 4.0	95 ± 4.0
100	100	100 ± 5.0	104 ± 3.0
100	30	99 ± 9.1	30 ± 3.1
200	0	194 ± 1.7	19 ± 1.6

Stability. Owing to the low stability of (neo)sulfalepsine in solution under certain conditions, stability tests were carried out under various conditions. Blood and urine samples containing 1000 ng/ml of unchanged (neo)sulfalepsine at the start of a test showed only insignificant losses after 32 days when kept at −22°. However, as soon as the samples are thawed they must be analyzed immediately. An aqueous test solution containing 200 ng/ml of (neo)sulfalepsine was kept at 4° for 15 days and re-analyzed. The solution contained only about 17% of the original concentration.

For the preparation of calibration graphs, stock solutions of (neo)sulfalepsine were prepared in ethanol, in which the compound is fairly stable. Solutions in methanol are highly unstable owing to methanolysis (formation of the succinic acid methyl ester).

Chlorthalidone

Methods for the determination of chlorthalidone in biological materials by GC have been reported by Brändström and Gustavii[3] and later by Ervik and Gustavii[4], but both methods lack a suitable internal standard. In order to obtain a method that can be used on a routine basis for various types of biological materials, an appropriate internal standard was sought. The addition of a known amount of N-butylchlorthalidone prior to any manipulations improved the method considerably. Both chlorthalidone and N-butylchlorthalidone are extracted from either plasma, blood or urine using methyl isobutyl ketone. After back-extraction into dilute sodium hydroxide solution, extractive alkylation is applied in order to convert chlorthalidone and N-butylchlorthalidone into tetramethyl and trimethyl derivatives, respectively (Fig. 3).

The initial extraction with methyl isobutyl ketone is not pH dependent for plasma or urine, but for whole blood the optimal pH was found to be between 5 and 6 (Fig. 4).

The sensitivity of the method is about 25 ng in 0.5 ml of plasma or urine and about 50 ng in 0.1 ml of whole blood. The lower sensitivity for whole blood is due to a higher background signal obtained with blood extracts. However, as the major part of the chlorthalidone is found in the erythrocytes, blood samples of 0.1 ml are sufficient for the determinations.

Extraction and derivatization. A 1-ml volume of urine or plasma (or 1 ml of

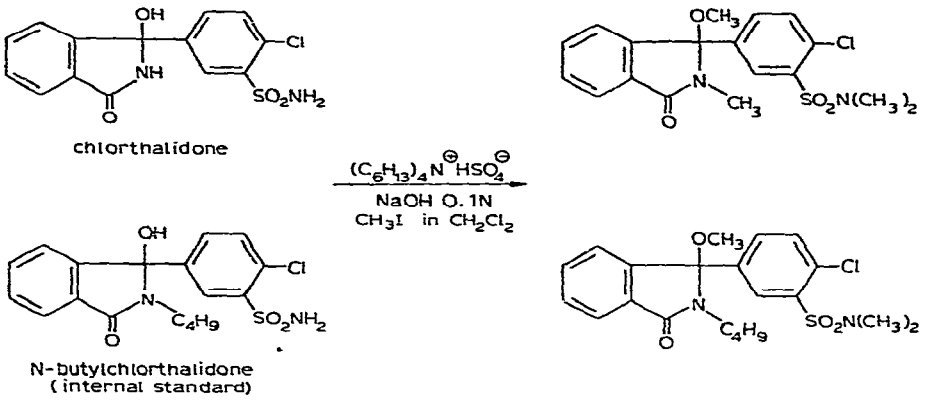

Fig. 3. Formation of the tetramethyl derivative of chlorthalidone and the trimethyl derivative of the internal standard.

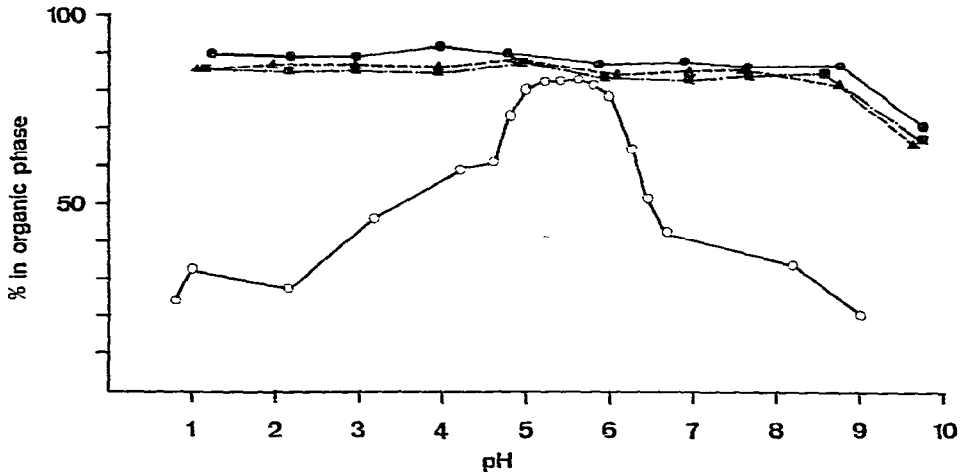

Fig. 4. pH dependence of the extractability of chlorthalidone from water (■), urine (●), plasma (▲) and whole blood (○). Percentage of 200 ng of [^{14}C]chlorthalidone in the organic phase following equilibration between 4 ml of methyl isobutyl ketone and 4 ml of aqueous phase containing buffer and the biological material.

diluted blood, corresponding to 50–250 μl of original whole blood), 0.3 ml of internal standard solution (containing 300 ng of N-butylchlorthalidone in water), 4 ml of 0.25 *M* citrate buffer (pH 5.4) and 4 ml of methyl isobutyl ketone are combined and shaken for 20 min on a rotary mechanical shaker at 150 rpm. After brief centrifugation, a maximum aliquot of the organic phase is transferred into a clean tube. Then, 2.5 ml 0.1 *N* sodium hydroxide solution are added and the mixture is shaken for 10 min at 150 rpm, followed by brief centrifugation.

The organic phase is discarded and the aqueous phase combined with 50 μl of a 0.05 *M* solution of tetrahexylammonium hydrogen sulfate and 3 ml of a 0.1 *M* solution of iodomethane in dichloromethane and shaken for 60 min at 80 rpm on a rotary mechanical shaker. After brief centrifugation, the organic phase is removed and evaporated to dryness under a stream of nitrogen.

To the dry residue 0.2 ml of toluene, 1.8 ml of *n*-hexane and 2.0 ml of 1 *N* sodium hydroxide solution are added and the mixture is shaken for 10 min at 220 rpm. After brief centrifugation, the organic phase is transferred into a clean tube and evaporated to dryness under a stream of nitrogen.

The residue is re-dissolved in 100 μl of toluene plus 0.9 ml of *n*-hexane. Of this final extract, about 3 μl are injected into the gas chromatograph.

GC conditions. A Hewlett-Packard Model 5736A instrument, equipped with a linear electron-capture detector (^{63}Ni, 15 mCi) was used with a column (1.4 m $\times$ 2 mm I.D.) of 3% OV-101 on Supelcoport (80–100 mesh). The carrier gas was argon–methane (19:1) at a flow-rate of 60 ml/min. The temperatures used were: column oven, 235°; detector, 350°; injector, 250°.

The retention times under the given conditions are 4 and 6 min for the tetramethyl derivative of chlorthalidone and for the trimethyl derivative of N-butylchlorthalidone, respectively.

Accuracy and precision. Using separate calibration graphs for each type of biological fluid, test samples were analyzed. Samples of 0.5 ml of urine, 0.5 ml of plasma and 0.1 ml of blood were spiked with known amounts of chlorthalidone and analyzed. The recoveries were 101.1 $\pm$ 3.6% (standard deviation) for concentrations between 25 and 290 ng per sample in urine, 100.4 $\pm$ 6.2% for concentrations between 35 and 142 ng per sample in plasma and 100.7 $\pm$ 3.9% for concentrations between 60 and 300 ng per sample in whole blood.

Chlorquinaldol

A method for the quantitative determination of clioquinol using extractive alkylation and GLC was published recently[9].

For the determination of chlorquinaldol, clioquinol is used as an internal standard, but the general procedure remains as reported for clioquinol (Fig. 5).

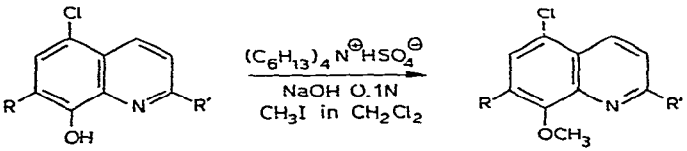

Fig. 5. Formation of the methyl derivative of chlorquinaldol and internal standard. R = Cl, R′ = CH_3, chlorquinaldol; R = I, R′ = H, clioquinol.

The sensitivity is about 10 ng in 0.5 ml plasma. The retention times using the conditions given for clioquinol are 2.6 and 4.4 min for the O-methyl derivatives of chlorquinaldol and clioquinol, respectively. Recoveries of spiked plasma samples with concentrations between 22 and 210 ng per 0.5 ml were 94 $\pm$ 12% (standard deviation).

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